# Canine and Feline Nutritional Immunology

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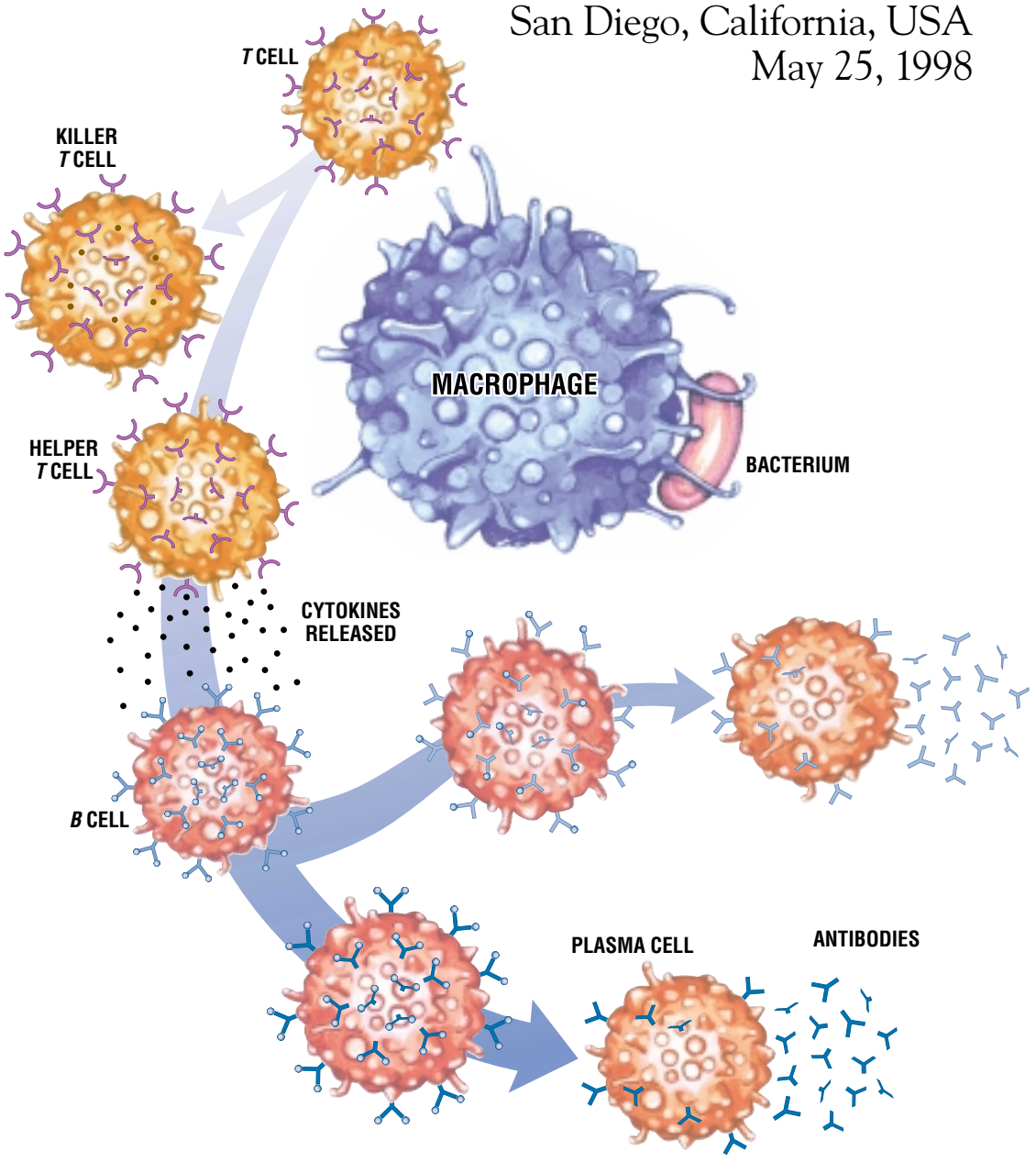
American College of
Veterinary Internal Medicine
San Diego, California, USA
May 25, 1998



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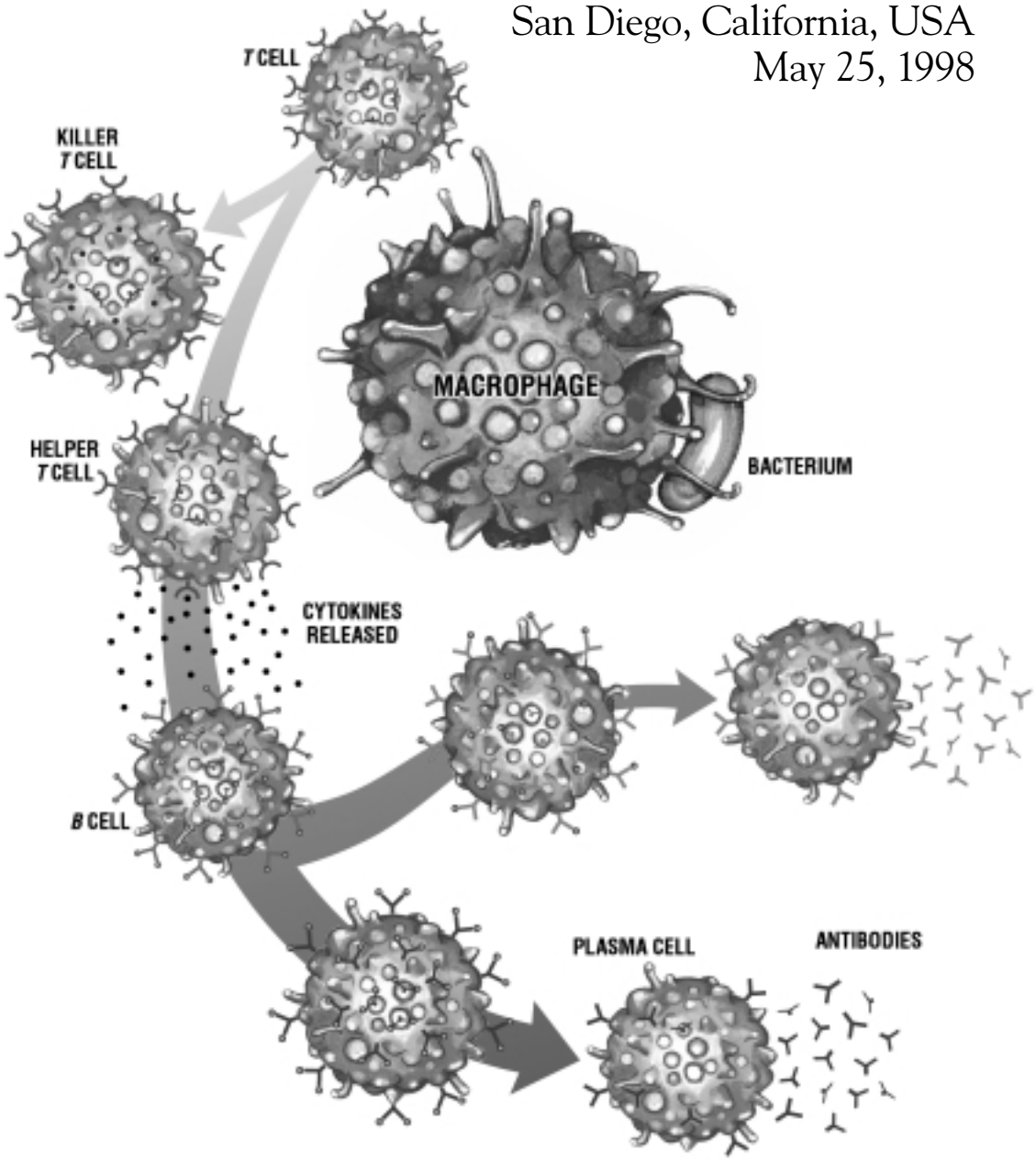

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# Participants

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## Catherine J. Field, PhD, RD
## University of Alberta

Dr. Catherine Field received a MSc in Nutrition from the University of Toronto in 1984 and a PhD in Nutrition and Metabolism from the University of Alberta in 1988. After postdoctoral work in Medicine at McGill University she accepted a Faculty position at the University of Alberta in Edmonton, Canada. Dr. Field is currently an Associate Professor in Nutrition in the Department of Agricultural, Food and Nutritional Science with a cross-appointment in Medicine at the University of Alberta. Her research program centers on the effect of nutrition on the immune system. Current areas of research are: effect of lipids on the development of the immune system; dietary modulation of the gut-associated immune system; the role of diet in the initiation of autoimmune-mediated insulin dependent Diabetes Mellitus (Type I Diabetes); dietary fat effects on membrane composition, and immune function during trauma and cancer.

## Jean A. Hall, DVM, PhD, DACVIM (Internal Medicine)
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Dr. Hall is currently an Assistant Professor at the College of Veterinary Medicine at Oregon State University. She is a graduate of Washington State University and received her DVM in 1982. She later attended Colorado State University, which awarded her an MS in 1987 and a PhD in physiology in 1989. Current research interests include idiopathic hyperlipoproteinemia of the Miniature Schnauzer and the effects of omega-3 fatty acids on immune system function, eicosanoid metabolism, lipid peroxidation, and vitamin E status in Beagles.

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Dr. Hayek received his BS degree in Biology from Villanova University, his MS degree in Animal Science from the University of Kentucky in 1989, and his PhD degree in Nutritional Science from the University of Kentucky in 1991. Dr. Hayek joined the Nutritional Immunology Laboratory at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University as a Research Associate in 1991. His research emphasis was in the interaction between nutrition and the aging immune system. In 1995 Dr. Hayek accepted a position as Research Nutritionist in the Research and Development Department of The Iams Company. Dr. Hayek's current research interests include geriatric nutrition, longevity, and the interaction between nutrition and the immune response. Dr. Hayek has published over 30 scientific papers and abstracts.

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Dr. Nelson received his DVM degree from Tuskegee Institute in 1979 and his PhD in Veterinary Sciences with an emphasis in immunology from North Carolina State University in 1993. Academic appointments have included Instructor and Assistant Professor at Mississippi State University from 1979 to 1984, Assistant and Associate Professor at Tuskegee University (1984-1989 and 1993-1994), and Associate Professor at Mississippi State University from 1994 to present. He is currently serving as the Associate Dean of the College of Veterinary Medicine and is a faculty member in the Research Program at Mississippi State University.

## John J. Turek, PhD
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Dr. Turek is a graduate of the University of Illinois where he received his BS degree in Biology in 1974, and his MS and PhD in Veterinary Microbiology/Immunology in 1976 and 1979, respectively. In 1980 he joined Purdue University. His academic appointments at Purdue include: Director, Electron Microscopy Facility (1980-present); Assistant Professor, Dept of Anatomy, School of Veterinary Medicine (1986-1991); Associate Professor, Dept. of Basic Medical Sciences, School of Veterinary Medicine (1991-present). He is also an Adjunct Associate Professor with Indiana University School of Medicine, Indianapolis (1994-present). His research is focused on how dietary lipids modulate the immune system in infectious diseases and in tissue injury and repair. He also has broad interests in the use of electron microscopic and image analytical methods to study cell structure and function. Dr. Turek has published over 100 scientific papers and abstracts in peer reviewed journals.

# Interactions of Nutrition and Immunology

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This paper summarizes the history of nutritional immunology and provides an overview of the immune response. The mechanisms responsible for decreased resistance to infection and the specific nutrient deficiencies that affect them are also summarized. Nutrients discussed include protein, vitamins (A, ß-carotene, B, C, and E), minerals (iron deficiency, iron excess, zinc, copper, magnesium, and selenium), over nutrition and fatty acids. Immune function tests that are used to assess the immune response are highlighted. The role of nutrients in influencing the outcome of various disease processes is briefly reviewed at the end of the paper.

## HISTORY OF NUTRITIONAL IMMUNOLOGY[1]

Nutritional immunology is a newly recognized subdiscipline based on observations that the immune system does not perform optimally if malnutrition is present. Unlike AIDS, Nutritionally Acquired Immune Deficiency Syndrome (NAIDS) can be reversed by correcting the underlying nutritional problems. The history of nutritional immunology began in 1810 with the recognition of lymphoid tissue atrophy due to malnutrition. The discovery of vitamins in the early 1900s, along with increasing knowledge about nutrition, aroused much medical and public interest in the 1920s and 1930s. Before the discovery of antibiotics, the outcome of infections often depended on excellent supportive care, including a fully nutritious diet. Progress in immunonutrition was slowed during World War II and the "antibiotic era." With the increasing availability of antibiotics and breakthroughs in other medical sciences, interest in the supportive and nutritional aspects of medical care waned in the 1940s and 1950s. A worldwide rebirth of interest began in the 1960s and early 1970s with continued work on vitamins and also on the effects of protein-energy malnutrition and of iron deficiency (or excess). The introduction of total parenteral hyperalimentation techniques for malnourished patients and the demonstration that NAIDS could be reversed by nutritional therapy also stimulated progress in immunonutrition. The current logarithmic phase of growth was stimulated by a number of major meetings on nutritional immunology in the late 1970s and 1980s. A number of books were published; e.g., the relationship of immunonutrition to infection, the roles of specific micronutrients, and immunonutrional problems in elderly people. An International Nutritional Immunology Group was formed at the FASEB meeting in 1980. The quarterly *Journal of Nutritional Immunology* debuted in 1991. New concepts and improved research methodologies from both parent sciences continue to propel the subdiscipline.

The 1968 WHO monograph *Interactions of Nutrition and Infection*,[2] suggested for the first time that the relationship between infection and malnutrition is synergistic. The monograph showed extensive evidence for both the adverse effect of infections on nutritional status and the increased susceptibility to infection of malnourished individuals. Foregoing a discussion on the multiple ways in which infections can affect nutritional status (e.g., anorexia, decreased intestinal absorption, fever), the following is an overview of the reciprocal relation between nutrition and immunology; i.e., focusing on how malnutrition relates to reduced resistance to infection.[3,4] Much of the evidence relates to the deficiencies of specific nutrients.

## OVERVIEW OF THE IMMUNE RESPONSE

Nutritional immunology has evolved from clinical evaluation to cellular and molecular observations. Before discussing specific nutrient deficiencies, it is important to have a general understanding of the immune response (Figure 1). Because present evidence suggests that cytokines, as intercellular mediators, play a key role in the nutrition-infection complex, they will be emphasized.[5] The successful initiation of an immune response to foreign antigens depends on several cell types including antigen presenting cells (e.g., macrophages) and T cells. The communications between different cells are mediated by soluble factors: the cytokines. The production of most cytokines requires the transcription of cytokine genes and the subsequent translation of mRNA into protein. Once released into the microenvironment, the cytokines transmit their biological signals to responsive targets interacting with specific high affinity cell surface receptors. Cytokines act on different organs, tissues, and cells in an endocrine, paracrine or autocrine fashion showing systemic and/or local effects. Thus, cytokines play a key role as communication signals during both normal immunologic responses and pathological conditions leading to infectious, inflammatory and neoplastic diseases. Nutritional factors may act by influencing the synthesis and release of

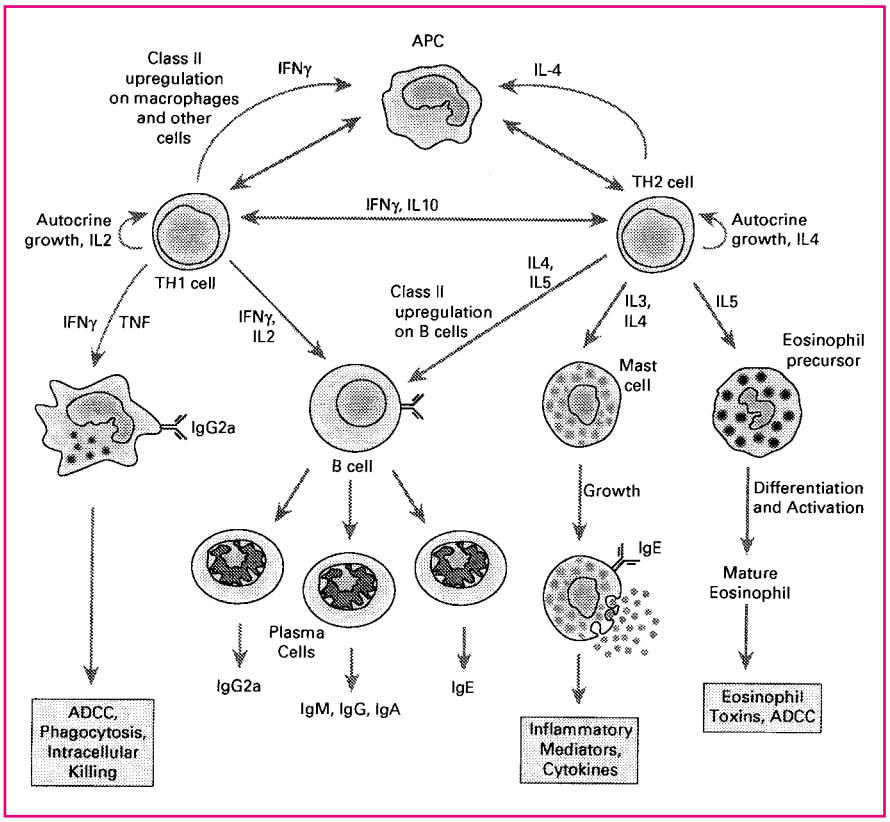


**Figure 1.** *The central role of helper T cells (TH) in cell-mediated immunity. Antigen-presenting cells (APC) present processed antigen to TH cells, which are central to the development of immune responses. These cells recognize particular epitopes. They then select and activate the appropriate effector mechanisms. They can help B cells to make antibody and activate or suppress the actions of a variety of other effector cells. They include cytotoxic T cells (TC), natural killer (NK) cells, macrophages, granulocytes and antibody-dependent cytotoxic (K) cells resulting in antibody-dependent cellular cytotoxicity (ADCC). Many of these effects are mediated by lymphokines, but cytokines from other cells, particularly macrophages, are also important. Both T and B cells may in turn be influenced by suppressor (TS) or regulatory cells. Not only does their lymphokine output drive different effector pathways, but TH1 cells tend to switch off TH2 cells, and vice-versa. (Reprinted with permission from: Bradley J, Xu X. Diet, age and the immune system. Nutr Rev 1996;54(11):S43-S50).*

cytokines and/or by affecting their direct or indirect actions on target tissues and subsequent responses.

The potential mechanisms responsible for decreased resistance to infection include:[6] interference with the production of humoral antibodies and of mucosal secretory antibodies, cell-mediated immunity, bactericidal capacity of phagocytes, complement formation, numbers of thymus-dependent T lymphocytes and T cell subsets (helper, suppressor-cytotoxic, and natural killer cells), and nonspecific defense mechanisms. These nonspecific defense mechanisms include intestinal flora; anatomical barriers (skin, mucosa, and epithelium); secretory substances such as lysozymes, mucus, and gastric acid; the febrile response; endocrine changes; and binding of serum and tissue iron. All of the antimicrobial systems in the neutrophil are potentially affected by malnutrition. These include both oxygen-dependent systems (responsible for the respiratory burst), and oxygen-independent systems (lactoferin, lysozymes, hydrolase, and proteases).

## Nutrient Deficiencies

Evidence for the effects on immunity of specific nutrient deficiencies (occasionally excesses) are summarized below.

## Protein

Many studies in experimental animals have shown adverse effects of protein deficiency on immunity.[6-8] These studies are confirmed by clinical and field studies in humans. As most immune mechanisms are dependent on cell replication or the production of active protein compounds (e.g., cytokines), it is easy to understand why protein deficiency interferes with resistance to infection. Because protein synthesis is dependent on a balance of essential amino acids, deficiencies of specific amino acids have similar effects. Depending on the severity of protein deficiency, effects on immune function include: the lymphoid organs, including the thymus, are smaller; there is reduced lymphocyte proliferation; the total peripheral blood lymphocyte count may be reduced; CD4 T cells are markedly reduced and there is a smaller reduction in CD8 T cells; there is reduced production of cytokines by T cells (e.g., interleukin-2 [IL-2] and interferon gamma); activated macrophages produce less IL-1; there is impaired antibody formation; serum immunoglobulin concentration is decreased; secretory immunoglobulin A is decreased; the delayed hypersensitivity skin test responses are reduced; there is decreased complement formation; and there are effects on nonspecific mechanisms that include anatomic barriers and secretory substances such as lysozymes and mucus. Certainly the nature and severity of protein insufficiency determine whether a demonstrable effect will be noted. The specific immunoglobulins used and the presence and nature of concurrent infections also affect the immune response. Interest in supplementing specific amino acids (e.g., arginine) to improve the immune response is in vogue. Arginine administered in clinical studies has been shown to enhance phagocytosis by alveolar macrophages, depress T suppressor cells, and stimulate T helper cells.[9]

## Vitamin A

Deficiency in experimental animals is associated with increased susceptibility to infection. These animals have decreased thymus and spleen sizes, reduced natural killer cell activity, lower production of interferon, impaired delayed cutaneous hypersensitivity, less effective fixed fat

macrophage activity, and lower lymphocyte response to stimulation by mitogens. Phagocytic activity may also be affected. Repletion of retinoic acid effectively reestablishes the number of circulating lymphocytes and may stimulate natural killer cell function.[10] High intakes of vitamin A are mixed in their effects, enhancing some immune responses and suppressing others. Recent studies in chickens suggest that vitamin A-deficient chickens developed a $T_H2$ immune response, whereas chickens fed highly enriched vitamin-A diet showed a $T_H1$ immune response.[11]

## β-Carotene

Carotenoids exhibit biological activities as antioxidants, affect cell growth regulation and modulate gene expression and immune response.[12] Administration *in vivo* has been shown to stimulate rat lymphocyte mitogenesis and increase human natural killer cell and T helper cell numbers. β-Carotene added to human lymphocytes *in vitro* stimulated natural killer cell activity, but did not affect other T cell subsets.

## B Vitamins

Pyridoxine, vitamin $B_6$, deficiency has been associated with reduced cell mediated immunity in experimental animals. Pantothenate deficiency combined with pyridoxine deficiency results in impaired antibody formation. Experimental folic acid and cobalamin, vitamin $B_{12}$, deficiencies interfere with both antibody formation, replication of stimulated leukocytes, and cell-mediated immunity. These as well as choline deficiency are associated with thymic atrophy.

## Vitamin C

Deficiency in experimental animals results in decreased neutrophil function, impaired delayed cutaneous hypersensitivity, and abnormal serum complement concentrations. In addition reduced phagocytic response and killing power as well as reduced antibody response have been described in clinical studies. There is not conclusive evidence to support the hypothesis that ascorbic acid deficiency in humans leads to either altered cell-mediated or humoral immunity. A favorable effect on infection of massive doses of vitamin C has not been confirmed in studies with acceptable experimental designs. In rats, ascorbic acid has been reported to have a corrective influence on the alteration of immunological functions that occurs during aging, including phagocytic function.[13]

## Vitamin E

Deficiency results in reduced lymphocyte and leukocyte killing power in experimental animals. It also interferes with antibody formation, plaque-forming cells, and other aspects of cell-mediated immunity. Vitamin E is one of the few nutrients for which supplementation at higher than recommended levels has been shown to enhance immune response and resistance to disease. Supplementation enhances both humoral and cell-mediated immunity and augments the efficacy of phagocytosis, particularly in healthy elderly persons with altered T-cell functions. Long-term feeding of a high vitamin E diet in rats improved the decrease in cellular immune functions caused by aging, and appeared to be associated with the enhancement of both macrophage functions and lymphocyte responsiveness.[14] Oxidant by-products of normal metabolism likely contributes to immune-system decline associated with aging. Antioxidant defenses against this damage include vitamin E, vitamin C, and carotenoids.[15]

## Iron Deficiency

This is the most common nutrient deficiency in the world in humans, and supplementation of iron-deficient populations results in decreased infectious episodes. Iron deficiency results in impaired phagocytic killing of bacteria, less response to lymphocyte stimulation, decreased T-cell numbers, fewer natural killer cells associated with reduced interferon production and depressed delayed cutaneous hypersensitivity. B cell and antibody formation are not affected. Iron-deficient rats with experimental salmonella infection had more viable intracellular and extracellular bacteria in the macrophages and intestinal walls than in iron-replete rats.

## Iron Excess

Iron is needed not only by the host but also by infectious agents. Transferrin, conalbumin, and lactoferrin have stronger iron-binding properties than do most bacteria siderophores and are normally highly unsaturated. In an iron deficient host with reduced immune function, lack of available iron for agent replication is protective. When individuals whose resistance to infection is compromised by iron deficiency are given parenteral iron or large doses of iron, a disastrous exacerbation of the infection and death may occur. This occurs because the agent is supplied with iron for replication before the host immune system has had time to recover. Supplementation of poorly nourished adults with daily physiologic amounts of iron, on the other hand, consistently results in decreased morbidity from infectious disease.

## Zinc

Deficiency results in extensive changes in T-cell indices, including thymic atrophy, impaired lymphocytosis, and impaired response to stimulation. Although iron deficiency sufficient to affect immunity in undernourished human populations is much more common than zinc deficiency, the effects of experimental zinc deficiency on immunity are currently more commonly studied and reported. Zinc administration alone has been shown to correct some of the T cell-mediated immune functions in children with protein–calorie malnutrition.[16] Excessive zinc intake also impairs immune responses.

## Copper

Deficiency in experimental animals results in both B cell- and T cell-related deficiencies. Impaired antibody formation, inflammatory response, phagocytic killing power, and lymphocytes stimulation responses, as well as

thymic atrophy, have been documented. Researchers consider these changes significant enough to impair the immune response to an intracellular pathogen in the bovine.[17] Copper deficiency alone or coupled with high dietary molybdenum resulted in decreased bactericidal activity of neutrophils and affected tumor necrosis factor (TNF) and perhaps other cytokines in calves.[18] An early clinical sign of copper deficiency in humans is a reduction in the number of circulating neutrophils.[19]

### Magnesium

Necessary as a cofactor for DNA synthesis, magnesium deficiency in experimental animals results in similar alterations of T and B cell functions as described for copper and zinc deficiencies.

### Selenium

Deficiency can also affect all components of the immune system. Sera collected from dogs previously fed a vitamin E and selenium deficient diet markedly suppressed the *in vitro* response of lymphocytes to mitogenic stimulations. The authors concluded that *in vivo* suppression of immunocompetent cells to antigenic stimulations may impair the capacity of the host to control infections.[20]

### Over Nutrition

It is clear from whole-animal studies that obesity and consumption of diets high in fat, particulary unsaturated fat, depress immunocompetence and enhance the risk for serious infectious disease and cancer.[21] Obese infants have higher incidences of lower respiratory tract infections than nonobese infants. Obese beagles challenged with distemper virus had an increased incidence of encephalitis and mortality. Obese dogs also showed increased morbidity and mortality from *Salmonella*. Feeding dogs diets high in fat also increased the severity of infectious viral canine hepatitis. Immune impairment may be reversible with adequate weight reduction.[22]

### Fatty Acids

Several mechanisms by which fatty acids might affect the functions of cells of the immune system exist.[23,24] For example, once incorporated into membrane phospholipids they could alter the fluidity of those membranes. They could affect signal transduction processes by altering their ability to act as substrates for phospholipases, and they could act as precursors for the synthesis of eicosanoids, thus affecting cytokine production.

## IMMUNE FUNCTION TESTING

Most nutrients are directly or indirectly involved in protein synthesis and most immune responses involve the production of proteins. There are a limited number of possible alterations of the immune system; thus, the lists of observed effects for different nutrient deficiencies tend to be quite similar. T cell functions are more sensitive than B cell functions to most nutrient deficiencies. Thus, a few specific immune tests can be used to assess the immune response. Depression of cell-mediated immune response is demonstrated by diminished cutaneous delayed hypersensitivity response, reduction in lymphocyte reactivity to mitogens and antigens, and changes in the ratio of helper and cytotoxic lymphocyte subpopulations. Also, an impairment in the nonspecific defense mechanisms such as interferon production, complement levels, and phagocytic and bactericidal capacity of polymorphonuclear leukocytes can be useful indicators of immune system impairment.[5,6,25]

## NUTRITIONAL MANAGEMENT OF VARIOUS DISORDERS

The role of nutrients in influencing the outcome of various disease processes is currently receiving much attention. The possible ways in which dietary lipids may influence immunologic function are areas of active research.[26,27] For example, the effects of dietary omega-3 (n-3) fatty acid supplementation on gentamicin-induced nephrotoxicosis in healthy dogs has been studied.[28] The ratio of dietary n-6 to n-3 fatty acids has been shown to influence immune system function, eicosanoid metabolism, lipid peroxidation, and vitamin E status in aged dogs.[29] Feeding diets supplemented with arginine plus glutamine, fish oil plus arginine, or fish oil plus glutamine have been shown to improve resistance to infection in a gut-derived sepsis model.[30,31] The benefits of early postoperative feeding with a nutritionally complete enteral diet supplemented with the nutrients arginine, ribonucleic acid, and omega-3 fatty acids on immune function in human patients undergoing surgery for upper gastrointestinal malignancies has been noted. These nutritional supplements improved postoperative immunologic responses and helped to overcome more rapidly the immunologic depression after surgical trauma.[32,33]

**The possible ways in which dietary lipids may influence immunologic function are areas of active research.**

Food allergy is defined as an immunologically based adverse reaction to a food antigen. Nutritional management of food allergy in dogs and cats has been reviewed.[34] There may be a modified immune response in Irish Setters with increased intestinal permeability in gluten-sensitive enteropathy.[35] Dietary nucleotides may up-regulate the $T_H1$ immune response in systemic immunity.[36-39] Dietary fiber may also have an immunoregulatory effect on the intestinal immune system, at least in rats.[40]

Clinical trials are needed to define the diet composition that will have an anti-inflammatory effect but maintain a normal immune response. For example, dietary supplementation with n-3 polyunsaturated fatty acids is generally recommended in elderly human subjects for

reducing or preventing inflammatory disease and to down-regulate cytokine production. Secondarily they suppress cell-medicated immunity, which may not be desirable.[5] The function of the immune system is known to decline in elderly humans. While the primary age-dependent intrinsic decline of immune responsiveness in the elderly is not presently influenced by therapeutic measures, secondary alterations of immune function, e.g., those caused by diet, offer possibilities for corrective measures.[41,42]

**The function of the immune system is known to decline in elderly humans.**

A better understanding of nutrition-cytokine interactions may be important for dietary treatment of cancer patients. The modulation of immune function by vitamins and trace elements remains important in cancer patients and affects survival.[43]

Nutritional factors may also regulate genes in the gastro-intestinal epithelium. Subsequent proteins expressed by the epi-thelium may act on the immune system and constitute a signaling mechanism from the intestinal lumen to the body's defenses.[44] Dietary lectins are metabolic signals for the gut and modulate immune and hormone functions.[45] The interaction between dietary lectins and the gut are predictable and exploration of these for possible use in medical (nutritional) clinical practice is attractive.

Studies of the effect of diet restriction on the canine immune system are currently underway.[46] The overall pattern of data suggests that the effects of diet restriction on the immune system may differ, depending on gender.

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# Ingestion of Oxidized Lipids Alters Canine Anti-Oxidant Status and Immune Function

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A variety of environmental and normal and pathological metabolic conditions will result in the formation of reactive oxygen (ROS) and nitrogen species (Figure 1).[1] For example, molecular oxygen is reduced by oxidasis to form superoxide, a reactive free radical. Free radicals are atoms or molecules that contain one or more unpaired electrons. These free radicals can react with and damage proteins, nucleic acids, and lipids. As a protective mechanism, there are enzymes and

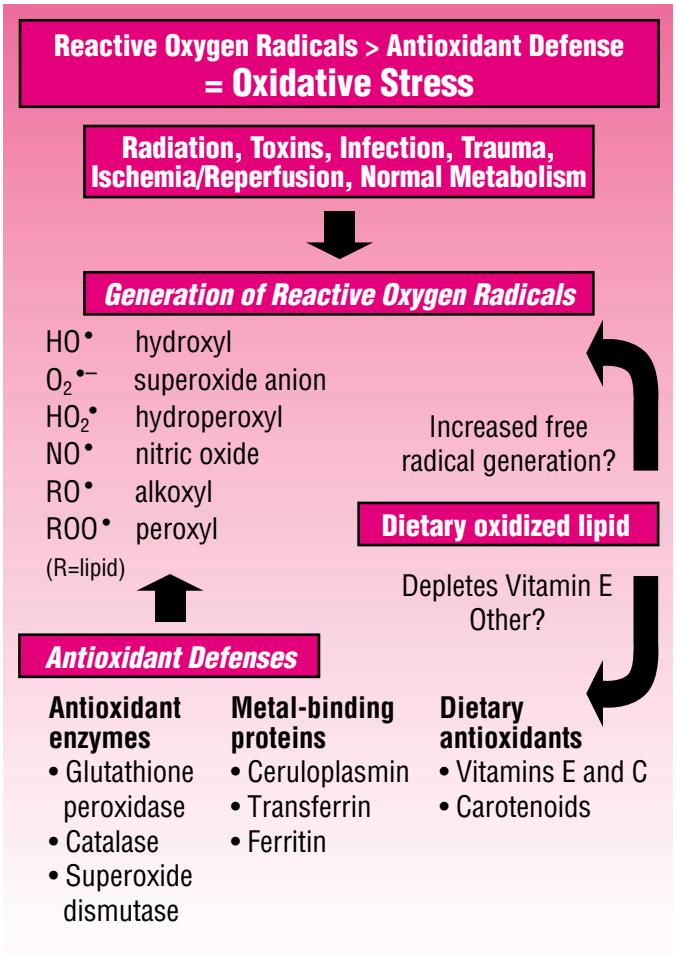


**Figure 1.** *Free radical generation and antioxidant defenses. A variety of environmental and biological processes can generate reactive oxygen species in the body. When the generation of free radicals is greater than the antioxidant capacity of the body, the result is oxidative stress. Dietary oxidized lipid contributes to oxidative stress via depletion (oxidation) of vitamin E and they may also enhance the production of free radicals.*

biomolecules that alter or trap these ROS to render them non-toxic. The superoxide anion is enzymatically dismutated to hydrogen peroxide, which is then broken down to water and oxygen by catalase. However, the generally accepted theory of free radical damage holds that some superoxide anions escape dismutation and drive an iron-catalyzed Fenton reaction, which ultimately results in the generation of hydroxyl radicals. There is no native scavenging system for hydroxyl radicals and these highly reactive molecules can remove a hydrogen atom from a fatty acid side chain in the cell membrane, which leaves an unpaired electron on a carbon atom. The carbon atom then reacts with oxygen to create a peroxyl radical, and the peroxyl radical in turn reacts with adjacent fatty acid side chains to form new carbon radicals. This lipid peroxidation is controlled by antioxidants such as vitamin E. Vitamin E provides a hydrogen atom with an unpaired electron that is able to pair up with the unpaired electron on the free radical, and trap the peroxyl radical and stop the chain reaction of lipid peroxidation.[2] In the process, however, vitamin E is oxidized. Currently, free radical damage to proteins, nucleic acids, and membrane lipids is thought to be a contributing factor in a variety of human diseases or conditions such as aging, cancer, atherosclerosis,

arthritis, inflammatory bowel disease, neurodegenerative disease, and some eye diseases.[3]

When there is an imbalance between the oxidants and antioxidants in favor of the oxidants, a condition of oxidative stress exists that can lead to tissue damage. Ingestion of oxidized lipids can contribute to oxidative stress by making additional demands upon the antioxidant system of the body. Dietary oxidized lipids are absorbed through the digestive tract and incorporated into membrane phospholipids[4] where they alter membrane fluidity.[5] Studies in chickens have demonstrated that feeding products with a low level of oxidation can suppress growth in broilers,[6,7] and highly oxidized lipids can cause a nutritional encephalopathy.[8] Another study in chickens demonstrated that feeding thermally oxidized lipids lowered vitamin E levels in tissues, and made the tissues more susceptible to peroxidation with iron-ascorbate.[9] Oxidized lipids thus increase the need for vitamin E, and animals deficient in vitamin E have increased plasma concentrations of lipid peroxidation products such as malondialdehyde.[10]

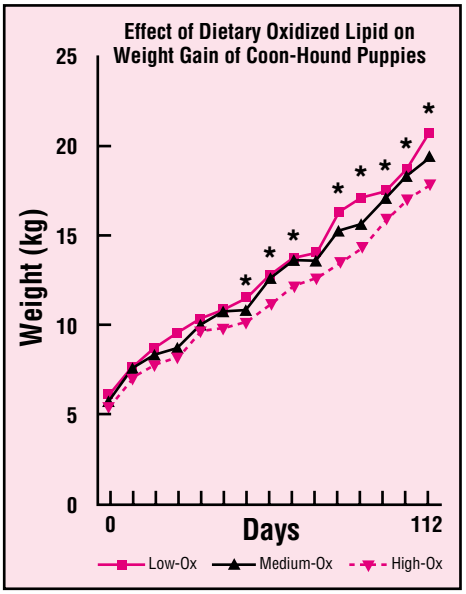


**Figure 2.** *Effect of dietary oxidized lipid on weight gain in coon-hound puppies. The asterisk (*) indicates a statistically significant difference between the low oxidation group and the high oxidation group at the P<0.05 level.*

Synthetic antioxidants such as ethoxyquin are effective in blocking oxidation reactions in food. Natural antioxidants like vitamin E are also effective in blocking oxidation, but they are less stable over time and are more susceptible to adverse storage conditions such as high heat.[11] There have been no adverse effects of ethoxyquin observed in short term feeding studies of chickens,[12] and indeed, there appear to be some benefits to their presence in the diet.[13,14] Ethoxyquin increased intestinal glutathione concentration in chickens fed either normal or oxidized fat.[13] This is particularly significant since one of the target organs for vitamin E depletion by oxidized fat appears to be the intestine.[9] However, *in vitro* studies have demonstrated that ethoxyquin can alter some metabolic reactions such as the inhibition NADH dehydrogenase in the mitochondrial respiratory chain and renal ATPase.[15] This has resulted in some controversy concerning the use of synthetic antioxidants such as ethoxyquin. Consequently, there has been a desire by some human consumers and pet owners for diets free of synthetic products such as ethoxyquin. However, diets formulated using natural antioxidants could potentially expose pets to higher levels of oxidation products, especially if the diets are stored for long periods or under less than optimal conditions.

The goal of the present study was to determine the effects of three levels of dietary oxidized lipid on the growth and immune system of growing dogs. Twenty-four, two-month-old purpose bred coon-hounds (mean weight of 5.7 kg) were randomly assigned to one of three groups. After a 2 week conditioning period, the dogs were pair fed one of three test diets for a period of 16 weeks. The diets contained lipid at 3 levels of oxidation: (1) low oxidation (LO) (control), targeted at < 50 PPM aldehydes, (2) medium oxidation (MO), targeted at 100 PPM aldehydes, (3) high oxidation (HO), targeted at 500 PPM aldehydes. All of the diets met or exceeded the NRC requirements for the dog. Fatty acid analysis of the diets indicated that the oxidation process decreased the concentration of 16:1n-7, 18:2n-6, and 18:3n-3 fatty acids and increased the concentration of 14:0, 18:1n-7, 18:0, 20:1n-9 and 22:6n-3 fatty acids.

At the start of the experiment, there was no significant difference in the mean body weight of the dogs of each group. However, after seven weeks there was a significant difference in body weight between the low and medium oxidation groups and the high oxidation group. This was maintained through the end of the study, and after 16 weeks the mean weights were as follows: LO, 20.43 kg; MO, 19.35 kg; HO, 17.7 kg (Figure 2). The body composition of the dogs was measured using dual energy x-ray absorptiometry (DEXA). There was no significant difference in bone mineral density between the groups or in the ratio of lean body mass to bone mineral content. However, the HO group had significantly less (5% less) body fat than the LO group. Bone biopsies from the iliac crest contained lower levels of the essential fatty acids 18:2n-6 and 18:3n-3 in the MO and HO group compared to the LO group.

Blood samples were collected from the dogs at the start of the experiment and after 8 and 16 weeks on the test diets. Analysis of the serum indicated that oxidation decreased the concentration of 18:2n-6 and 22:5n-3 fatty acids and increased 20:3n-6 fatty acids. Serum vitamin E levels decreased as oxidation levels in the diet increased (Figure 3). At 8 weeks the MO and HO groups had mean vitamin E serum concentrations that were 41% and 87% lower respectively than the low oxidation group. At 16 weeks, the MO group continued to have a serum vitamin E level 41% lower than the LO group,

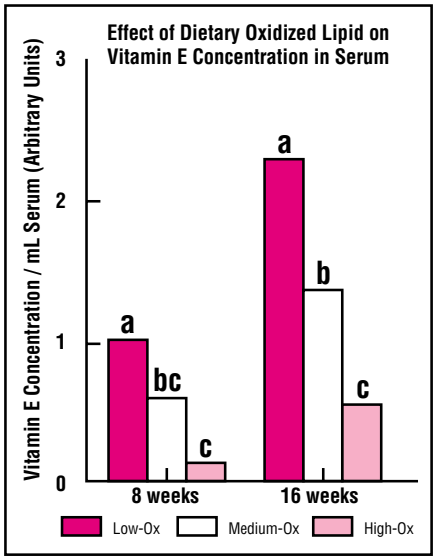


**Figure 3.** *Effect of dietary oxidized lipid on serum vitamin E concentrations. There was a significant linear trend for decreasing serum vitamin E concentration with increasing oxidation level in the diet. Mean values with a different superscript are significantly different (P<0.05).*

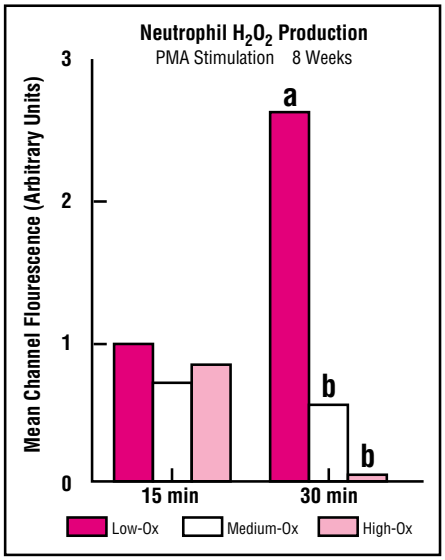


**Figure 4.** *Effect of dietary oxidized lipid on neutrophil hydrogen peroxide production in response to phorbol myristate acetate (PMA). Hydrogen peroxide production peaked at 15 minutes in dogs fed the medium and high oxidation diets, and did not increase thereafter. Mean values with a different superscript are significantly different (P<0.05).*

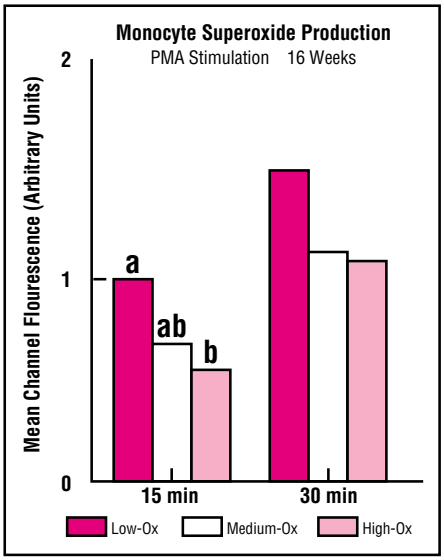


**Figure 5.** *Effect of dietary oxidized lipid on monocyte superoxide production. Superoxide production was significantly lower in the high oxidation group 15 minutes after phorbol myristate actetate (PMA) stimulation. After 30 minutes there were no significant differences between the groups but the mean levels of superoxide production were lower in the medium and high oxidation groups. Mean values with a different superscript are significantly different (P<0.05).*

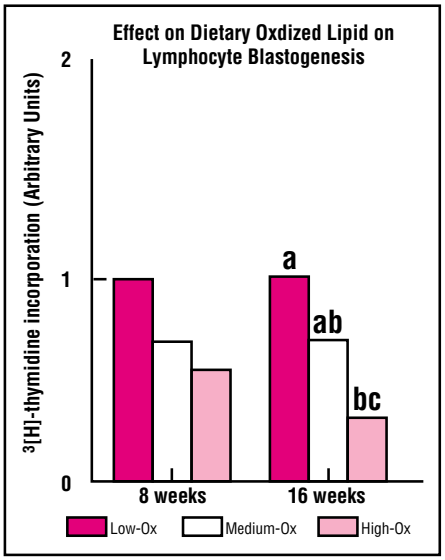


**Figure 6.** *Effect of dietary oxidized lipid on lymphocyte blastogenesis in response to concanavalin A. After 8 weeks there was no difference in mitogenic response, but by 16 weeks the response in the HO group was significantly lower than the LO group. Mean values with a different superscript are significantly different (P<0.05).*

and the HO group had a level 76% lower than that of the LO group. Serum osteocalcin levels were within normal parameters and ranged from 3.2 to 6.2 ng/ml with no differences between the groups. The blood clinical chemistry and hematology data were all within the normal value range for dogs and were not affected by the oxidized lipids. However, the HO dogs had significantly elevated platelet counts and decreased triglyceride levels compared to the LO dogs.

**After 8 weeks neutrophil production of hydrogen peroxide 30 minutes after phorbol ester stimulation was reduced by 79% in MO dogs and 99% in HO dogs compared to LO dogs.**

The oxidative burst capacity of the peripheral blood mononuclear cells was determined using flow cytometry. After 8 weeks neutrophil production of hydrogen peroxide 30 minutes after phorbol ester stimulation was reduced by 79% in MO dogs and 99% in HO dogs compared to LO dogs (Figure 4). Similarly, neutrophil superoxide was reduced 85% in MO dogs and 83.7% in HO dogs. By 16 weeks, there was a recovery of oxidative burst capacity in the neutrophil and there was no difference between the dietary groups. For the monocyte, there was no difference in oxidative burst capacity after 8 weeks, but after 16 weeks there was a significant linear trend for decreasing superoxide production with increasing oxidation level in the diet. The monocytes from HO dogs produced significantly less (29.4% less) superoxide than LO dogs 15 minutes after stimulation with phorbol esters (Figure 5). Hydrogen peroxide production in the monocyte was not significantly affected. Lymphocyte blastogenesis in response to concanavalin A was suppressed by the oxidized lipid. After 16 weeks, $^3$[H]-thymidine incorporation was reduced 17.5% (MO dogs) and 52% (HO dogs) compared to LO group (Figure 6). Monocyte production of tumor necrosis factor (TNF), interleukin-1 and interleukin-6 were also measured by bioassay. There were no significant differences in TNF or IL-1 production between the groups. After 16 weeks, the MO group produced significantly more IL-6 than the LO group.

## SUMMARY AND CONCLUSIONS

Dietary oxidized lipids reduced weight gain in the dog. The difference between the low oxidation group and the medium oxidation group was small, but the mean difference between the low oxidation group and the high oxidation group was significant. The difference in mean weight may be due in part to a difference in body composition. The HO dogs had significantly less body fat compared to the LO dogs. A skeletal difference between the groups was not apparent as measured by bone mineral density or the ratio of bone mineral content to lean body mass. It is unknown if the HO dogs would have compensatory weight gain and eventually reach the same mean weight as the LO and MO dogs at skeletal maturity.

The dietary oxidized lipids decreased the antioxidant status as measured by serum vitamin E levels. There was a significant linear trend for decreasing serum vitamin E levels with increasing oxidation level in the diet. This effect is similar

to what has been observed in chickens [9] and rats.[16]

In addition to altering the concentrations of different lipids in the serum, the dietary oxidized lipids also reduced the essential fatty acid concentrations of 18:2n-6 and 18:3:n-3 in bone. The consequences of this change in essential fatty acid content, especially in animals chronically exposed to oxidized lipid, remains unknown. However, these fatty acids are the parent compounds for arachidonic acid (AA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3) which are substrates for cyclooxygenase and lipoxygenase enzymes. Alteration of these essential fatty acid concentrations could therefore affect eicosanoid production in the bone.[17]

The dietary oxidized lipids altered various monocyte, lymphocyte, and neutrophil functions. Neutrophil superoxide and hydrogen peroxide production were impaired after 8 weeks, but appeared to recover by 16 weeks. Monocyte oxidative burst was unaffected at 8 weeks, but superoxide production was impaired at 16 weeks. Although there was no statistically significant difference in mitogenic response at 8 weeks, $^{3}$[H]-thymidine incorporation was lowest in the HO group. At 16 weeks lymphocyte blastogenesis was significantly reduced in the HO group. These results may be related to the half-life and turnover of the various cell types. The neutrophil has a shorter half-life and more rapid turnover in the body than the monocyte or lymphocyte. Consequently, any effects of the oxidized lipid would be expected to be evident earlier in the feeding trial. The same principle would apply for the longer lived monocytes and lymphocytes, with the oxidized lipid effect being evident at a later time point (16 weeks). However, the apparent recovery in neutrophil function by 16 weeks may indicate that with chronic exposure to oxidized lipid there is a physiological adaptation. If that is true, then monocyte and lymphocyte function may have eventually recovered. There is evidence that oxidative stress will alter gene expression in some cells via activation of NF-kappa B, an oxidative stress-responsive transcription factor.[18] Although there may be an adaptation to the oxidized lipid via altered gene expression or up-regulation of antioxidant defenses, our data indicates that there are acute effects on some immune system functions that could render a dog more susceptible to infection or other disease. Therefore, to maintain optimum health in our companion animals, minimizing oxidation products in the diet should be a definite objective.

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# Influence of Dietary Omega 6:3 Fatty Acid Ratio on the Immune Response of Young and Old Fox Terriers and Labrador Retrievers

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## INTRODUCTION

The interaction between dietary fat and the immune response has been an area of growing interest. Omega-3 (n-3) fatty acids have been used in veterinary medicine as supplements for pruritic patients to control the inflammatory response that is associated with the disease.[1-3] The influence of n-3 fatty acids on inflammation has been suggested to be through the ability of these fatty acids to be incorporated into cellular membranes and act as substrates for eicosanoid metabolism resulting in production of eicosanoids with lower inflammatory potential than those produced from omega-6 (n-6) fatty acids[4] (Figure 1). Recent studies have demonstrated that eicosanoid metabolism can be altered in the dog with dietary adjustments of n-6 to n-3 fatty acids.[5] These studies have led to widespread incorporation of n-3 fatty acids in commercial pet foods.

## OMEGA-3 FATTY ACIDS AND THE IMMUNE SYSTEM

Increased utilization of n-3 fatty acids has heightened the interest in the interaction between these fatty acids and the immune response. Studies examining this interaction have reported mixed results. While some studies have demonstrated an increase in T cell mitogen response due to n-3 feeding,[6,7] others reported a decline in T cell mediated immunity and production of interleukin (IL)-1, IL-2, IL-6 and tumor necrosis factor (TNF)-α.[8,9] In dogs it has been demonstrated that feeding diets containing an n-6:n-3 ratio of 1.4:1 results in a decline in delayed type hypersensitivity responsiveness (DTH), which is an *in vivo* measure of T cell immunity.[10] Feeding diets containing n-3 fatty acids has been reported to result in decreased serum vitamin E concentrations and increased serum lipid peroxidation markers.[8,10] These changes have been suggested to contribute to the decline in T cell responsiveness in the presence of n-3 fatty acids. Studies have suggested that the

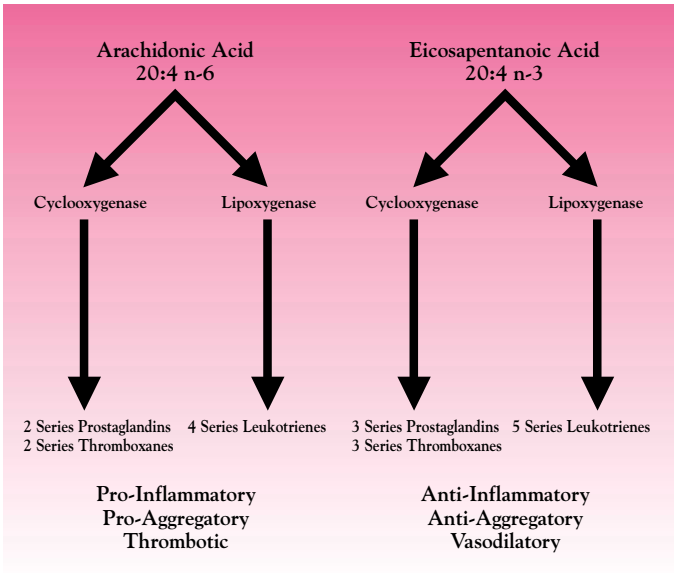


**Figure 1.** *Eicosanoid metabolism from membrane n-6 or n-3 fatty acids*

negative aspects of n-3 fatty acid supplementation may be negated by vitamin E supplementation.[11]

A negative interaction between n-3 fatty acids and the immune response would be of particular concern for the geriatric population. The negative influence on the immune response may be more pronounced in the elderly.[6] The aging process has been associated with a decline in immune responsiveness in a variety of species. The age-associated changes in the immune response have mainly been attributed to a decrease in responsiveness to T cells.[12,13] A decline in immunological vigor has been demonstrated in the dog. Laboratory studies have observed a decrease in mitogen stimulation,[14,15] chemotaxis, and phagocytisis.[16] A recent longitudinal study has reported age-related changes in clinical immunological parameters such as decreased numbers of white blood cells and immature neutrophils and increased numbers of mature neutrophils and concentrations of immunoglobulin G.[17] Together, these data demonstrate an age-related decline in immunity in dogs that may increase the susceptibility of the geriatric animal to infection.[18]

Few studies have been conducted to examine the influence of various n-6:n-3 ratios on the immune response of young and old dogs.[10] The purpose of this study was to examine the influence of diets formulated to contain n-6:n-3 ratios of 25:1 or 5:1 on the immune response and oxidative status of young and old Fox Terriers and Labrador Retrievers. A secondary objective of this study was to determine if the effect of age on the immune response differs between different breeds.

## CANINE STUDY

Eighteen young (9 Labrador Retriever, mean age 1.5 years; and 9 Fox Terrier, mean age 1.8 years) and 18 old (9 Labrador Retriever, mean age, 9.6 years; and 9 Fox Terrier, mean age, 11.5 years) dogs were utilized for this study. Dogs were fed a basal diet (25% protein, 12% fat) containing an n-6:n-3 ratio of 25:1 for 8 weeks. Half of the dogs were switched to a diet containing an n-6:n-3 ratio of 5:1 and all dogs were fed their respective diets for the following 8 weeks. Omega-3 fatty acids were supplied in the diet with equal concentrations of α-linolenic acid, eicosapentanoic acid, and docosahexanoic acid. Blood was drawn twice at the end of each respective feeding period for determination of plasma fatty acid profiles, plasma vitamin E levels, plasma oxidative stress markers malondialdehyde (MDA), and 4-hydroxynonenal (4-HNE). Peripheral blood mononuclear cells (PBMC) were isolated from blood and were examined for proliferative response to three concentrations of Concanavalin A (Con A), Phytohemagglutinin A (PHA) and Pokeweed mitogens (PWM). Macrophages from the peritoneal cavity were isolated and stimulated for the production of cytokines (IL-1, IL-6 and TNF-α and eicosanoids. Cytokine levels were determined by bioassay and eicosanoids via GCMS. At the end of each feeding period DTH was tested with PHA. At the end of the study dogs were challenged with sheep red blood cells and antibody titers were determined on days 7, 14, and 21.

Age and breed differences were noted for the percentage of certain serum fatty acid levels during the 25:1 feeding period.[19] At baseline it was noted that arachidonic acid levels were higher in serum of old Labrador Retrievers and lower in old Fox Terriers when compared to their young counterparts. Conversely, eicosapentanoic acid was lower in old Labrador Retrievers and higher in old Fox Terriers (Figure 2). After switching half of the dogs to the 5:1 diet, a breed by diet interaction for serum levels of α-linoleic acid, arachidonic acid, eicosapentanoic acid, docosahexanoic acid, total n-3 and n-6 fatty acids was noted. Data for n-3 and n-6 fatty acids are presented in Figure 3. The general trend was increased n-3 fatty acids and decreased n-6 fatty acids. However, the degree of these changes was greater in the old or young Fox Terriers than the Labrador Retrievers. These data may suggest that metabolism of fatty acids is influenced by both age and breed.

Several parameters of the immune response were assessed in this study. *In vivo* immune responsiveness was measured by DTH response to PHA. Humoral immunity was measured by the dog's ability to produce antibody titers

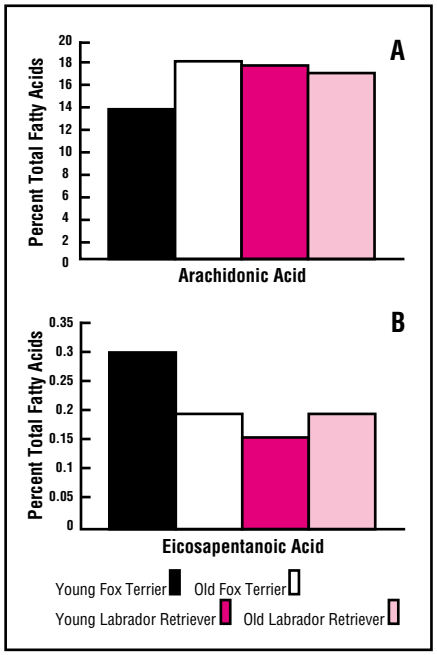


**Figure 2.** *Serum arachidonic acid (A) and eicosapentanoic acid (B) levels in young and old Fox Terriers and Labrador Retrievers fed a diet containing an omega-6:omega-3 ratio of 25:1. Data expressed as percent total fatty acids.*

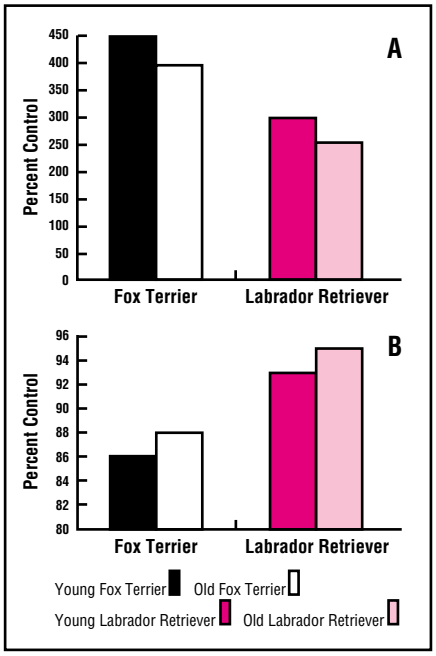


**Figure 3.** *Serum total n-3 (A) or n-6 (B) concentrations in young and old Fox Terriers and Labrador Retrievers fed diets containing n-6:n-3 ratios of 25:1 or 5:1. Data expressed as percent control (serum fatty acid percent in 5:1 diet/serum fatty acid percent in 25:1 diet x 100).*

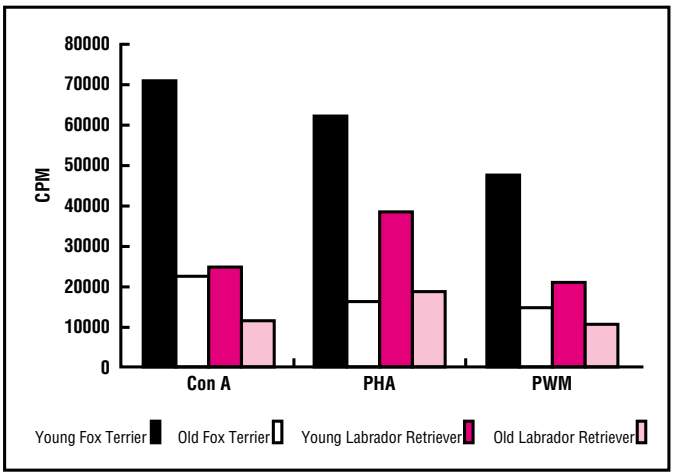


**Figure 4.** *Effect of age and breed on T and B cell mitogen. Blood was collected from young and old Fox Terriers and Labrador Retrievers (n=per age per breed). 1.0 x $10^5$ cells were isolated from peripheral blood, incubated for 54 hours in the presence of Concanvalin A (Con A), phytohemagluttinin A (PHA) and pokeweed mitogen (PWM), pulsed with $^3$[H]-thymidine for 18 hours and harvested. Data is expressed as counts per minute (CPM). (Reprinted with permission).*[23]

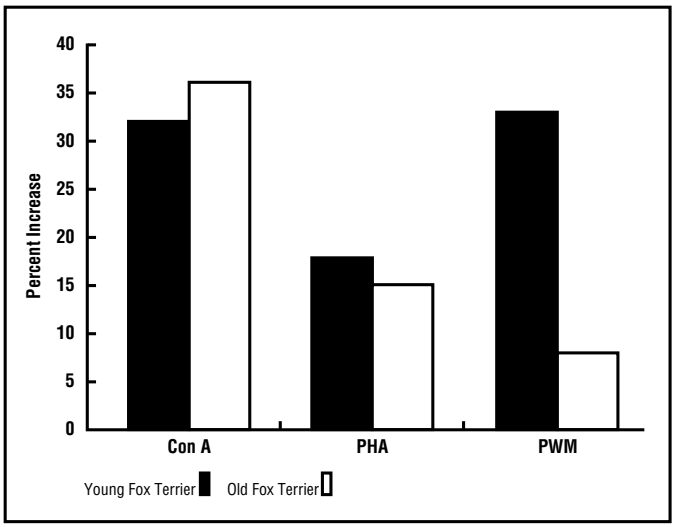


**Figure 5.** *Effect of diet on T and B cell mitogen response in young and old Fox Terriers and Labrador Retrievers. Culture conditions were the same as explained in the Figure 4 caption. Percent increase = [(average cpm of 5:1 cultures – average cpms of 25:1 cultures)/ average cpms of 25:1 cultures] x 100.*

to sheep red blood cells. T cell and B cell activity was measured by the PBMC's ability to proliferate to mitogens Con A and PHA (T cell activation) or PWM (B cell activation). The macrophage's ability to produce cytokines was assessed by IL-1, IL-6 and TNF-α production.

Examination of these data at baseline revealed an age-associated decline in several of these immune parameters. While DTH response and cytokine production did not differ with age, sheep red blood cell titers were significantly lower in old dogs on days 7, 14, and 21. Both breeds demonstrated an age-associated decline in their ability to respond to the different mitogens (Figure 4). It is interesting to note that the degree of decline was greater for the Fox Terriers than the Labrador Retrievers (-216% vs -114%; -292% vs -106%; -234% vs -102% Fox Terriers vs Labrador Retrievers, ConA, PHA, and PWM, respectively). Also, thymidine incorporation was greater in lymphocytes isolated from Fox Terriers than Labrador Retrievers. This suggests a decrease in cellular proliferative capacity in lymphocytes isolated from the larger breed. A similar reduction in cellular proliferative capability in large canine breeds has been reported using skin fibroblasts.[20]

**Both breeds demonstrated an age-associated decline in their ability to respond to the different mitogens.**

Feeding a diet containing an n-6:n-3 ratio of 5:1 did not affect IL-1, IL-6 or TNF-α production from peritoneal macrophages or peripheral blood mononuclear cells. Omega-3 supplementation also did not influence sheep red blood cell antibody titer response. Feeding a diet containing a ratio of 5:1 resulted in an increase in T cell and B cell mitogen response (Figure 5). Together these data suggest that feeding a diet containing an n-3 fatty acid ratio of 5:1 does not have a negative effect on the canine immune response.

Previous research has demonstrated that feeding a diet containing an n-6:n-3 fatty acid ratio of 5:1 resulted in lower production of $LTB_4$ and higher production of $LTB_5$ in neutrophils and skin compared to dogs fed a 25:1 diet.[5] To determine if feeding a 5:1 ratio affects eicosanoid production from immune cells, peritoneal macrophages and peripheral blood cells were stimulated with LPS to generate prostaglandin production. Neither age nor diet affected $PGE_2$ or $PGE_3$ production from either macrophages or PBMC.

Feeding dietary n-3 fatty acids has been suggested to increase the oxidative stress of the animal, possibly by decreasing vitamin E levels in the blood and tissue.[21] Oxidative stress was assessed by measuring MDA and HNE. Both of these compounds are by-products of arachidonic acid metabolism and serve as markers for oxidative status of animals.[22] Neither MDA nor HNE were affected by diet. Serum vitamin E levels were not affected by diet but it was observed that levels were 12% lower in the serum of old dogs compared to young dogs. It should be noted that these parameters were corrected for total lipid concentrations (triglycerides + cholesterol) since total serum lipids were observed to be higher in older dogs.

## SUMMARY AND CONCLUSIONS

The main conclusion from this study was that feeding a diet containing an n-6:n-3 ratio of 5:1 did not have a negative effect on the immune response of young or geriatric dogs. If anything, there was an increase in T and B cell activation in the cells isolated from the dogs fed a 5:1 n-6:n-3 ratio. Several interesting breed and age differences were observed in this study. First, the metabolism of n-3

fatty acids appears to be differentially regulated by breed and age in the dog. These breed-associated differences in fatty acid metabolism may explain why not all atopic dogs respond to fatty acid therapy. This suggests that consideration of specific dietary fatty acid profiles may be warranted for different breeds and stages of life. Secondly, T and B cell proliferative ability appears to be lower in Labrador Retrievers than Fox Terriers. How this effects the health of these two breeds will require further study.

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# Developing Characteristics of CD4+ and CD8+ Populations in the Pediatric Dog and Cat

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## INTRODUCTION

Our understanding of the immune system has advanced dramatically over the past decade, thanks to significant advances in biotechnology and the impetus provided by the threat of human AIDS. In almost every immunological study, the effect of age must be considered, particularly in geriatric and pediatric subjects. Cellular and humoral immune responses of the juvenile mammal are known to become more effective over time as maturity approaches,[1-4] while a decline in immune function has been documented in several species in advanced ages.[5-11]

The effect of advanced age on the immune system (immunosenescence) is generally characterized by depressed helper T-cell activity, and changes in signal transduction.[4,11-13] Several investigators have published results that indicate this change may be due to both functional changes of immune cells as well as a shift in phenotype subsets.[10,14] Functional changes include reports of changes in patterns of cytokine gene expression,[14] a decline in cytotoxic activity,[6] and decreased proliferative responses to mitogen stimulation.[12] Age-related T-cell dysfunction in particular is associated with increased autoimmunity and tumor incidence.[4] Age-related subset changes in various species include clonal expansion of $CD4^+$ cell clones, and variable changes in $CD4^+$, $CD8^+$, $\gamma\delta^+$, and/or B cell populations.[4,9,15] The reported characteristics of immunosenescence also differ according to gender, species, breed or race.[9] These confounding parameters make it impossible to make broad sweeping assumptions from human or mouse studies and apply them to the dog and cat.

Similar information concerning the developing immune system in the neonatal and pediatric patient is not as complete. Though it is generally accepted that cellular immunity improves from birth to maturity, little is known about lymphoid maturational changes in the dog or cat specifically. Our knowledge concerning lymphocyte distribution, and the development of CD4 or CD8 receptor expression in the major lymphoid organs in the dog and cat is largely extrapolated from murine and human studies. The acquisition of such species specific knowledge would provide clinicians valuable information concerning the effects of immaturity on the *in vivo* and *in vitro* analysis of the immune system.

## LYMPHOCYTE DEVELOPMENT

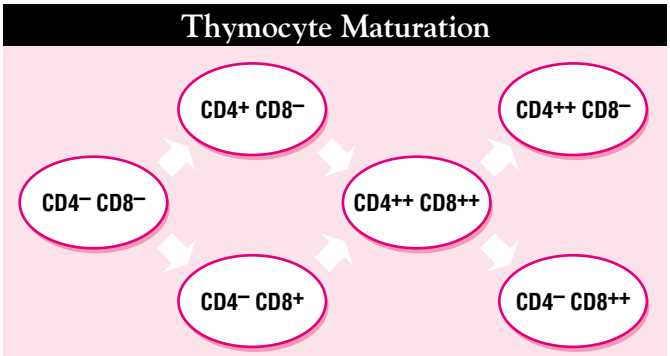
Some knowledge of normal lymphocyte development is a prerequisite for any investigation concerning the normal proportions and/or distributions of lymphocyte phenotypes. It is important to understand that particularly in pediatric patients, the expression of certain markers may be age- and tissue-dependent.

Present knowledge of lymphocyte development suggests that most T-cell development and instruction occurs in the thymus.[16-21] At least in the human fetus, thymic precursors initially reside in the fetal liver and migrate via the blood to seed the thymus during the first 8 weeks of gestation.[22] During the sixteenth week of gestation the fetal liver colonizes the bone marrow and approximately 6 weeks later, thymic precursors are derived from the bone marrow. These bone marrow derived, thymic precursors are generally assumed to have the capability to develop into either T-cells, natural killer cells, or dendritic cells. These cells can also develop into B cells in the mouse.[22]

A small number of B cells have been reported to exist in the thymus of normal mice and human cells.[23] Though these cells are generally progenitor cells, it has been reported that they can mature in the thymus, and do not migrate peripherally.[23] The developmental pathways resulting in a mature B-cell in the thymic environment is unknown. It is speculated that they play some role in antigen presentation to T-cells, and may be involved in negative selection.[23,24]

With respect to the CD4 and CD8 receptors, the $CD4^{-}CD8^{-}$ precursor T-cells differentiate and mature to a $CD8^{+}4^{low}$ or $CD4^{+}8^{low}$ cell. The cell then becomes $CD8^{+}4^{+}$ double positive (DP). Most DP thymocytes express a T-cell receptor (TCR) that cannot recognize self major histocompatibility complex (MHC), and are sentenced to a programmed cell death within 3-4 days. Those DP thymocytes that are self-MHC restricted are positively selected to develop into functionally mature $CD4^{+}$ or $CD8^{+}$ single positive (SP) cells[25,26] (Figure 1).

The progressive expression of the CD4 and CD8 co-receptors in the maturing lymphocyte represent a factor that must be considered when ascertaining subpopulations of the thymus and, possibly, other lymphoid organs in the immature mammal.




Phenotypic changes during normal maturation of the thymocyte as they are described for the CD4 and CD8 molecules in the mouse and human.

**Figure 1.** *Graphic representation of CD4 and CD8 receptor expression during lymphocyte development in the thymus of mice and humans.*

## LYMPHOCYTE SUBSET DISTRIBUTION

### Peripheral Blood

**Canine:** In puppies 1-9 weeks of age, T-cell percentages gradually increase so that by 9 weeks of age T-cells comprise nearly 80% of the lymphocyte population whereas B-cells comprise approximately 10-15%.[27,28] This pattern is paradoxically similar to that seen in the aged human in peripheral blood lymphocytes.[28] As the dog ages from 2 to 6 years there is a reported increase in T-cells accompanied by an increase in the percentage of $CD8^{+}$ lymphocytes, while B-cells decreased during the same period.[9]

**Feline:** Cytometric analysis of T-lymphocyte subsets in adult SPF and random source cats reveal that T-lymphocytes, $CD4^{+}8^{-}$ cells, $CD8^{+}4^{-}$ cells, and $Ig^{+}$ (or B) cells comprise 21-64%, 6-33.9%, 19-23% and 23-45% of the peripheral lymphocyte population, respectively.[29-31] There were no significant differences between the two sources of cats (SPF vs. random source) in the study by Dean et al. Reported ranges for peripheral blood $CD4^{+}$ and $CD8^{+}$ population numbers in adult random source and SPF cats are approximately 900-2600 and 700-1000 cells/μL, respectively.[32,33]

Normal SPF kittens have quite different blood lymphocyte profiles. In kittens (0-8 weeks of age) lymphocyte subset populations (T-lymphocytes, $CD4^{+}8^{-}$ cells, $CD8^{+}4^{-}$ cells, and $Ig^{+}$ (or B) cells) are reported to change over time from 17 to 50%, 25 to 35%, 6 to 10%, and 31 to 45%, respectively.[34] As in the dog, feline lymphocytes with pan T-cell markers increase in number and proportion during the first 8 weeks of life. In contrast to canine reports, feline peripheral blood T-cell subset populations increase in total numbers over the first 8 weeks of age, while Ig+ cells also increase in number and proportion from birth to 4 weeks of age.[34] These T-cell changes are similar to changes in the aged human and the early post-natal canine. However, peripheral blood B-cell dynamics appear to differ in the kitten from that exhibited by the puppy.

### Thymus

**Canine:** Canine values (FACS) of lymphocyte subset distributions in the thymus of dogs 4-8 weeks of age have been reported as follows: T-cells, 95%; $CD4^{+}8^{-}$ cells, 10%; $CD8^{+}4^{-}$ cells, <3%; $CD4^{+}8^{+}$ cells, 75%; and $CD4^{-}8^{-}$ cells, 10%.[27] Using immunohistochemical techniques on adult dogs, Rabanal et al. reported that 90% of thymic lymphocytes were T-cells, whereas an anti- B-cell monoclonal antibody recognized 5% of lymph cells near trabeculae. $CD4^{+}$ and $CD8^{+}$ cells were described in the cortex and in the medulla at a proportion of 3:2 respectively. It should be noted that the anti-CD4 antibody also reacted with cells thought to be thymic epithelial cells.[35]

**Feline:** Feline lymphocytes with T-cell properties (rosettes) and/or markers make up 35-45% of thymic lymphocytes, while 2.9% are B-cells. $CD4^{+}8^{-}$ and $CD8^{+}4^{-}$ cells have been found to comprise 52% and 63-76% of thymic lymphocytes, respectively (reviewed by Lin).[30] The finding of B-cells in the canine and feline thymus is consistent with reports that small numbers of B cell progenitors ($sIg^{-}$, $B220^{med}$, and $CD43^{+}$) have been found in the thymus of mouse and humans.[23]

### Other Lymphoid Organs

Similar information is not available for bone marrow and/or other secondary lymphoid organs during the early post-natal period. The need for characterizing the canine and feline immune systems is important if appropriate immunological models of human diseases are to be identified, or for pathogenic investigations of the effect of disease on the immune system, or if immunologic evaluations of novel therapies are to be studied.[27,28,36-38] Using 2-color FACS analysis, we have conducted studies to determine the T-cell, $Ig^{+}$, CD4, CD8, and CD48 populations of secondary lymphoid tissues during the first 9 weeks of age and during adulthood.

**Canine:** Recent studies in our laboratory indicate there is a 2:1 B-cell to T-cell ratio in the canine spleen.[39] Adult values for T-cells (14%), $CD4^{+}$-cells (17%), and $CD8^{+}$-cells (8%) were generally higher than pediatric values from 1 day to 9 weeks of age. Interestingly, the $CD4^{+}8^{+}$ (15%) double positive cell was found at all ages tested, including adults.

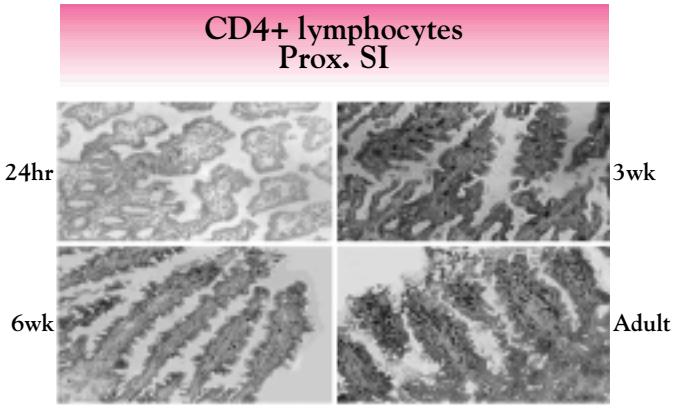



**Figure 2.** *Representative photomicrographs of villi stained with anti-CD4 monoclonal antibody. Samples shown were taken from dogs at 24 hours, 3 weeks, 6 weeks and adult ages. The dark staining cells are CD4+ cells.*

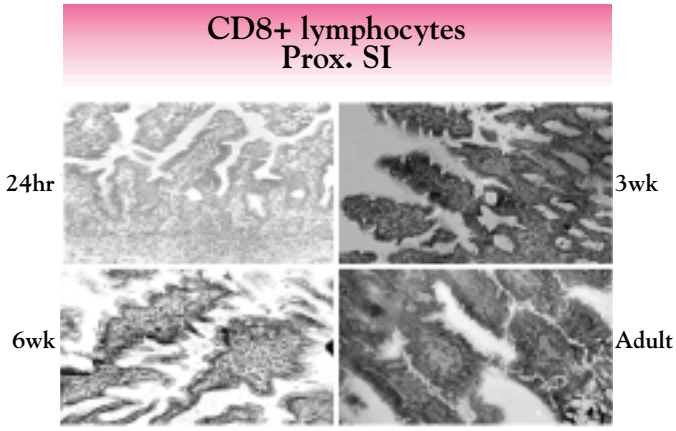



**Figure 3.** *Representative photomicrographs of villi stained with anti-CD8 monoclonal antibody. Samples shown were taken from dogs at 24 hours, 3 weeks, 6 weeks and adult ages. The dark staining cells are CD8+ cells.*

The canine mesenteric and popliteal lymph nodes exhibited similar lymphoid makeup during the neonatal and adult periods. Both organs demonstrated an increase in T-cells (5% to 57%), $CD4^+$-cells (11% to 40%), and $CD8^+$-cells (4% to 18%) between 1 day of age and adulthood. By 9 weeks of age, lymph node T-cell subpopulation values were found to be approximately half (54%) of adult values. $Ig^+$ populations remain relatively constant (~35%) between birth and adulthood in both lymph nodes.

**Feline:** Lymphoid subpopulation changes in the spleen, mesenteric lymph node and popliteal lymph nodes during the first 9 weeks of age are similar in the cat and dog. Exceptions are noted below.

The B:T ratio in the feline spleen is approximately 5:1. The splenic CD4:CD8 ratio is often inverted due to a greater percentage of CD8 lymphocytes. Finally, the T-cell population of feline lymph nodes (mesenteric and popliteal) were generally lower at all ages (12%-20%) when compared to age matched canine values.

## LYMPHOCYTE DISTRIBUTION IN THE GI TRACT

Several immunohistological studies of the immune system have been devoted to the description of lymphocyte subset distribution in the intestines of various species.[35, 40-45] Results have been generally reported by phenotype (CD4, CD8, IgA, etc.) and their structural location (villi vs. crypts, lamina propria vs. epithelium, follicle vs. inter-follicular area, small intestines vs. large intestines). The recognition of normal distribution patterns should provide clues to functional mechanisms of defense, and abnormal distribution in disease should shed some light on immunopathologic mechanisms.

In humans, T-cells comprise up to 15% of cells in the intestinal epithelium and over 40% in the lamina propria.[46] IELs are predominantly $CD4^-CD8^+$ whereas, at least in the human, T-cells in the lamina propria express CD4 (60%) or CD8 (40%) molecules similar to that in peripheral blood.[45]

Canine studies of the gut using immunohistochemical staining have been reported in dogs aged from 2 months to 13 years.[35,42,43,47] Follicles of the gastric mucosa were reported to have an organized distribution of lymphocytes with a predominantly B-cell area containing some $CD4^+$ and $CD8^+$ cells. Follicles in the fundus and body regions possessed similar percentages of lymphocytes averaging 42% (B-cells), 22% ($CD4^+$), and 3% ($CD8^+$). The predominance of $CD4^+$ T-cells is similar to that reported for jejunal PP in the lamb.[48] Age related changes in lymphocyte distribution were not found.[42]

In another investigation (HogenEsch et al.), regional differences in morphology and lymphocyte distribution within canine Peyer's patches (PP) were reported. The distinct types of canine PP is similar to that known to exist in lambs, calves, and pigs. Duodenal and jejunal PP contain more T-cells than the ileal PP. However, the analysis performed in this study examined the distribution of immunoglobulin isotypes within the domes of PP. The plasma cells in the canine lamina propria of the distinct PP were $IgA^+$, while the plasma cells in the domes of the PP were principally $IgG^+$.[47] Lymphocytes in the paracortical and interfollicular areas of PP are predominantly T-cells that express the CD4 molecule (70-80%), whereas a minority of the T-cells express the CD8 molecule (20-30%).[35]

Using immunohistochemical techniques, we have been able to determine that $CD4^+$ and $CD8^+$ distribution in the gastrointestinal mucosa of the juvenile canine is similar to that of humans and pigs.[49] Specifically, recent investigations have demonstrated that small intestinal villi have very few $CD4^+$ or $CD8^+$ cells at 1 day of age, and that the number increases dramatically during the first 3 and 6 weeks of age for each phenotype respectively. Additionally, as noted in other species, $CD4^+$ cells are almost exclusively located in the LP of the villus when compared to IEL's. However, though the predominant phenotype of the IEL was $CD8^+$, numerous $CD8^+$ lymphocytes were found in the LP as well. (Figures 2 and 3)

## LYMPHOPROLIFERATIVE RESPONSES TO MITOGENS

The lymphocyte blastogenesis assay measures the

degree of cell replication (DNA synthesis) after mitogen stimulation. Mitogens used in lymphocyte blastogenesis assays deliver a signal that is transmitted across the membrane via the release of two messenger molecules, diacylglycerol and inositol 1,4,5-tris-phosphate ($IP_3$), involved in the activation of protein kinase C (PK-C) and the increase in cytosolic free $Ca^{2+}$. The ability of the lymphocyte population to replicate in response to mitogen stimulation is used as an indirect measure of the intrinsic ability of the lymphocyte to respond to antigen.

*In vitro* response to mitogen stimulation appears to be affected by the tissue source. Human lymphocytes collected from intestinal mucosa have shown a depressed response to mitogen stimulation (reviewed by Khoo et al.).[46] However, discounting possible species differences, age may have a more profound effect on mitogen response capability in that IELs from 4-week-old calves are capable of blastogenic responses and interferon-γ production at levels similar to peripheral blood lymphocytes.[50] This is inconsistent with the expectation of depressed mitogen response noted in IELs of mature humans,[46] and underscores the importance of potential age effects.

Lymphoproliferative responses to mitogens have been found to be significantly depressed in older dogs when compared to young or middle aged dogs. The mean CPM responses of older dogs to Concanavalin A (Con A), staphyloccal enterotoxin B, phytohemagglutinin (PHA) and pokeweed mitogen (PWM) were found to be significantly depressed when compared to that of younger dogs.[9] This data is consistent with age-related data noted in other species.

Feline lymphocytes respond to Con A, PHA, PWM, and marginally respond to lipopolysaccharide (LPS).[30,51] Feline lymphocytes have been found to be sensitive to the induction of apoptosis when cultured overnight in medium.[52] Holznagel et al. suggests this sensitivity to apoptotic induction may be based on the high constitutive expression of MHC II (indicating activation) on a B cell subpopulation. The loss of such cells may explain the limited response of feline lymphocytes to lipopolysaccharide stimulation.

Using ConA, PHA, PWM, and LPS to stimulate lymphocytes obtained from juvenile cats of various ages, we have been able to determine the mitogen response effects exerted by both tissue source and age. Generally, our findings suggest that both tissue source and age affect the feline lymphocyte's capability to respond to mitogenic stimuli. For example, thymic lymphocytes demonstrated significant mitogenic responses to mitogens (including LPS) during the first 9 weeks of age, while becoming non-responsive to virtually all mitogens during adulthood. In contrast, lymphocytes from the mesenteric and popliteal lymph nodes demonstrated limited response to mitogenic stimulation at birth (ConA) and became consistently reactive to ConA, PHA, and PWM during adulthood (Figure 3).

## DISCUSSION

The data cited in this article suggest that the development and age-related change of lymphocyte subset populations in various lymphatic organs may reflect species specific patterns. Using two-color FACS analysis, we have determined that peripheral blood subpopulation values are similar in the dog and cat, although $CD4^+$ populations were generally higher in the dog. Feline peripheral blood lymphocytes were consistently responsive to ConA (1 day to adult) and PWM (6 weeks to adult) stimulation.

**Both the canine and feline spleen are predominantly B-cell lymphoid organs.**

Both the canine and feline spleen are predominantly B-cell lymphoid organs. Within the T-cell population, however, the spleenic CD4:CD8 ratios differ markedly between the two species. The canine spleen in particular was found to contain a persistent population of the immature $CD4^+CD8^+$, even in the adult. Considering the low number of mature T-cells identified in our study, and the presence of the immature double positive cell, the canine spleen may serve as an extra-thymic site of T-cell maturation. Feline spleenic lymphocytes exhibited variable responses to the mitogens tested. Generally, by 3 weeks of age spleenic lymphocytes were found to be responsive to all mitogens tested,

**Summary of Lymphocyte Blastogenesis**

● ConA ● PHA ● LPS ● PWM

| | 0 hr | 24 hrs | 3 wks | 6 wks | 9 wks | Adults |
|---|---|---|---|---|---|---|
| Blood | | ●● | ● | ●●● | ●● | ●● |
| Bone Marrow | | | | | | ●● |
| Mes LN | ● | ● | ● | ●●●● | ●●● | ●●● |
| Pop LN | | | ● | ● | ●● | ●●● |
| Spleen | ●● | ●●● | ●●●● | ●● | ●● | ●●● |
| Thymus | ●● | ●● | ●● | ●● | ● | |

**Figure 4.** *Lymphocytes were isolated from feline organs listed in the table and stimulated with various concentrations of ConA, PHA, LPS, and PWM in triplicate. Each oval indicates when significant stimulation indexes were obtained when compared to control (non-stimulated) samples of similar origin. Samples were obtained from 3-5 cats for each age represented.*

including LPS. However, significant stimulation indexes could only be acquired with ConA and PHA in adult cats.

The canine mesenteric and popliteal lymph nodes are predominantly T-cell organs whereas, the corresponding feline lymph nodes were found to be comprised of equivalent percentages of T- and B-cells by the time the animal becomes an adult. In the dog mesenteric and popliteal lymph nodes and the cat popliteal lymph node, the $CD4^+$ and $CD8^+$ cell populations increase gradually with age. The resulting CD4:CD8 ratio is 2:1 for the respective lymph nodes in each species. However, in the mesenteric lymph node of the cat, the CD4-cell population is decreased in the adult by as much as 40% from levels measured at birth. The different subpopulation dynamics in the two species result in an adult CD4:CD8 ratio of 1:1 in the cat. These findings in the cat are consistent with the mitogen stimulation results obtained on samples from the same tissues. During the first 6 weeks of life, lymphocytes from these lymph nodes were responsive only to ConA. Samples from adult cats continued to be responsive to all mitogens except LPS.

Generally, age was found to affect T-cell numbers in various organs (blood, mesenteric lymph nodes) exhibiting a gradual increase in numbers, while B ($Ig^+$) cells remained relatively constant in number from birth to adult. T-cell numbers and the T-cell subpopulations do not reach adult values by 9 weeks of age, indicating immaturity of the lymphoid organs (at least in reference to population percentanges). B-cell numbers remained constant from birth onward (blood, mesenteric lymph node, popliteal lymph node).

Immunohistochemical evaluation of the canine small intestines during the first 9 weeks of life indicates dramatic changes in the first 9 weeks of life with reference to CD4 and CD8 populations. Still, as with the FACS analysis of other lymphoid organs, the canine gut associated lymphoid tissues appear to be immunologically immature at 9 weeks of age. As reported in humans and pigs, the CD4-cell seems to be confined to the lamina propria of the villus while the CD8-cell is found as an IEL as well as in the villus LP.

**The influence of age, gender, species, breed, and organ of interest present a complex problem in describing the immune system.**

The influence of age, gender, species, breed, and organ of interest present a complex problem in describing the immune system. The problem is further complicated by the use of various agents, techniques, and cell lines when different investigators seek to characterize a particular immunologic response. These factors provide ample justification for delineation of various immune parameters and processes specific for the dog and cat. Animal models of human diseases that affect the immune system are often based on similar phenotypic expression in the immune response. However, immunophenotypic differences may simply reflect operational differences between two species (as evidenced by the canine and feline spleen). Utilizing newly developed agents and biotechniques, it is now possible to more definitively describe the canine and feline immune systems, and potentially provide insight that may result in the identification of new animal disease models.

The need for characterizing the canine and feline immune systems is important if appropriate immunological models of human diseases are to be identified, or for pathogenic investigations of the effect of disease on the immune system, or if immunologic evaluations of novel therapies are to be studied.[27, 28, 36-38] The complex effects of species, age, and gender on various immunologic measured responses further strengthens this need.

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# Effect Of Fermentable Fiber On The Canine Gastrointestinal Immune System

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The gastrointestinal tract in dogs continually encounters enormous antigenic stimuli in the form of both food and microbes. It is, therefore, critical that protective immune responses are made to prevent the entry of potential pathogens and equally important that hypersensitivity responses to dietary antigens are minimized. There are many important non-immunological barrier defenses in the gut. These include: the anatomy of the gut (intact microvillus and tight junctions between cells), peristalsis and mucus which make it difficult for bacteria/virus to attach or enter cells, the low pH of gastric secretions and the digestive and bactericidal enzymes secreted by the stomach, pancreas, and epithelial cells that inhibit the attachment and growth of bacteria.[1]

## THE GUT-ASSOCIATED IMMUNE SYSTEM

Despite the non-immunological barriers in the small and large intestine, under normal circumstances, small amounts of immune-active antigens (usually proteins) cross the epithelium. The intestine is protected by an extensive immune system that make it the largest immune organ in the body.[2-3] Although there is little anatomical data available, it is often stated that the gut associated lymphoid tissue (GALT) represents approximately 40% of an animals' immune effector cells,[2] 80% of the body's immunological secreting cells,[4] and accounts for as much as 25% of mucosal mass.[2] GALT (Figure 1) is composed of the cells residing in the lamina propria regions of the gut, those interspersed between epithelial cells (intraepithelial lymphocytes; IEL) and the immune cells residing in organized lymphatic tissue such as Peyer's patches and mesenteric lymph nodes.[3,5] These immunocompetent cells distributed throughout the intestine are critical in maintaining the mucosal barrier.[6] The integrity of this immune barrier in the gut is essential for maintenance of intestinal health.

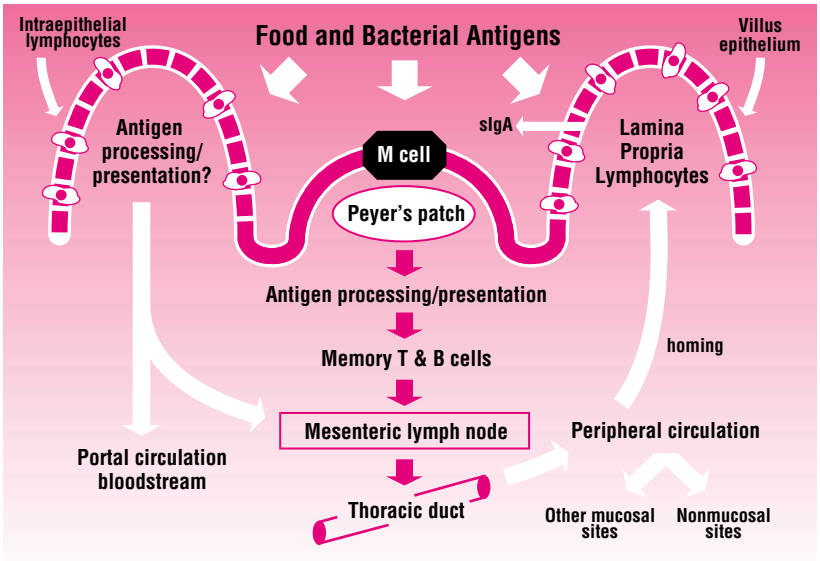


**Figure 1.** *The gastrointestinal immune system. Adapted from Weiner, 1997.*[5]

| Nutrient | Non-Fermentable Fiber | High Fermentable Fiber |
|---|---|---|
| Chicken by product meal | 460 | 460 |
| Fish Meal | 120 | 120 |
| Egg | 40 | 40 |
| Chicken digest | 25 | 25 |
| Chicken fat | 160 | 160 |
| Menhaden Oil | 3 | 3 |
| Pregelled starch | 110 | 80 |
| IAMS Vitamin premix [1] | 3.2 | 3.2 |
| IAMS Mineral premix [2] | 2.4 | 2.4 |
| Potassium chloride | 2.1 | 2.2 |
| Calcium chloride | 1.1 | 1.9 |
| Choline chloride | 0 | 1.1 |
| Sodium chloride | 0.3 | 0.3 |
| Cellulose | 70 | 0 |
| Beet pulp | 0 | 60 |
| Fructooligosaccharide powder | 0 | 15 |
| Gum arabic | 0 | 20 |

[1] *Proprietary vitamin premix provided the following per kg diet: 25 KIU vitamin A, 124 IU vitamin E, 1561 IU vitamin D3, 14 mg thiamin, 59 mg riboflavin, 90 mg niacin, 32 mg d-pantothenic acid, 10 mg pyridoxine, 0.6 mg biotin, 1.9 mg folic acid, 2,067 mg choline, 23 mg inositol, 0.31 µg vitamin B12.*

[2] *Proprietary mineral premix provided the following per kg diet: 41 mg manganese, 217 mg zinc, 168 mg iron, 47 mg copper, 4 mg iodine, 0.08 mg magnesium, 4.8 mg sulfur, 0.62 mg selenium.*

**Table 1.** *Ingredient composition of the diets (g/kg diet as mixed)*

## NUTRITION, DIETARY FIBER AND IMMUNE FUNCTION

It has been clearly demonstrated that nutritional status and specific nutrients in the diet can modulate immune function.[7-9] Alterations in GALT function have been observed with protein-calorie malnutrition, vitamin A deficiency, and glutamine deprivation.[9-10] Nutrients and their digestion products are in direct contact with GALT and it has been suggested that the presence of food in the small intestine is necessary for the development of GALT. [11]

## EFFECT OF DIETARY FIBER ON GALT

The effect of plant fibers and their fermentation products on the structure and function of the gut is well known.[12-13] However, less is known about the role of dietary fiber on immune function. Recently it was reported that feeding a diet high in fermentable fiber to rats increased cytokine production by mesenteric lymph node cells.[14] This observation suggests an immunostimulatory effect of fermentable fiber, or its fermentation products, on the gut. In support of this we have demonstrated, also in a rat model, that adding the end products of fiber fermentation, short chain fatty acids, to total parenteral nutrition improved parameters of host immune defense after gastrointestinal resection.[15]

The role of fiber and its fermentation products (short chain fatty acids) is of current interest in maintaining intestinal health in companion animals.[16-18] As little is known about the effect of fiber types on immune function we conducted a study to determine the role of feeding dogs two different types of dietary fiber, on the composition and function of GALT. Sixteen adult mongrel dogs (23 ± 2 kg) were randomly assigned to receive one of two diets, with the same amount of dietary fiber, but differing in fiber fermentability, for 2 weeks. The experimental diets were designed to be isonitrogenous, isoenergetic (providing approximately 4,650 kcal/kg) and made to provide similar amounts of total dietary fiber (6% w/w) but differing in fiber fermentability (Table 1). After feeding the diets for 2 weeks, the dogs were anesthetized and several mesenteric lymph nodes and a 1m segment of jejunum removed. Immune cells from the Peyer's patches, lamina propria and intraepithelial regions of the gut were isolated.

**The role of fiber and its fermentation products (short chain fatty acids) is of current interest in maintaining intestinal health in companion animals.**

## MESENTERIC LYMPH NODES

Although not always included as part of GALT, the mesenteric lymph nodes are composed of immune cells (mainly T and B lymphocytes) that are leaving and entering the gut and those that are part of peripheral circulation (Figure 1).[5] Mature lymphocytes are found to recirculate continuously between blood and lymphoid organs.[5] This recirculation (homing) between the periphery and the gastrointestinal tract is a special feature of GALT and does not appear to be a random process. Cells drain to the intestinal lymphatics after differentiation in Peyer's patches and pass through mesenteric lymph nodes on route to peripheral blood via the thoracic duct and then again on route back to the lamina propria regions of the gut.[5]

The ability of cells to incorporate [$^3$H]-thymidine *in vitro* in response to mitogens is a frequently used assay to estimate cell-mediated immunity.[19] Of all the cell types in GALT studied, the response to mitogens by the immune cells from mesenteric lymph nodes was most affected by the

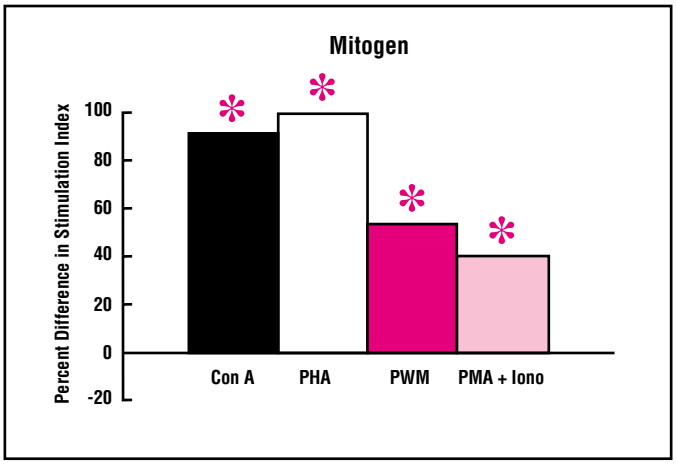


**Figure 2.** *Bars represent the percent difference in [$^3$H]-thymidine incorporation by mesenteric lymph node cells from dogs fed the high fermentable fiber diet as a percent of the response by dogs fed the non-fermentable fiber diet. ✻ indicates that the response to the high fermentable fiber diet is significantly (P<0.05) different from dogs fed the non-fermentable fiber diet.*

amount of fermentable fiber in the diet. Feeding the high fermentable fiber diet, as compared to the diet containing cellulose, resulted in a significantly higher response to all tested mitogens: Concanavalin A (Con A), Phytohemagglutinin (PHA), Pokeweed mitogen (PWM) and the combination of Phorbol Myristate Acetate (PMA) plus Ionomycin (Iono, Figure 2). The response by mesenteric lymphocytes to the T cell mitogens Con A and PHA was 100% higher in cells from dogs fed the high fermentable fiber diet. Feeding the high fermentable fiber diet increased the proportion of T-helper ($CD4^+$) T cells in mesenteric lymph nodes which may have contributed to their higher response. An increase in the proportion of $CD4^+$ cells is often associated with an increase in the response to mitogens.[20-22] The results of our study suggest that feeding fermentable fiber increases the ability of cells in the mesenteric lymph nodes to respond to immune challenges. Recently it was reported that feeding fermentable fiber to rats enhanced the production of IFN-γ by mesenteric lymph node lymphocytes stimulated by Con A.[14] This suggests that feeding fermentable fibers may promote a Th1 type response which would favor stimulation of the innate immune system (macrophages and neutrophils).

## PEYER'S PATCHES

The classical mucosal defense in the gut is the production of IgA. Microfold/membranous (M) cells that overlay the Peyer's patches at the interface with the lumen are specially designed to take up antigens (peptides) by endocytosis (Figure 1).[23] In the epithelium overlying Peyer's patches are goblet cells that enable intestinal content to reach the M cell.[23] Thus M cells serve as a portal of entry for selected pathogens and potential dietary antigens to the cells residing in the Peyer's patches. Peyer's patches are the site of antigen priming that will ultimately result in IgA secretion into the gut. The Peyer's patches are densely

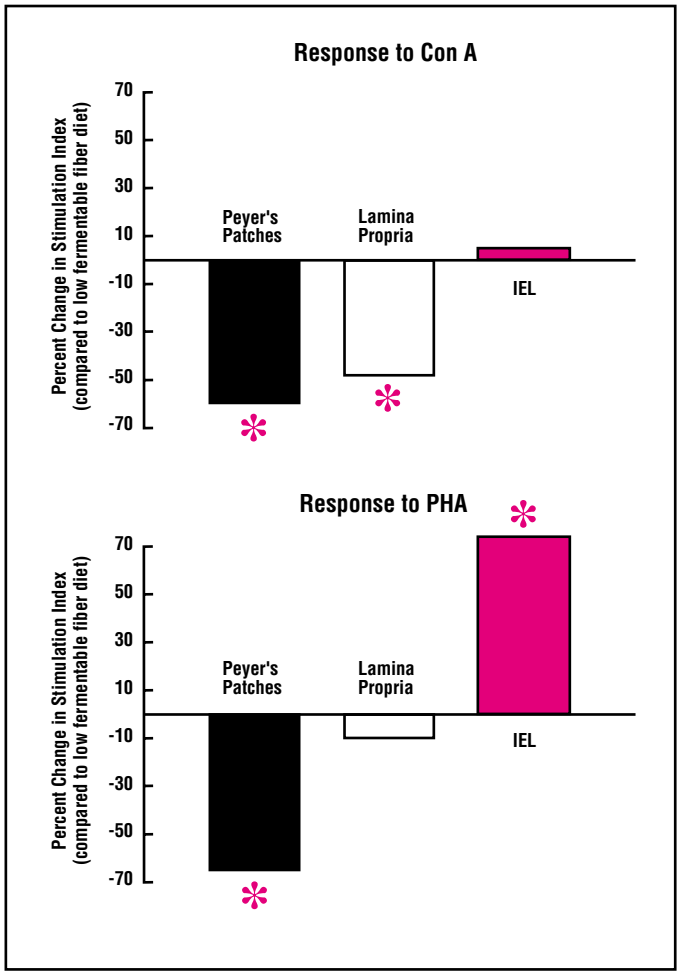


**Figure 3.** *Bars represent the percent difference in [$^3$H]-thymidine incorporation by gut derived immune cells from dogs fed the diet high in fermentable fiber. ✻ indicates that the response is different (P<0.05) from the low fiber group.*

populated with lymphocytes (primarily mature $CD4^+$ T cells and B cells).[24] Antigens are processed and presented directly to T and B lymphocytes residing in Peyer's patches or are transferred to the "professional" antigen-presenting cells such as macrophages, langerhans cells or dendritic cells.[5] Presentation of antigen induces lymphocyte activation and proliferation. Proliferation results in T cells (primarily $CD4^+$) producing cytokines that include interleukin (IL)-4, IL-5, IL-6 and transforming growth factor (TGF-β) that stimulate B cells to differentiate to plasma cells which will ultimately produce IgA.[2]

Feeding a fermentable fiber diet resulted in a lower (60%) response (estimated by the incorporation of [$^3$H]-thymidine) to T cell mitogens by Peyer's patch lymphocytes, as compared to feeding a diet containing non-fermentable fibers (Figure 3). These results suggest a lower T cell response in stimulated cells from dogs fed the high fermentable fiber diet. Regulation of B cell function in Peyer's patches is dependent on the balance between the cytokines produced by Th1 and Th2 $CD4^+$ T cells.[25] In the current study, the proportion of $CD8^+$ (T suppressor/cytotoxic) cells was significantly higher in dogs fed the high fermentable diet. This increase in

suppressor cells may have contributed to the lower T cell response in these dogs.

## LAMINA PROPRIA

Plasma cells, originating in the Peyer's patches, return to the gut from the periphery to reside in the lamina propria region (Figure 1).[5] This is a poorly understood process of specific homing that involves a number of specialized adhesion molecules and is a special feature of GALT.[26] B cells in the lamina propria primarily secrete IgA as compared to those in peripheral circulation which primarily produce IgG and IgM. The secretatory component of sIgA originates as a transmembrane protein found on the basal and lateral plasma membrane of luminal epithelial cells.

IgA is one of the most important defense factors in the mucosal immune system. Dogs that are unable to produce IgA demonstrate increased susceptibility to intestinal infections.[27-28] Secretatory IgA (sIgA), unlike other immunoglobulins, does not fix complement, opsonize bacteria nor is it efficient in killing microorganisms. These features are beneficial as its prevents sIgA from initiating an inflammatory response. The unique role of sIgA in the gut is to inhibit the attachment and penetration of bacteria and toxins in the lumen, bind undigested protein to prevent absorption (thereby increasing the time for digestive enzymes to work) and to increase mucus secretion.[2]

In the current study, increasing the amount of fermentable fiber in the diet of dogs significantly decreased [$^3$H]-thymidine incorporation by immune cells from the lamina propria of the gut after stimulation with Con A (Figure 3). The amount of fermentable fiber did not significantly alter the proportion of B cells in this predominantly B tissue but did result in a significantly higher proportion of CD8$^+$ cells and a lower CD4/CD8 T cell ratio. Further studies are required to determine the effect of feeding fermentable fiber on the balance between Th1 and Th2 cytokines and IgA secretion in both the lamina propria and Peyer's patches regions of the gut.

## INTRAEPITHELIAL LYMPHOCYTES

Although the immune cells in Peyer's patches are important in the initial contact of food and bacterial antigens in the gut, the number of Peyer's patches is small in relation to the total mucosal surface. In contrast there is an average of 1 intraepithelial T cell for every 6-10 epithelial cells, making IEL the largest immunocompetent cell pool in the body.[29] Although IEL line both the small and large intestine from the crypt base to the villus tip, their exact biological function in the mucosal immune system is not known. IEL are situated in a central location for host defense against numerous orally encountered foreign antigens. They are in continuous contact with luminal antigens through the epithelial layer and are likely involved in several aspects of mucosal immune defense. It has been suggested that they may be the first compartment of the immune system that responds to gut-derived antigens (both pathogens and dietary antigens; Figure 1).[2] Phenotypically, IEL are quite different from immune cells elsewhere in the body, suggesting a specific biological function. IEL are comprised primarily of CD8$^+$ cells, suggesting that IEL cells may be functional suppressor cells and may be involved in processes such as oral tolerance.[5]

Although they express the CD3 T cell marker, IEL have low expression of CD5, CD6, LFA-1 and VLA-4 markers seen on most peripheral T cells. Few of these cells express regular activation markers such as CD25 (IL-2 receptor) or CD71 (transferrin receptor).[31] In some species there are a number of CD4$^+$CD8$^+$ (double positive) T cells, cells that are usually only seen during maturation in the thymus.[32] In the present study, we did not find these double positive cells in the IEL of adult dogs.

Natural killer cells are large granular lymphocytes, found predominantly in the spleen and peripheral blood, that exhibit spontaneous cytotoxicity against various targets.[38] Peripheral blood cells from the dog have been demonstrated to exhibit considerable natural killer cell cytotoxic activity.[37] In some species, but not in others, IEL have been shown to also exhibit natural killer cell activity. Little is known about the potential cytotoxic activity of canine IEL. In the dog we demonstrated that canine IEL have cytotoxic activity against a canine natural killer sensitive cell line (CTAC; Figure 4). The physiological importance of this cytotoxic activity in the gut has not been established but is suggested for other species to provide an important first line defense against invasion by enteric pathogens or the elimination of transformed (pre-carcinogenic) epithelial stem cells.[31] Natural killer activity did not differ in dogs fed the diets containing different amounts of fermentable fiber (Figure 4).

Canine IEL are large and granular and respond well to mitogen stimulation. Adding fermentable fiber to the diet increased the [$^3$H]-thymidine response to the T cell mitogen PHA (Figure 3). IEL from dogs fed the high fermentable fiber diet contained a higher proportion of CD8$^+$ cells (the major lymphocyte subset in this region of the gut). The physiological significance of these changes are not known, but may be important in IEL

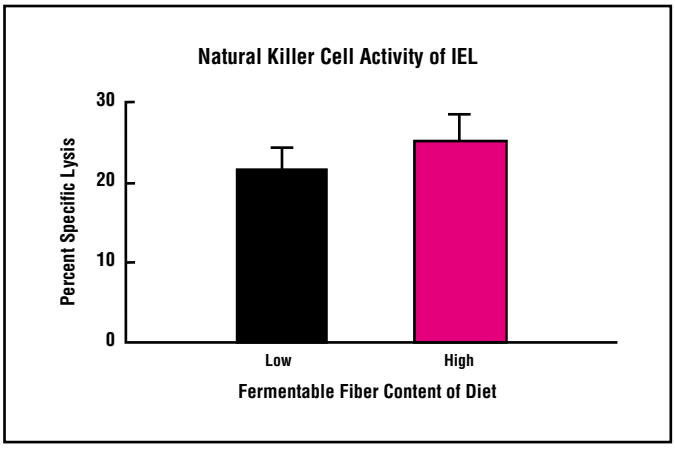


**Figure 4.** *Bars represent the percent specific lysis against a canine-sensitive cell line. There was no significant difference in the response by dogs fed the diets with different amounts of fermentable fiber.*

immunosurveillance against bacterial invasion.[33] The majority of IEL cells ($CD8^+$) produce IL-5 and TNF-γ, [31,36] two cytokines believed to be involved in oral tolerance.[34] Oral tolerance describes a state of hyporesponsiveness following exposure to a previously fed antigen.[5] During the last 20 years there has been increased interest in IEL, not only as a component of GALT but also the possible role in disease states both within and outside the GI tract. Specific cells from regions of GALT (primarily IEL) have been suggested to be involved in autoimmune diseases, inflam-matory bowel disease, and food allergies.[4,36] Additional work is needed to determine the role of fiber fermentability on the function of IEL not only in healthy animals but also in clinical conditions.

**GALT is a unique and important part of the canine immune system located in an organ that is chronically exposed to antigens.**

## CONCLUSIONS

GALT is a unique and important part of the canine immune system located in an organ that is chronically exposed to antigens. Adding fermentable plant fibers to the diet of dogs changed the composition and function of the immune cells in GALT. More specifically, feeding fermentable fiber produced a higher mitogen response in the predominantly T cell tissues (mesenteric lymph nodes and IEL) and a lower response in cells isolated from areas involved in B-cell mediated functions (Peyer's patches and lamina propria). The role of reduced T cell function in the areas involved in IgA secretion is not known, however, it may be important to have a higher response in dogs fed the non-fermentable fiber diet as low fiber diets containing no supplemental fiber have been shown to increase the permeability of the gastrointestinal tract.[35] Further work is needed to identify the physiological consequences of diet-induced changes in the composition and function of GALT.

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# Carotenoid Supplementation Enhanced Canine and Feline Immunity

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## INTRODUCTION

Over 600 carotenoids can be found in nature. Large species differences occur in the absorption and, very likely, the functions of these pigmented compounds. Carotenoids found in significant concentrations in blood and tissues include β-carotene, α-carotene, lutein, zeaxanthin, lycopene and astaxanthin (Figure 1). These carotenoids are known to play important roles in modulating immunity and promoting health in human and certain animal species. Both *in vitro* and *in vivo* studies showed that ß-carotene can increase the number of T helper cell number, IL-2 receptor expression on natural killer cells, lymphocyte proliferation, humoral response and blood neutrophil function.[1,2] Studies with other carotenoids like lutein, astaxanthin and canthaxanthin also showed enhanced immmune function.[3] Unfortunately, little is known about the uptake and possible function of these carotenoids in the canine and feline.

## Carotenoid Uptake

Early studies have reported that both the canine and domestic feline are unable to absorb β-carotene from the diet.[4] Only one recent study indicated the presence of β-carotene in the blood of domestic cats.[5] Carotene was undetectable in the blood of exotic canids but was very high in exotic felids.[6] Recently, several systematic studies were conducted to study the absorption of β-carotene and lutein by the canine and feline. Results show a dose-dependent increase in concentrations of plasma β-carotene[7] and lutein[8] in dogs fed the respective carotenoids. Furthermore, β-carotene and lutein were taken up by circulating blood lymphocytes and neutrophils and distributed into the cytosol, mitochondria, microsomes, and nuclei. Studies with the domestic feline similarly showed a dose-dependent increase in plasma β-carotene[9] and lutein[10] and uptake by the mitochondria, microsomes, cytosol, and nuclei of blood lymphocytes. The feline seems to absorb β-carotene more efficiently than the canine. The reverse was true for lutein.

Therefore, both the canine and feline readily absorb dietary β-carotene and lutein. These carotenoids are subsequently distributed into the subcellular organelles of important immune cells. It is tempting to postulate that the β-carotene and lutein in the lymphocytes may serve to optimize lymphocyte function by (1) acting as antioxidants in protecting the lymphocytes from harmful oxygen free radicals or (2) directly regulating cellular events. If this is

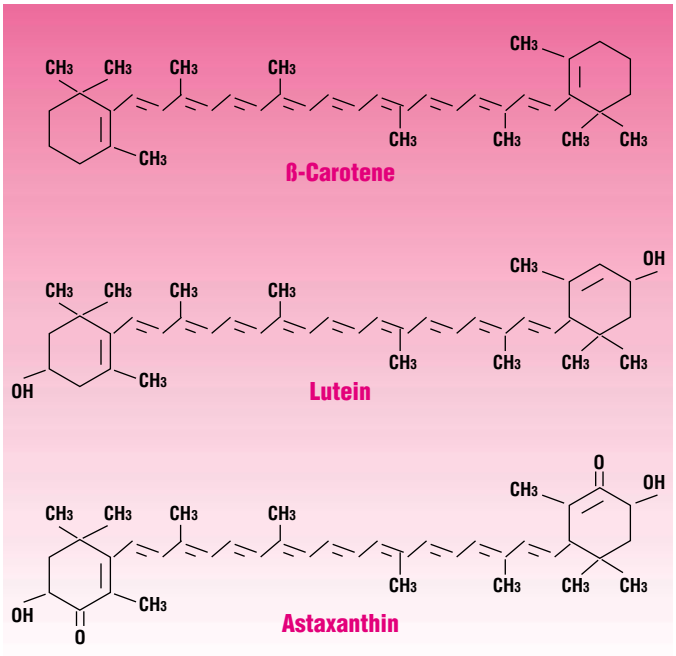


**Figure 1.** *Structure of carotenoids*

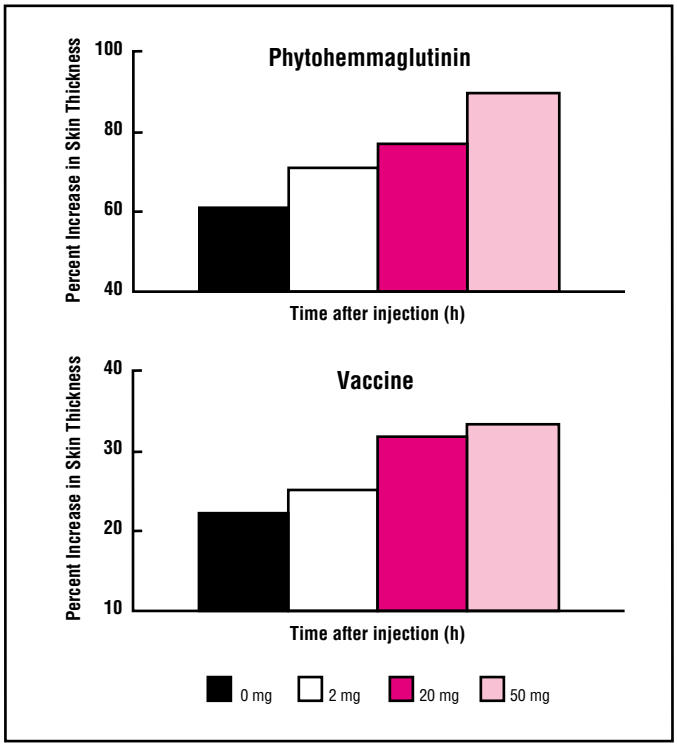


**Figure 2.** *DTH response (at 48 h) in dogs fed 0, 2, 20 or 50 mg of β-carotene daily for 7 wk and challenged with phytohemagglutinin or vaccine.*

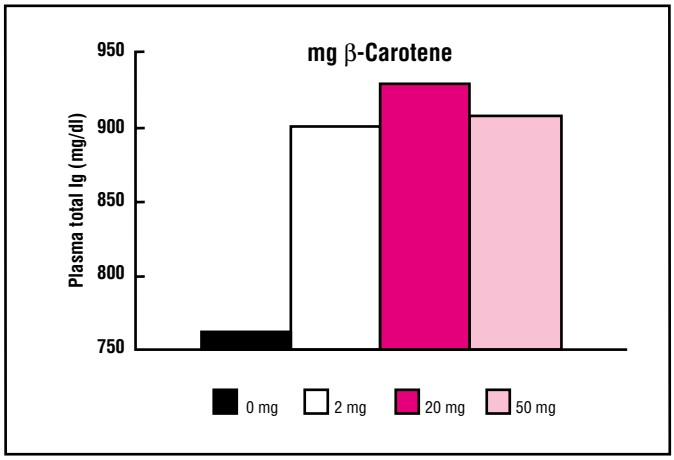


**Figure 3.** *Changes in plasma total Ig in dogs fed 0, 2, 20 or 50 mg of β-carotene daily for 2 wk.*

the case, then one can expect enhanced immune function and improved health in dogs and cats fed adequate amounts of these carotenoids. Indeed, very recent studies in our laboratory showed an important role played by β-carotene and lutein in regulating immune response and reproduction in the canine and feline.

## Carotenoid and Immune Response

The immune system is comprised of cell-mediated and humoral immunity. Several immune parameters commonly measured include: lymphocyte proliferation, delayed-type hypersensitivity (DTH) response to specific and nonspecific antigens, and changes in blood lymphocyte populations and immunoglobulin (Ig) concentrations. Lymphocytes are involved in cell-mediated immunity. Upon recognizing an antigen, lymphocytes will divide rapidly, thereby cloning themselves in preparation for combating a potential invasion. T cells can be classified according to the expression of CD4 membrane molecules. The CD4 functions as an adhesion molecule and as a co-signaling co-receptor. It plays a role in T cell activation. The CD4+ T lymphocytes recognize antigen in association with the class II MHC molecules and largely function as helper cells. The DTH response is strictly a cellular reaction involving T cells and macrophages without involving an antibody component. Antigen-presenting cells (e.g., macrophages) present the antigen or allergen to T cells that become activated and release lymphokines. These lymphokines activate macrophages and cause them to become voracious killers of the foreign invaders. In humoral immune response, IL-2 stimulates both T helper cells and B cells to proliferate in response to antigens. It is required for the clonal expansion of antigen- or mitogen-activated T cells. In cell-mediated immune response, IL-2 activates natural killer cells, stimulates thymocyte proliferation and induces cytotoxic T cell activity. Another major function of the immune system is the production of antibodies that circulate freely to protect the body against foreign materials. Antibodies serve to neutralize toxins, immobilize certain microorganisms, neutralize viral activity, agglutinate microorganisms or antigen particles, and precipitate soluble antigens.

**Canine:** The role of dietary β-carotene and lutein in modulating the cell-mediated and humoral immune systems was studied in female Beagle dogs. The following cell-mediated and humoral immune parameters were assessed: DTH response to vaccine (specific) and phytohemagglutinin (PHA; non-specific) antigens, blood lymphocyte proliferation, changes in blood lymphocyte subsets, and blood Ig G and M concentrations. β-Carotene supplementation increased plasma concentrations of the carotenoid in a dose-dependent manner.[11] There was a dose-dependent increase in DTH response to both the specific and non-specific antigens in dogs supplemented with β-carotene (Figure 2). β-Carotene feeding also produced significant changes in lymphocyte subset populations. Dogs fed β-carotene had elevated populations of $CD4^+$, $CD5^+$, and $CD8^+$ cells. Dietary β-carotene also increased mitogen-induced lymphocyte blastogenesis. The increase in T helper cell population in this study can explain the corresponding increase in DTH response in dogs fed β-carotene. Besides increasing cell-mediated immune response, dietary β-carotene also increased concentrations of IgG, IgM and total IgG (Figure 3) in a dose-dependent

**There was a dose-dependent increase in DTH response to both the specific and non-specific antigens in dogs supplemented with β-carotene.**

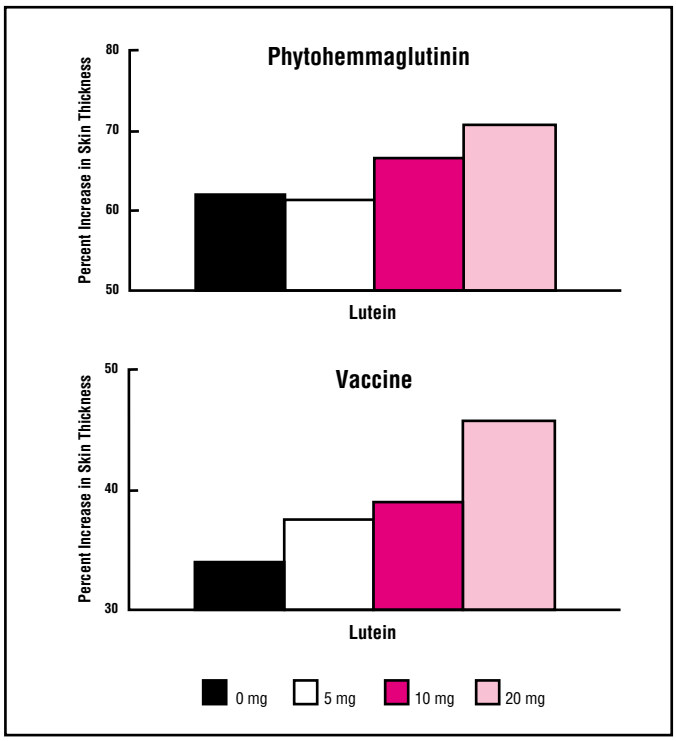


**Figure 4.** *DTH response (at 48 h) in dogs fed 0, 5, 10 or 20 mg of lutein daily for 6 wk and challenged with phytohemagglutinin or vaccine.*

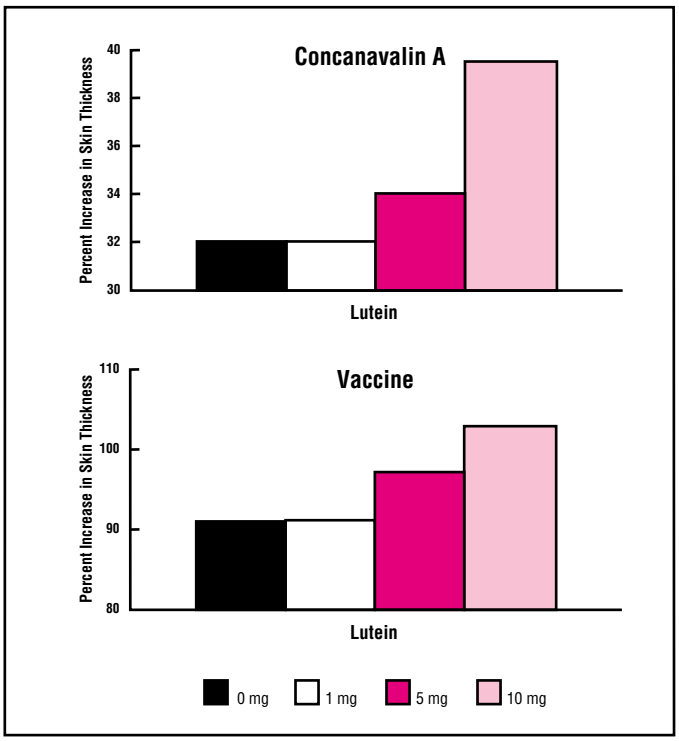


**Figure 5.** *DTH response (at 72 h) in cats fed 0, 1, 5 or 10 mg of lutein daily for 6 wk and challenged with Concanavalin A or vaccine.*

manner. Therefore, our data show a heightened cell-mediated and humoral response in dogs fed β-carotene.

A similar study[12] also showed immuno-enhancement of certain immune responses in dogs fed lutein. Dogs fed lutein had heightened DTH response to both PHA and vaccine after 6 weeks of feeding (Figure 4). Also, pokeweed mitogen-induced lymphocyte proliferation was increased in lutein-fed dogs compared to unsupplemented animals. Therefore, dietary lutein heightened cell-mediated immune response in dogs.

**Feline:** We also studied the role of dietary β-carotene and lutein in regulating cell-mediated and humoral immunity in the feline. Female Tabby cats supplemented with dietary β-carotene or lutein showed a dose-dependent increase in the concentration of each carotenoid in plasma. Dietary β-carotene did not influence immune response (lymphocyte proliferation, DTH response and the production of IgG and IL-2) in cats.[13] In contrast, dietary lutein increased DTH response to both specific and non-specific antigens (Figure 5). Data on the efffect of lutein on other aspects of immunity is not available at the present time. Therefore, even though cats readily absorb both β-carotene and lutein, only dietary lutein significantly enhanced immune response in the domestic cat.

**Dietary lutein increased DTH response to both specific and non-specific antigens.**

## OVERALL SUMMARY AND CONCLUSION

In summary, the canine and feline readily absorb ß-carotene and lutein from the diet and transfer the ß-carotene and lutein into immune cells and phagocytes. Cats are relatively more efficient in absorbing dietary ß-carotene than are dogs. However, the reverse is true with dietary lutein. Uptake of these carotenoids into the subcellular organelles of lymphocytes suggests possible cell regulatory/protective functions. β-Carotene enhanced cell-mediated and humoral immune response in the canine but not in the feline. However, dietary lutein enhanced immune response in both the canine and feline. Therefore, species (canine versus feline) differences exist in the relative uptake of the carotenoids and in immune responsiveness. The exciting information on carotenoid nutrition in companion animals should lead to considerations for including these important components in the diet to optimize health.

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