

Functions and Actions of Retinoids and Carotenoids: Building on the Vision of James Allen Olson

Carotenoid Action on the Immune Response¹

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ABSTRACT Early studies demonstrating the ability of dietary carotenes to prevent infections have left open the possibility that the action of these carotenoids may be through their prior conversion to vitamin A. Subsequent studies to demonstrate the specific action of dietary carotenoids have used carotenoids without provitamin A activity such as lutein, canthaxanthin, lycopene and astaxanthin. In fact, these nonprovitamin A carotenoids were as active, and at times more active, than β -carotene in enhancing cell-mediated and humoral immune response in animals and humans. Another approach to study the possible specific role of dietary carotenoids has used animals that are inefficient converters of carotenoids to vitamin A, for example the domestic cat. Results have similarly shown immuno-enhancement by nonprovitamin A carotenoids, based either on the relative activity or on the type of immune response affected compared to β -carotene. Certain carotenoids, acting as antioxidants, can potentially reduce the toxic effects of reactive oxygen species (ROS). These ROS, and therefore carotenoids, have been implicated in the etiology of diseases such as cancer, cardiovascular and neurodegenerative diseases and aging. Recent studies on the role of carotenoids in gene regulation, apoptosis and angiogenesis have advanced our knowledge on the possible mechanism by which carotenoids regulate immune function and cancer. *J. Nutr.* 134: 257S–261S, 2004.

KEY WORDS: • antioxidants • carotenoids • immunity

Immune system and reactive oxygen species

The immune system is comprised of innate (natural) and acquired (adaptive) immunity. Acquired immunity is composed of lymphocytes; these are highly active cells that constantly generate reactive oxidative products (ROS) as a part of their normal cellular activity. Oxidizing pollutants and many viruses also can induce ROS production by normal cells. The ROS are highly reactive and can destroy cellular membranes, cellular proteins and nucleic acids. One mechanism by which the innate branch of the immune system protects the animal is by phagocytosis and subsequently killing antigens through an oxidative bactericidal mechanism termed respiratory burst. Phagocytosis of a foreign particle by a macrophage or neutrophil activates NADP oxidase, resulting in the production of a

large amount of superoxide anion ($O_2^{\cdot-}$) from molecular oxygen. The $O_2^{\cdot-}$ is then rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. Neutrophils contain myeloperoxidase that converts H_2O_2 to the highly potent bactericidal component, hypochloride ions (OCl^-). Macrophages do not possess myeloperoxidase and, instead, depend on a myeloperoxidase-independent mechanism to generate other biological oxygen-derived free radicals. The latter include the generation of the hydroxyl radical (OH^{\cdot}) through Fenton and/or Haber-Weiss chemistry. Even though the ROS are produced as part of the killing mechanism, nevertheless excessive phagocytic activity can lead to ROS-induced tissue damage.

As a defense mechanism, the body produces a number of endogenous antioxidants capable of scavenging these harmful ROS to maintain an optimal oxidant:antioxidant balance, thereby maintaining normal cellular function and health. However, under conditions of high oxidative stress, the ability of these antioxidants to eliminate ROS are often exceeded and, therefore, dietary sources of antioxidants or drugs are required. The most widely used dietary antioxidants include vitamin E, vitamin C, carotenoids, flavanoids, zinc and selenium. The use of N-acetylcysteine as an antioxidant drug has gained popularity in recent years due to its ability to inhibit proinflammatory molecules and HIV replication. The harmful affects of ROS are not unique to immune cells but affect all cell types. However, immune cells are particularly sensitive to oxidative stress because their plasma membranes contain a high percentage of PUFA and they generally produce more ROS (1).

Studies on the role of carotenoids on immune response

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³ Abbreviations used: DTH, delayed type hypersensitivity; Ig, Immunoglobulin; PBMC, peripheral blood mononuclear cells; ROS, reactive oxygen species; Th, helper T cells.

have generally used several key immune function assays. These include:

(i) Immunoglobulin (Ig) production. Production of Ig has traditionally been used to assess B cell function in a humoral immune response. B cells produce Ig that circulate freely to protect the body against foreign materials. The Ig serve to neutralize toxins, immobilize certain microorganisms, neutralize viral activity, agglutinate microorganisms or antigen particles and precipitate soluble antigens. B cell function requires the help of helper T (Th) cells.

(ii) Lymphoblastogenesis. Antigen-stimulated lymphocyte proliferation normally occurs in lymphoid tissues. However, the ability of isolated lymphoid cells to proliferate when cultured in the presence of certain mitogens has given researchers an important tool to assess both T and B cell function *in vitro*. Commonly used mitogens include concanavalin A that stimulates T cells, lipopolysaccharide that stimulates B cells, and pokeweed mitogen that stimulates T and B cells. This *in vitro* immune response correlates well with that observed *in vivo* (2).

(iii) Lymphocyte cytotoxic activity. NK cells are a critical component of innate resistance against viruses, bacteria, fungi and parasites. They regulate the adaptive immune system and hematopoiesis, and serve as an immuno-surveillance system against tumors. The T cell cytotoxicity assay determines the efficiency of killing of target cells (e.g., cancer cells) by effector cells (lymphocytes) when cultured *in vitro*.

(iv) Cytokine production. Cytokines are soluble molecules that mediate cell-to-cell interactions. Cytokines commonly measured include IL-2, TNF α and IFN- γ produced by the CD4+Th1 cell subset, and IL-4, IL-5, IL-6 and IL-10 produced by the Th2 subset. The Th1 cells mediate cytotoxic and local inflammatory reactions, and therefore play important roles in combating intracellular pathogens including viruses, bacteria and parasites. The Th2 cells are more effective in humoral immunity, i.e., they stimulate B cells to proliferate and produce antibodies against free-living microorganisms. Therefore, a normal immune response will require a balance between the Th1 and Th2 subsets.

(v) Delayed type hypersensitivity (DTH). This is a cellular reaction involving T cells and macrophages without involving an antibody component. Antigen-presenting cells (e.g., dendritic cells) 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. The DTH response is simple to conduct. More importantly, it is a good indicator of *in vivo* cell-mediated immune response and is a predictor of morbidity and mortality in elderly human (3).

(vi) Modern advances in flow cytometry techniques have enabled researchers to phenotype blood lymphocyte subsets by identifying cell surface molecules. Identifying the population shifts for a given cell subset will provide additional supporting evidence for the immune responses assessed using the functional assays described earlier. Also, flow cytometry can be used to identify cell activation through the induction of certain surface markers. Availability of reagents has further extended the application of flow cytometry to include apoptosis, cell cycle progression and cell signaling.

(vii) Last, but not least, molecular bioscience techniques have provided exciting new research tools for studying mechanism of action of carotenoids in regulating intracellular events.

Carotenoids, ROS and immunity

The traditional concept of ROS function is that they indiscriminately destroy cell components. However, exciting

research has more recently elucidated the role of these reactive species in signal transduction, gene regulation, and disease etiology. This has infused new excitement and challenges into research on the possible role of carotenoids as antioxidants in disease prevention. This discussion will attempt to address the complex interaction of carotenoids and the immune response, and how this interaction may relate to cancer etiology.

Early studies demonstrated that dietary β -carotene prevented bladder, kidney, ear and gut infection in vitamin A-deficient rats (4) and reduced ear infection in young children (5). Because of the provitamin A activity of β -carotene, these studies raised the possibility that the action of the carotenoid is due to its prior conversion to vitamin A. To circumvent this problem, the specific role of carotenoids can be demonstrated either by using carotenoids without provitamin A activity (e.g., lutein, lycopene, canthaxanthin, astaxanthin) or by using animals that cannot convert or are poor converters of carotenoids to vitamin A (e.g., cats). Numerous studies using nonprovitamin A carotenoids and, more recently, using cats as the animal model have demonstrated the immuno-modulatory action of dietary carotenoids. It is recognized that cats can convert carotenoids to vitamin A, albeit very inefficiently.

Many earlier studies focused on β -carotene (6). Seifter et al. (7) reported a marked stimulatory action of β -carotene on the growth of the thymus gland and a large increase in the number of thymic small lymphocytes. The stimulatory activity of β -carotene on lymphocyte blastogenesis has similarly been demonstrated in rats (8), pigs (9), and cattle (10). Increased numbers of Th and T inducer lymphocytes have been reported in human adults given oral β -carotene supplementation (11,12). The number of lymphoid cells with surface markers for NK cells and for IL-2 and transferrin receptors also was increased substantially in peripheral blood mononuclear cells (PBMC) from individuals supplemented with β -carotene (12,13). Enhanced NK cell cytotoxicity was observed in human subjects given oral β -carotene (14). Similarly, long-term β -carotene supplementation to elderly but not middle-age men increased NK cell activity (15). *In vitro*, β -carotene induced hamster macrophages to produce TNF α (16). Activation of TNF α by ROS increases the dissociation of I κ B from NF κ B, and the subsequent translocation of this transcription factor to the nucleus, resulting in the production of cytokines, chemokines, cell adhesion molecules, and acute phase proteins; this activation also produces an anti-apoptotic effect. Alternatively, intracellular ROS may directly increase NF κ B. Therefore, ROS are important in primary immune response; conversely, antioxidants can produce the opposite effect. In fact, Verhasselt et al. (17) reported that the antioxidant molecule N-acetyl-L-cysteine can inhibit NF κ B, and consequently down-regulate the production of cytokines (IL-6, IL-8, IL-12, and TNF α), as well as down-regulate the expression of surface molecules (HLA-DR, B7-2 and CD40) in human dendritic cells. Therefore, an antioxidant may impair the generation of primary immune responses through its inhibitory action on dendritic cells. While this scenario occurs in a normal cell, under conditions of high oxidative stress, excess ROS may be produced, resulting in the inhibition of NF κ B. Excess ROS is known to cause abnormal cell proliferation and to decrease apoptosis; both are undesirable responses in tumor cells. Therefore, antioxidants are desirable under conditions of high oxidative stress. Analogous to this situation, high concentrations of intracellular nitric oxide induced oxidative killing of isolated rat hepatocytes while low nitric oxide concentrations was protective (18).

Besides cell-mediated and humoral immune responses, β -carotene has been shown to regulate nonspecific cellular

host defense. Blood neutrophils isolated from cattle fed β -carotene had higher killing ability during the peripartum period (19). The increased bacterial killing could be accounted for partly by increased myeloperoxidase activity in the neutrophils. Tjoelker et al. (20) reported that dietary β -carotene stimulated phagocytic and bacterial killing ability of neutrophils from dairy cows during the stressful drying off period. In contrast, retinol and retinoic acid generally decreased phagocytosis and had no effect on killing activity.

A specific role of carotenoids on immune response was first reported by Bendich and Shapiro (8). They showed that rats fed canthaxanthin, a carotenoid with no provitamin A activity, had a heightened mitogen-induced lymphocyte proliferation; dietary β -carotene showed similar action. Subsequent studies have similarly reported the immuno-enhancing action of carotenoids without provitamin A activity, notably lutein, lycopene, astaxanthin and canthaxanthin. Canthaxanthin enhanced the expression of activation markers for Th and NK cells in human PBMC *in vitro* (21). Jyonouchi et al. (22) reported that lutein and astaxanthin increased the *ex vivo* antibody response of mouse splenocytes to T-cell antigens. Schwartz et al. (23) reported increased cytochrome oxidase and peroxidase activities in macrophages incubated with canthaxanthin, β -carotene, and α -carotene compared with incubation with 13-*cis* retinoic acid. The stimulatory activity of canthaxanthin was greater than that observed with β -carotene and α -carotene. Phagocytosis also was stimulated by these carotenoids, even though to a lower degree. All of these changes indicate increased respiratory bursts by the macrophages when they are exposed to carotenoids.

The domestic dog and cat have recently been used in parallel studies using similar experimental designs to compare the immuno-modulatory role of carotenoids. These studies thus provide direct comparisons between carotenoids with (β -carotene) or without (lutein) provitamin A activity, and also between species that can (dogs) or that are very inefficient converters (cats) of β -carotene to vitamin A. Dietary β -carotene (24) and lutein (25) stimulated DTH response, the number of CD4+Th cells, and IgG production in dogs, thus demonstrating that lutein, a carotenoid without provitamin A activity, exerts a similar immuno-modulating action as β -carotene. In contrast, lutein but not β -carotene enhanced mitogen-induced lymphocyte proliferation in dogs, indicating species differences in the lymphocyte proliferation response to a given dietary carotenoid. Cats fed β -carotene (unpublished data, Park et al.) or lutein (26) also showed heightened DTH response, higher Th and B cell subpopulations, and increased plasma IgG concentrations. It can be concluded that the actions of both β -carotene and lutein in cats are not due to their prior conversion to vitamin A because cats are poor converters of β -carotene to vitamin A.

Carotenoids and tumor immunity

The immuno-regulatory action of carotenoids has also been demonstrated through their role in tumor immunity. An elegant study by Tomita et al. (27) reported that mice fed β -carotene had augmented tumor immunity against syngeneic fibrosarcoma cells. They further demonstrated that the action of β -carotene was specific against the antigens. *In vitro*, β -carotene inhibited the growth of MCF-7 and Hs578T while lycopene inhibited MCF-7 and MDA-MB-231 human breast cancer cells; canthaxanthin had no inhibitory effect (28). The authors concluded that the presence of estrogen receptors is an important, even though not essential, factor in the action of carotenoids on tumor cell growth. We (29) compared the

antitumor activity of various carotenoids against the growth of a transplantable mammary tumor in mice. Even though astaxanthin, canthaxanthin, and β -carotene inhibited tumor growth, astaxanthin showed the highest anti-tumor activity. In a parallel study (30), astaxanthin and β -carotene but not canthaxanthin stimulated phytohemagglutinin-induced splenocyte proliferation. The antitumor activity of astaxanthin against the growth of a fibrosarcoma cell has similarly been reported (31). Furthermore, the latter authors showed a parallel increase in Tc cell activity and IFN γ production by splenocytes and tumor-draining lymph node.

Through a series of experiments, our laboratory has used a transplantable mammary tumor model in BALB/c mice to provide a more detailed study on the mechanism of action of dietary lutein against mammary tumor growth. Dietary lutein consistently inhibited the growth of mammary tumors in mice (32–35), and even lowered the incidence of tumor development when a lower tumor cell challenge load was used (33). How lutein may regulate tumor immunity was examined next. We reported that the presence of a mammary tumor suppressed the populations of total T, Th, and Tc cells, but increased the populations of IL-2R α + T cells and B cells compared to mice not carrying tumors (34). However, dietary lutein prevented these tumor-associated lymphocyte subpopulation changes. In addition, dietary lutein increased IFN- γ mRNA expression but decreased the expression of IL-10 in splenocytes of tumor-bearing mice; these changes paralleled the inhibitory action of lutein against tumor growth (34). IFN- γ has several immuno-regulatory actions. It is produced by activated T cells and NK cells, and is a potent inducer of macrophage activation and of class II molecules. IL-10 inhibits IFN- γ production, antigen presentation, and IL-1, IL-6 and TNF α production by macrophages. The anti-tumor and immuno-modulatory actions of lutein suggest the involvement of subcellular events such as apoptosis, angiogenesis and gene regulation. We (35) reported that dietary lutein decreased apoptosis in blood leukocytes from tumor-bearing mice compared to unsupplemented mice, suggesting a heightened immune status. On the other hand, apoptosis in tumor cells was increased by dietary lutein, suggesting increased death of tumor cells. These results demonstrate a selective action of lutein by decreasing apoptosis in immune cells but increasing apoptosis in tumor cells. Sumantran et al. (36) similarly showed lutein to selectively induce apoptosis in transformed but not normal human mammary cells *in vitro*.

Suggestions for the possible gene regulatory action of carotenoids in hematopoietic cells were first provided by Park et al. (37). We reported that lutein but not β -carotene or astaxanthin up-regulated the pim-1 gene in mouse splenocytes. Pim-1 is expressed in normal lymphocytes and is involved in hematopoietic cell proliferation, differentiation and apoptosis.

Because of the possible involvement of lutein in apoptosis, we recently used the BALB/c mouse model to study the ability of lutein to regulate the expression of genes involved in apoptosis. We (35) showed that dietary lutein decreased mammary tumor growth, increased the mRNA expression of the proapoptotic genes p53 and BAX, decreased the expression of the anti-apoptotic gene Bcl-2, and increased the BAX: Bcl-2 ratio in tumors. Again, this selective action of lutein was similarly observed in human mammary cells (36). The p53 tumor suppressive gene can induce cell cycle arrest to allow DNA repair or apoptosis. Its pathway is independent of the mitochondria, and therefore of ROS. On the other hand, Bcl-2 functions as a suppressor of apoptotic death and is negatively regulated by wild type p53. The predominance of BAX over Bcl-2 accelerates apoptosis. Bcl-2 resides in the

outer mitochondria membrane and prevents cytochrome c release. BAX is inactive until it is translocated to the mitochondria where it binds to Bcl-2 to induce cytochrome c release. Once released, cytochrome c activates caspases to bring about apoptosis. Apoptosis or programmed cell death is important in normal development and health. Uncontrolled cell proliferation can lead to cancer and autoimmune diseases whereas excessive cell death can lead to neurodegenerative diseases and AIDS.

The functionality of a carotenoid is determined by its subcellular localization. Studies in cats (38) and dogs (39) have shown significant uptake of orally fed lutein by the mitochondria, nuclei and microsomes of circulating lymphocytes, with the mitochondria showing the highest uptake in the cat. Similar studies in cats (40), dogs (41), cattle (42) and pigs (43) reported uptake of β -carotene by all lymphocyte subcellular fractions. In the case of cats and cattle, the mitochondria again took up the greatest proportion of β -carotene. The mitochondria electron transport system utilizes ~85% of the oxygen consumed by the cell to generate ATP; therefore, they are the most important source of ROS (44). Cytochrome c located between the inner and outer mitochondria membranes plays a critical role in the apoptotic process. The release of cytochrome c is regulated by the pro-apoptotic proteins BAX, BID and BIM, and by the anti-apoptotic proteins Bcl-2, Bcl-XL and BFL-1. Therefore, the mitochondria is likely a key player in immunity and disease, and the localization of the carotenoids in the mitochondria is therefore of particular relevance. The presence of these carotenoids in subcellular organelles can protect the immune cells against oxidative injury, and ensure optimal cellular functions, including apoptosis, cell signaling and gene regulation.

Besides acting through the various mechanisms described earlier, carotenoids can also influence immune function through their ability to regulate membrane fluidity, and gap-junctional communication. Of course, all these actions are most likely interrelated in their modulation of an immune response. Evidence has suggested that the action of carotenoids on immunity and diseases may be mediated, at least in part, by their ability to quench ROS. However, the action of ROS is multifaceted: on the one hand, they are toxic to cellular components, but on the other hand, intracellular and extracellular ROS are important signaling molecules involved in the regulation of gene expression, cell growth and cell death. Therefore, to simply conclude whether carotenoids are heroes or villains is to oversimplify their true role in the body. The action of carotenoids on immune response hangs in a delicate balance with the intra- and extracellular milieu, the outcome of which depends not only on the type and concentration of the carotenoid but also on the cell type and animal species involved. Even though studies to date have provided evidence for a specific action of carotenoids, much has yet to be done to truly understand their molecular action. The use of molecular bioscience technics can provide the necessary research tool to probe into the complex interaction of carotenoids with cell systems.

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