Carotenoids
Volume 5: Nutrition and Health

Chapter 17

The Immune System

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A. The Immune System and Disease

1. Introduction

The immune system plays an essential role in maintaining the body's overall health and resistance to diseases. It comprises two branches, known as the innate or antigen-nonspecific branch, and the adaptive or antigen-specific branch. A truly effective immune defence is based on a balance of the different arms of the whole immune system. The human immune response system is very complex and carotenoids have been reported to have effects on many different aspects. To understand the significance of this it is necessary to have a working knowledge of the immune system. An outline of the main features and principles is given below, but the non-specialist reader is recommended to consult a modern biology or biochemistry textbook, or an introductory book on immunology.

Stimulation of a particular immune response does not necessarily translate into improved immune defence or health; it must be taken in context with changes in other immune responses and with the physiological state in question. Any immune stimulation must be significant, yet within the range of a normal response; hyperactivity can mean an autoimmune or immune-mediated disease whilst a hypoactive response can result in immune suppression and incompetence, such as in human AIDS and feline immunodeficiency syndrome.

Inflammation, increased temperature and swelling of the tissue, is a localized non-specific respone to infection or injury. The increased temperature and blood flow facilitate the migration in of neutrophils, monocytes and macrophages to attack the infection. The acute inflammatory response is mediated by the cytokine TNF (tumour necrosis factor).

2. Features of the immune system

a) The innate or antigen-non-specific immune system

The innate immune system is the first line of defence and is composed of barriers such as the skin, gastrointestinal tract and lungs, as well as phagocytic cells and non-cellular components such as lysozymes and complements. If the physical barriers are breached, the body then employs a collection of non-specific cellular and chemical defences that respond to any microbial infection without the need to recognize and identify it. These cells and chemicals circulate throughout the body in the blood and the lymphatic system. The most important of the cells are several forms of white blood cells (leukocytes), which fight invading microorganisms in different ways. In this process, the leukocytes first migrate along a chemical gradient toward the microorganism that has been opsonized (coated with immunoglobulins (Ig) or complements), adhere to it, and attack and destroy it.

Macrophages are large irregular cells that engulf the invader by phagocytosis and kill it with oxygen free radicals produced by lysosomal enzymes. At the site of infection, undifferentiated leukocytes (monocytes) are transformed into additional macrophages. Neutrophils, the most abundant circulating leukocytes, also ingest and kill bacteria by phagocytosis but, in addition, they release oxidizing chemicals that kill other bacteria in the neighbourhood. Eosinophils defend against invading parasites. Natural killer cells do not attack invading microbes directly, but kill cells of the body that have been infected. They kill by creating a hole in the plasma membrane of the target cell, not by phagocytosis. Natural killer cells also attack cancer cells, often before a detectable tumour develops.

These cellular defences are enhanced by the complement system, which consists of approximately twenty different proteins that circulate in the blood. These proteins aggregate into a complex that inserts into the membrane of the marked foreign cell, and forms a pore through which fluids can enter the cell. The complement proteins have various other effects. They can amplify the inflammatory response, attract neutrophils to the site of infection, or coat the surface of the foreign cell to facilitate the attachment of phagocytes. Other important proteins are the interferons (IFN). These are secreted by body cells that have been infected with a virus, and protect neighbouring cells from being infected.

Cell-to-cell adhesion occurs during leukocyte trafficking. The contact, through a pair consisting of intracellular adhesion molecule-1 (ICAM-1) and the leukocyte-function-associated antigens-1 (LFA-1) ligand receptor, serves to co-stimulate an immune response, thereby enhancing cell proliferation and cytokine production. The LFA-1 is a β_2-integrin protein expressed on leukocytes and is involved in the migration of lymphocytes, monocytes and neutrophils. LFA-1 binds to ICAM-1 and ICAM-2 expressed on the vascular endothelium, and controls the migration of lymphocytes into inflammatory sites. The endothelial expression of ICAM-1 is inducible, whilst that of ICAM-2 is constitutive.

b) The adaptive or antigen-specific specific immunity

The specific immune system is mediated by two types of cells that circulate in the lymphatic system, known as T cells and B cells, which direct the cell-mediated and humoural responses, respectively. These lymphocytes, also sometimes referred to as splenocytes, are not themselves phagocytic.

T cells originate in the bone marrow and then migrate to the thymus, where they develop the ability to identify microorganisms and viruses by the antigen molecules exposed on the surface of the invaders. There are different subsets of T cells. Inducer T cells mediate the development and maturation of other T cells in the thymus. Helper T cells (Th) detect infection and initiate both T cell and B cell responses. The Th cells can be sub-divided into Th1 cells, that are important in response to bacterial infection, and Th2 cells, that are important in response to parasite infection. Cytotoxic T cells (Tc) lyse cells that have been infected by viruses. Suppressor T cells terminate the immune response. Unlike T cells, B cells complete their maturation in the bone marrow and do not migrate to the thymus. B cells are specialized to recognize particular foreign antigens. When activated, a B cell becomes a plasma cell that produces specific antibodies.

c) Cell-mediated immune response

When an invading foreign particle, *e.g*. a virus, is taken into a body cell, it is partially digested and the viral antigens thus produced are processed and moved to the surface of the cell, which thus becomes an antigen-presenting cell (APC). At the membrane of the APC the processed antigens are complexed with major histocompatibility complex proteins MHC-II. MHC-II is found only on macrophages, B cells and helper T cells, also known as CD4+ T cells because they have the CD4 surface co-receptor, which interacts only with the MHC-II proteins of another lymphocyte. Cytotoxic T cells (CD8+) have the co-receptor CD8 and can interact only with the MHC-I proteins of an infected cell. The human form of MHC is also known as the human leukocyte-associated antigen (HLA) complex. The T-cell antigen receptor (TCR) recognizes a peptide antigen in conjunction with the MHCII molecule. Dendritic cells, derived from the bone marrow, are the most potent of the antigen-presenting cells. Immature dendritic cells in peripheral tissues capture and process antigens; the maturing dendritic cells then migrate to lymphoid organs where they stimulate naïve T cells *via* TCRs which recognize peptide antigens in conjunction with MHC-II molecules. The degree of immune response is proportional to the number of PACs that possess MHC-II molecules and the density of the latter on the cell surface. Dendritic cells are also highly responsive to inflammatory cytokines such as TNF-α or to bacterial products that induce phenotypic and functional changes.

Activation of a Th cell by such an APC is mediated by soluble regulatory proteins known as cytokines. The most important of these are the interleukins. Interleukin-1 (IL-1) is secreted by macrophages and signals Th cells to bind to the antigen-MHC protein complex. The Th

cells then release IL-2, which stimulates the multiplication of Tc cells that are specific for the antigen. Cytotoxic T cells can only destroy infected cells that display the foreign antigen together with their MHC-I proteins. Interleukin-4 (IL-4), secreted by T cells, stimulates the proliferation of B cells, and thus the humoural response. Cytotoxic T cells will attack any cells recognized as carrying a foreign version of MHC-I. This includes transplanted cells from another individual, and cancer cells that reveal abnormal surface antigens.

d) The humoural immune response

The B cells of the humoural immune system also respond to Th cells activated by IL-1. B cells recognize invading microbes but do not attack them; they mark the pathogen for destruction by macrophages and natural killer cells. The B cells recognize antigens and divide to produce plasma cells and memory B cells, resulting in the circulation of high titres of antibodies against those antigens. Antibodies are immunoglobulin (Ig) proteins, of which there are several subclasses with different structures and functions, namely IgM, IgG, IgD, IgA and IgE. IgM antibodies are produced first and they activate the complement system. Following this, large amounts of IgG antibodies are produced, and these bind to antigens on an infected cell thereby serving as markers that stimulate phagocytosis by macrophages; antibodies do not kill invading pathogens directly.

3. Nutritional intervention

Some physiological or environmental insults can weaken the immune system, resulting in increased risk of infection and disease. Under these conditions, nutritional intervention can be beneficial in modulating the immune response. A variety of immune response tests have thus been developed to assess the effect of nutritional intervention on different aspects of the immune response. These include: (i) gene expression and cell signalling associated with cell-cycle progression and apoptosis (see *Chapter 11*), (ii) lectin-induced lymphocyte proliferation, (iii) NK cell cytotoxic activity, (iv) cytokine production, (v) phenotyping, (vi) Ig production, (vii) delayed type hypersensitivity (DTH), and (viii) phagocytosis and killing ability. The assessment of these immune responses has been aided by the use of techniques associated with flow cytometric analysis, genomics, proteomics and metabolomics.

The interpretation of the results of nutritional intervention studies on immunity must, therefore, consider the whole immune system.

4. Immunity and oxidative stress

Cellular oxidative damage by reactive oxygen species (ROS) has been suggested to be a key factor in numerous chronic diseases (see *Chapter 12*). The ROS destroy cellular membranes, cellular proteins and nucleic acids. Immune cells are particularly sensitive to oxidative stress because their plasma membranes contain a high percentage of polyunsaturated acyl lipids,

which easily undergo peroxidation [1] (see *Chapter 12*). Immune cells rely on cell-to-cell communication *via* membrane-bound receptors; peroxidation of the polyunsaturated acyl chains in the cell membrane, therefore, can lead to the loss of membrane integrity and altered membrane fluidity, resulting in impairment of intracellular signalling and overall cell function. Indeed, exposure to ROS leads to decreased expression of membrane receptors [2].

The ROS can arise from several sources. Immune cells are very active cells and, therefore, generate ROS during normal cellular activity, mainly through their mitochondria. Oxidative stress on the membrane of normal healthy cells can also be caused by oxidizing pollutants and many viruses, factors that are capable of inducing excessive production of ROS. A third source of ROS is from the 'respiratory burst' used by phagocytic cells (macrophages and neutrophils) during the killing of invading antigens. In this oxidative bactericidal mechanism, the NADP oxidase system is activated, and a large amount of superoxide anion ($O_2^{\bullet -}$) is produced from molecular oxygen. The $O_2^{\bullet -}$ is rapidly converted into hydrogen peroxide (H_2O_2) by superoxide dismutase. Neutrophils contain myeloperoxidase that converts H_2O_2 into the highly potent bactericidal component, hypochlorite ion (OCl^-), whilst macrophages generate oxygen-derived free radicals such as the hydroxyl radical ($OH^{\bullet}$). Excess ROS can in turn destroy both the cells that produce them and surrounding cells. Excess ROS can be eliminated by endogenous or dietary antioxidants which together maintain an optimal oxidant:antioxidant balance that is critical for maintaining normal cellular function and health. Tipping this balance in favour of ROS is thought to be a major contributor to several age-related diseases such as cancer, and neurodegenerative, cardiovascular and eye diseases.

Even though ROS are usually portrayed as the villain, research has now demonstrated that they are important signalling molecules involved in the regulation of gene expression, cell growth and cell death. Therefore, the action of antioxidants, including carotenoids, on immune response is anything but straightforward; their actions hang in a delicate balance between the total elimination of toxic ROS on one hand, and the maintenance of an optimal ROS concentration for cell signalling on the other.

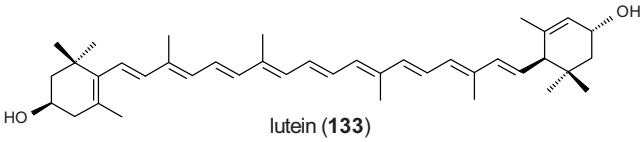
B. Carotenoids and the Immune Response

Immune cells are very active cells and, therefore, generate ROS during normal cellular activity. The mitochondrial electron transport system utilizes approximately 85% of the oxygen consumed by the cell to generate ATP, so mitochondria are the most important source of ROS [3]. Unfortunately, the mitochondria are also a target of the ROS.

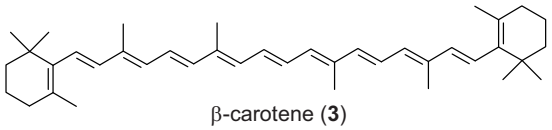
1. Effects of carotenoids

The localization of carotenoids in the mitochondria is of particular relevance as these carotenoids may serve to protect the subcellular organelles of immune cells against oxidative

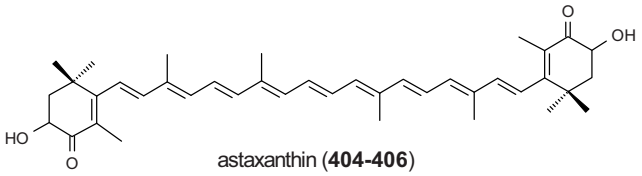
injury. Optimal function of the subcellular organelles ensures that cellular functions, including apoptosis, cell signalling and gene regulation, are optimal. Studies with cats and dogs have shown significant uptake of orally fed lutein (**133**) [4,5], β-carotene (**3**) [6,7], and astaxanthin (**404-6**) [8] into the mitochondria, nuclei, and microsomes of circulating lymphocytes, with the mitochondria showing high total uptake of these carotenoids. Uptake of β-carotene by human neutrophils [9], and by neutrophils and lymphocytes from calves [10] and pigs [11,12], has similarly been demonstrated.



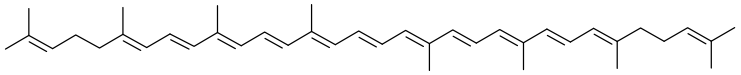
lutein (**133**)



β-carotene (**3**)



astaxanthin (**404-406**)



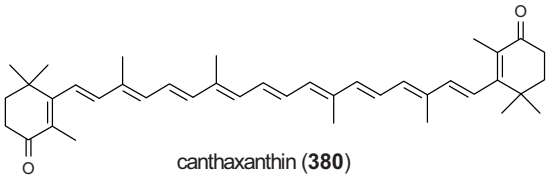
lycopene (**31**)

It is proposed that dietary carotenoids help to protect the immune system from oxidative damage and thereby enhance cell-mediated immune response [13]. Most studies have been with β-carotene, though more now deal with other carotenoids. Earlier reports that lycopene (**31**) prolonged the survival time of bacterially-infected mice [14] and that β-carotene markedly increased the growth of the thymus gland and the number of thymic lymphocytes [15] stimulated the study of the possible immune modulation action of carotenoids.

a) Specific effects

The ability of carotenoids to modulate the embryonic development (ontogenesis) of the immune system begins early during neonatal development. β-Carotene supplementation

significantly changed the percentage and total number of splenic CD3+, CD4+ and CD8+ cells, and IgG production, in mice between days 7 and 14 of age [16]. Lycopene also increased the number of splenic T and B cell subsets, but its immune modulation action occurred at a later time point. In humans, the sequence of events in T lymphocyte development starts during embryogenesis, and is thus comparable to that in mice, so it is possible that dietary carotenoids can influence the ontogenesis of the human immune system.



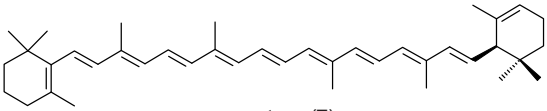
canthaxanthin (**380**)

Numerous studies have reported that high plasma β-carotene status or β-carotene supplementation enhance immune response [17-22]. Supplementation with β-carotene stimulated lymphocyte proliferation in several human intervention studies [19,23,24], and in rats [25], pigs [26], and cattle [27]. In mice, astaxanthin and β-carotene but not canthaxanthin (**380**) stimulated phytohaemagglutinin-induced splenocyte proliferation [28]. Higher lymphocyte proliferation after β-carotene supplementation was accompanied by an increase in specific lymphocyte populations. For example, human adults given β-carotene orally had increased numbers of Th and T inducer lymphocytes [17,29]. Subjects given 60 mg/day β-carotene for 4 weeks showed a slight increase in the number of CD4+ cells [30]. In contrast, another study [31] failed to show significant changes in the number of T cells, lectin-stimulated lymphocyte proliferation, or surface molecule expression, in older subjects (>65 years) who were given β-carotene or lycopene daily for 12 weeks. Short-term supplementation with β-carotene (30 mg/day [32] or 89 mg/day [33]) had no effect on T lymphocyte function in healthy women.

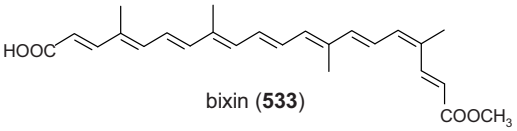
The number of lymphoid cells with surface markers for NK cells and for IL-2 and transferrin receptors also was increased substantially in peripheral blood from individuals after short-term supplementation with β-carotene [17,34]; the NK cells as a percentage of the total increased [17]. The lytic activity of NK cells was increased in elderly but not in middle-aged men on long-term β-carotene supplementation [21]. β-Carotene reversed the age-related decline in NK cell lytic activity in older (65-86 years) male subjects, restoring levels to those of younger (51-64 years) subjects. Similar studies [35] also reported higher NK cell cytotoxicity in human subjects given β-carotene orally. It was concluded that additional low amounts of β-carotene or lycopene are unable to enhance cell-mediated immune response in well-nourished healthy individuals.

Astaxanthin also possesses immune enhancing activity. In mice, astaxanthin stimulated splenocyte proliferation [28]. In a double-blind, placebo-controlled study [36], 2-8 mg

astaxanthin given daily to young healthy human female subjects stimulated mitogen-induced lymphoproliferation, and increased NK cell cytotoxic activity. Astaxanthin also increased the number of total T and B cells but did not influence the sub-populations of Th, Tc or NK cells. There was a heightened DTH response and a higher frequency of cells that expressed the marker LFA-1 in subjects given 2 mg astaxanthin. Delayed type hypersensitivity is a local inflammation occurring 24-48 hours after challenge with an antigen against which the person has previously been immunized. In response to inflammation, plasma concentrations of some proteins, known as acute phase proteins, may increase or decrease. Astaxanthin decreased DNA damage and plasma concentrations of acute phase proteins. In mice, lutein and astaxanthin increased the antibody response of splenocytes *ex vivo* to T-cell antigens [37]. *In vitro*, lycopene directly suppressed the antigen-presenting function of lipopolysaccharide-stimulated bone marrow-derived murine myeloid dendritic cells by down-regulating the expression of co-stimulatory molecules CD80 and CD86, and MHC-II molecules [38]. Dendritic cells treated with lycopene were poor stimulators of naïve allogeneic T cell proliferation and they showed impaired IL-12 production in responding T cells [39]. IL-12 is a pro-inflammatory cytokine and its expression is a specific marker of functionally activated dendritic cells [40,41]. Lycopene, therefore, may control chronic immune and/or inflammatory diseases through down-regulation of dendritic cell maturation.



α-carotene (**7**)



bixin (**533**)

There are also reports that carotenoids can modulate the activity of phagocytic cells, although this has been less well studied. Murine macrophages incubated with canthaxanthin, β-carotene or α-carotene (**7**) had higher cytochrome oxidase and peroxidase activities than did those incubated with (*cis*)-retinoic acid; the highest activity was observed with canthaxanthin [42]. Dietary β-carotene stimulated the phagocytic and killing ability of bovine blood neutrophils [27,43,44]. On the other hand, β-carotene, lutein, bixin (**533**), and canthaxanthin decreased luminol-dependent chemiluminescence generated from rat peritoneal macrophages stimulated by phorbol myristate acetate. This suggests that suppression by carotenoids of the respiratory burst of macrophages represents a way to protect host cells and tissues from the harmful effects of oxygen metabolites that may be overproduced during specific immune response [45].

Studies with dogs and cats have provided direct comparisons of the immune modulation action of several carotenoids. In dogs, dietary β-carotene [46], lutein [47] or astaxanthin [48] stimulated DTH response, the number of CD4+ Th cells, and IgG production, and lutein but not β-carotene enhanced mitogen-induced lymphocyte proliferation [47]. Cats fed astaxanthin [49], β-carotene [50] or lutein [51] also showed heightened DTH response, higher Th and B cell sub-populations, and increased plasma IgG concentrations. These results demonstrated that β-carotene, lutein and astaxanthin can have immune-enhancing activity, especially after antigenic challenge with a vaccine. However, the specific immune response factors modulated may be different for different carotenoids and for different species.

b) Effects of carotenoid-rich foods and extracts

The above studies used pure carotenoids, but the importance of a balance and interaction of different dietary components is recognized. Recent studies, therefore, have used whole foods to provide a more complete array of important carotenoids. Tomatoes and tomato products are rich in lycopene. Tomato intake is inversely related to the risk of diarrhoea and respiratory infections in young children [52]. Tomato juice improved T lymphocyte function in subjects who otherwise consumed low carotenoid diets [53]. In a blinded, randomized crossover study, healthy male subjects on a low carotenoid diet were fed 330 mL/day tomato juice (providing 37 mg/day lycopene) or carrot juice (27 mg/day β-carotene + 13 mg/day α-carotene) for 2 weeks followed by a 2-week depletion period [54]. There was a time-delayed modulation of IL-2, NK cytotoxicity, and lymphocyte proliferation during the depletion period. An earlier study [55] showed no stimulation of cell-mediated immune response in well-nourished elderly men and women fed tomato juice for 8 weeks. Male non-smokers on a low carotenoid diet were given three carotenoid-rich food sources sequentially, each for two weeks, namely first tomato juice (containing 40 mg lycopene + 1.5 mg β-carotene), then carrot juice (22 mg β-carotene + 16 mg α-carotene + 0.5 mg lutein), and finally dried spinach powder (11 mg lutein + 3 mg β-carotene) [56]. Tomato juice, but not carrot juice or spinach powder, enhanced IL-2 and IL-4 secretion. On the other hand, tomato oleoresin, when given to smokers and non-smokers in a double-blind, placebo-controlled randomized study, returned IL-4 production to normal in smokers but had no effect on lymphocyte proliferation, NK cell activity, IL-2 or TNF-α [57]. There was a decrease in DNA strand breaks in both smokers and non-smokers. High levels of circulating IL-4 led to increased susceptibility of smokers to viral or mycobacterial infections [58].

It must be emphasized, however, that these plant materials and extracts would contain a large collection of other phytochemicals, including antioxidants and phytosterols, besides a mixture of carotenoids, so the effects seen cannot safely be attributed to the carotenoids.

c) Model studies of health benefits

Whether the immune regulatory action of carotenoids translates into health benefits has been studied with several biological models such as exercise-induced oxidative damage, photoprotection and *Helicobacter pylori* infection.

i) Exercise-induced oxidative stress. During intense prolonged exercise, oxidative stress (originating from metabolism in the mitochondria, ischaemic-reperfusion injury and phagocytic cell activity) is greatly increased. Intense exercise in sled dogs suppressed T cell and B cell mitogenic response, suppressed the number of MHC-II+ cells, and increased Th cell and B cell populations [59]. Exercise also increased the concentration of acute phase proteins, suggesting a stress-induced response similar to inflammation or infection. Dietary β-carotene, lutein and α-tocopherol, supplemented together, returned to normal the exercise-induced changes in Tc and B cell sub-populations, and the concentration of acute phase proteins. Sled dogs supplemented with antioxidants also had decreased DNA oxidation and increased resistance of blood lipoproteins to oxidation compared to unsupplemented exercised dogs [60].

ii) Exposure to UV light. Exposure to UV light can suppress immune response, but carotenoids have photoprotective properties against this. In young male subjects fed 30 mg/day β-carotene for 28 days before periodic exposure to UV light, no DTH suppression by UV exposure was reported, and the DTH response was inversely proportional to plasma β-carotene concentration [18]. In healthy older males given 30 mg/day β-carotene for 28 days and exposed to UV light, UV-induced DTH suppression was also reduced, but β-carotene was not as protective as it was in younger male subjects [61], perhaps because of lower plasma β-carotene response or higher vitamin E status in the older individuals. Because UV light can inhibit expression of the human MHC protein HLA-DR, and the adhesion molecule ICAM-1 in human cell lines, a carotenoid-induced increase in cell surface molecules may help to explain the ability of β-carotene to prevent a decrease in DTH response after UV exposure.

iii) Helicobacter pylori *infection*. Infection by *Helicobacter pylori* is a major cause of chronic gastritis and is marked by an active inflammatory response due to neutrophilic infiltration. During *H. pylori* infection, the immune response is polarized to a Th1 cell-mediated immune response with the release of IFN-γ which activates phagocytic cells and contributes to mucosal damage [62,63]. Supplementation with astaxanthin led to a decrease in bacterial load and gastric inflammation in infected mice by shifting the T-lymphocyte response from a Th1 response dominated by IFN-γ to a Th1/Th2 response dominated by IFN-γ and IL-4 [64].

C. Carotenoids and Disease

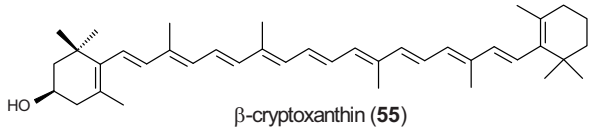
Considerable interest has been generated around the possible use of the immune-enhancing activity of carotenoids in prevention of inflammatory diseases, cancer, and human immunodeficiency disease.

1. Age-related diseases

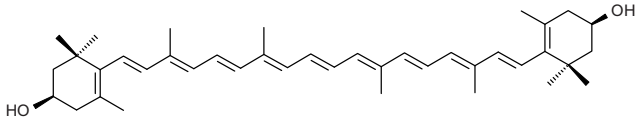
a) Age-related immunity decline

Overall immune response declines with advancing age, thereby increasing susceptibility to infection and a number of age-related conditions such as inflammatory, cardiovascular and neurodegenerative diseases, and cancer. The 'mitochondria theory of aging' states that oxidative damage to DNA, proteins and lipids accumulates in the mitochondria over the lifespan of the organism [3]. Indeed, numerous studies have reported increased oxidative damage to mitochondrial macromolecules with age, resulting in mitochondrial dysfunction and loss of ATP production. Mitochondrial dysfunction can lead to impaired immune response and to neurodegenerative conditions such as Alzheimer's, Parkinson's, Huntington's diseases and Amyotrophic lateral sclerosis. Conversely, antioxidants, which may include carotenoids, can alleviate the harmful effects of the ROS.

It has been reported that supplementation with carotenoids can restore the age-related decline in both cell-mediated and humoural immune responses, in some cases to the levels found in younger individuals. High β-carotene status was associated with decreased incidence of acute respiratory infection (incidence rate ratio = 0.71, 95% CI 0.54-0.92) in elderly individuals compared to those who had low β-carotene [65]. In that study, no similar improvement was observed with α-carotene, β-cryptoxanthin (**55**), lycopene, lutein, or zeaxanthin (**119**). Geriatric dogs had lower Th and B cell sub-populations, lower T cell proliferation, and lower DTH response than age-matched controls and young dogs [66]. However, supplementation with β-carotene and α-tocopherol restored these impaired immune functions in older dogs.



β-cryptoxanthin (**55**)



zeaxanthin (**119**)

b) Neurodegenerative conditions

A large body of evidence has emerged implicating impaired energy metabolism and oxidative damage in Alzheimer's disease [67]. In fact, oxidative damage occurs before the deposition of β-amyloid. In this disease, the inflammatory response is atypical in that there is an absence of an overt leukocyte infiltration [68]. Instead, the major factors include the resident cellular elements such as the microglia and astrocytes. The possible neuroprotective role of dietary antioxidants has been studied. Both β-carotene and vitamin E protected rat neurons against oxidative stress from ethanol exposure [69]. β-Carotene had a greater protective effect than vitamins E or C against neuro-vascular dysfunction [70].

c) Rheumatoid arthritis

Oxidative damage to the synovium can lead to the pathogenesis of rheumatoid arthritis [71]. Rheumatoid arthritis is an immune disorder in which lymphocytes accumulate and organize into lymphoid structures on the synovial surface of the cavities of small joints. CD4+ cells, activated B cells, and plasma cells are found in the inflamed synovium. In a large prospective population-based study with women aged 55-69 years [72], high intakes of β-cryptoxanthin were associated with protection against rheumatoid arthritis. A similar study recently reported that a modest increase in β-cryptoxanthin (equivalent to one glass of freshly squeezed orange juice per day) in human subjects was associated with a lower incidence of developing rheumatoid arthritis.

2. Cancer

Studies both *in vitro* and *in vivo* have reported effects of carotenoids in stimulating immunity against tumour growth. A specific action of β-carotene was reported [73] in augmenting immunity against syngeneic fibrosarcoma cells in mice. *In vitro*, β-carotene and lycopene inhibited the growth of human breast cancer cells; their action was related to the presence of oestrogen receptors [74]. Astaxanthin, canthaxanthin or β-carotene fed to mice injected with a transplantable mammary tumour cell line inhibited tumour growth, with astaxanthin having the highest inhibitory activity. Astaxanthin also inhibited the growth of fibrosarcoma cells and concomitantly increased Tc cell activity and IFNγ production by splenocytes and tumour-draining lymph node [75]. A transplantable mammary tumour model in BALB/c mice has been used to demonstrate the antitumour activity of dietary lutein and to study the mechanism involved. In several studies, dietary lutein consistently inhibited the growth of mammary tumours in mice [76-78]. When a lower tumour load was used, lutein decreased the incidence of tumour development [77]. The presence of a mammary tumour suppressed the populations of total T, Th, and Tc cells, but increased the populations of IL-2Rα+ T cells and B cells compared to those in mice not carrying tumours [78]. However, lutein prevented these

tumour-associated lymphocyte sub-population changes. In addition, lutein increased IFN-γ mRNA expression but decreased IL-10 expression in splenocytes of tumour-bearing mice; these changes were associated with the inhibitory action of lutein against tumour growth [78].

Tumour growth is highly dependent on angiogenesis, *i.e.* formation of small blood vessels to increase blood flow [79]. Without proper neovascularization, tumour cells will lack growth factors and therefore undergo apoptosis. Mice fed lutein had fewer blood vessels associated with their tumours than did unsupplemented mice [80]. Other studies have also demonstrated the ability of carotenoids to reduce tumour blood flow [81].

3. Human immunodeficiency: HIV and AIDS

The human immunodeficiency virus, HIV, is a retrovirus that circulates in the bloodstrean but will specifically infect only CD4+ cells. It has a surface glycoprotein that precisely fits the cell surface receptor protein CD4 on the surface of macrophages. It replicates in the macrophage and is released from the cell by budding. After some years of HIV infection, the surface glycoprotein may undergo mutation to a form that binds the surface receptor of CD4+ T cells. These T cells are destroyed and the immune response is blocked. The consequence of this is the onset of AIDS (Acquired Immunodeficiency Syndrome). The body's defences against infection, cancer, *etc.* are compromised, usually with fatal consequences.

Low levels of carotene and other carotenoids are common in HIV patients and are more marked than any other micronutrient deficiencies [82-84]. In HIV-infected subjects, there is a significant depletion of all carotenoids analysed (lutein, cryptoxanthin, lycopene, β-carotene, α-carotene) but not of vitamin A or E [82]. Low plasma carotenoid concentrations were associated with increased risk of death during HIV infection among infants in Uganda [85]. Also, low serum carotene concentration is positively correlated with severity of the disease; children with AIDS had a greater magnitude decrease in serum carotene than children with HIV infection.

A correlation was demonstrated between serum carotene and both CD4+ cell counts and CD4+/CD8+ ratio in HIV-infected individuals [84]. Healthy individuals given 180 mg β-carotene daily for 14 days had higher CD4+ populations [29]. A transient increase of 60% in lymphocyte counts was reported in AIDS patients given 60 mg β-carotene per day for 4 weeks. Patients with HIV who were given 60 mg β-carotene daily showed a significant increase in leukocyte counts and CD4+:CD8+ ratio [86]. Also, patients administered 60 mg/day β-carotene had higher CD4+ counts and alleviated symptoms of the disease over 24-36 months [87]. Another study [30] showed a slight but not significant increase in CD4+ numbers with supplementation with 60 mg β-carotene daily for 4 weeks.

The potential ability of carotenoids to increase CD4+ lymphocytes has led to studies on the use of carotenoids as immuno-enhancing agents in the treatment of HIV infection. Giving natural mixed carotenoids to patients who had advanced AIDS and were on antiviral therapy improved survival rate [88].

In contrast, elderly women supplemented with 89 mg β-carotene daily for 21 days, or elderly men given 50 mg/day on alternate days for 10-12 years showed no significant changes in lymphocyte subsets [33]. Similarly, there was no significant change in CD4, CD8, or CD11 sub-populations in HIV-positive veterans given 60 mg β-carotene daily for 4 months [34]. Treatment of HIV seropositive subjects with 60-120 mg β-carotene daily for 3-7 months resulted in no improvement in infection or lymphocyte counts [89]. Several clinical trials with β-carotene supplementation failed to show significant or sustained improvement in immune response of patients with HIV infection or AIDS. A combination of β-carotene and vitamin A given daily to women during pregnancy and lactation increased the risk of mother-to-child transmission of HIV [90]. In a smaller study in South Africa where women were given β-carotene and vitamin A daily during pregnancy and at delivery, no beneficial effect on mother-to-child transmission of HIV was observed with the supplements [91]. Multivitamins (B complex, vitamins C and E), administered during pregnancy and lactation, were effective in improving postnatal growth; β-carotene + vitamin A reduced this beneficial effect [92].

Whilst a consistent negative relationship is reported between blood concentrations of carotenoids and HIV infection, carotenoid intervention studies to date have not produced a consistent beneficial effect on the clinical course of the disease. β-Carotene doses used have been very high; perhaps carotenoids can be included at a more optimal dose or other carotenoids can be studied. Studies on the possible action of other carotenoids are lacking.

D. Mechanism of Action

Carotenoids may regulate cell cycle progression, apoptosis and signalling pathways by modulating genes and transcription factors. Mechanisms and the general significance of these effects are discussed in detail in *Chapter 11*. Here only a brief outline will be given of those aspects that seem most relevant to modulation of the immune system. Central to the regulation of redox-sensitive molecular signalling pathways are the mitochondria, which are critical for processing and integrating the pro-apoptotic and anti-apoptotic signals. Mitochondrial dysfunction, therefore, can lead to pro-oxidative changes in redox homeostasis, resulting in an efflux of mitochondrial components, further increasing oxidative stress.

The Bcl-2 protein family, comprising the pro-apoptotic members Bax, Bak, Bad, and Bid, and the anti-apoptotic members Bcl-2 and Bcl-xL, are important in the regulation of apoptosis. The main target site for both groups is in the mitochondria where they facilitate or inhibit the release of cytochrome *c* that is located between the inner and outer mitochondrial membranes. Bcl-2 resides in the outer mitochondrial membrane and prevents the release of cytochrome *c*. On the other hand, the predominance of Bax over Bcl-2 accelerates apoptosis; Bax is inactive until it is translocated to the mitochondria where it binds to Bcl-2 to induce the release of cytochrome *c*, which then activates caspases to bring about apoptosis. Singlet oxygen [93] and nitric oxide [94] activate caspase-8.

Uncontrolled cell proliferation can lead to cancer and autoimmune diseases whereas excessive cell death can lead to neurodegenerative diseases and AIDS. In human leukaemia, colon adenocarcinoma and melanoma cells, β-carotene altered mitochondrial membrane potential ($\Delta\Psi m$) and induced the release of cytochrome *c* [95]. The first evidence for a gene regulatory role of lutein came when mice fed lutein, but not ones fed astaxanthin or β-carotene, showed increased *Pim-1* gene expression in lymphocytes [96]. Mice fed lutein and injected with a mammary tumour cell line had smaller tumours, higher p53 and Bax mRNA expression, lower Bcl-2 expression, and higher Bax:Bcl-2 ratio in tumours [80]. In contrast, lutein down-regulated the tumour-suppressive p53 and Bax mRNA and up-regulated Bcl-2 expression in circulating leukocytes; p53 can induce cell cycle arrest to allow DNA repair or apoptosis. The regulation of apoptotic genes by lutein parallels the observations of apoptosis rate in tumour tissues and leukocytes, lutein decreasing apoptosis in blood leukocytes but increasing apoptosis in tumour cells [80]. These results demonstrate a differential action of lutein on apoptosis in tumour cells and immune cells. Other work has shown similarly that lutein selectively induced apoptosis in transformed but not in normal human mammary cells *in vitro* [97]. In colon cancer cells, β-carotene also decreased the expression of Bcl-2 caused by ROS production [98].

Cyclins are essential for cell cycle progression from G_1 to S-phase (see *Chapter 11*). The D cyclins bind to and activate the cyclin-dependent kinases cdk4 and cdk6; this is promoted by the proteins p21 and p27. Activation of the cyclin-dependent kinases results in the phosphorylation of the Rb protein leading to the release of the E2F transcription factors, resulting in proper G_1/S transition. Lycopene inhibited cell cycle progression in breast and endometrial cancer cells by decreasing cyclin D and retaining $p27^{Kip1}$ in cyclin E-cdk2 complexes [99]. Damage to DNA elicits a complex response mediated by various intracellular and extracellular factors such as p53, abl, Rb, E2F, and growth factors, resulting in cell cycle arrest and apoptosis [100,101]. An abundance of intra-cellular or extra-cellular ROS can result in the over-stimulation of cell signalling mechanisms such as NFκB, resulting in the production of inflammatory cytokines and in inflammatory diseases. NFκB is a redox-sensitive transcription factor induced by TNF-α and IL-1, leading to the generation of ROS [102,103]. β-Carotene inhibited the growth of HL-60 and colon carcinoma cells through sustained NFκB expression and the induction of ROS production and glutathione content [95]. β-Carotene also modulates the activation of another redox-sensitive transcription factor, Ap-1, that is involved in cell growth regulation [104]. Similarly, lycopene inhibited NFκB p65 translocation in murine myeloid dendritic cells, thereby preventing the maturation of these cells [38].

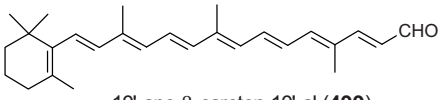
As discussed in *Chapters 11* and *18*, carotenoids can modulate cancer cell growth by modulating the expression of Cox-2 which is involved in carcinogenesis and tumour promotion [105]. Cox-2 is an inducible enzyme [106] and it can act as an anti-apoptotic factor. Its expression is regulated by peroxisome proliferation-activated receptor PPARγ that in turn

is regulated by carotenoids [107]. β-Carotene down-regulated Cox-2 expression in colon cancer cells [108] and this was accompanied by induction of apoptosis, decrease in intracellular ROS production, increase in the activation of ERK1/2, and decrease in production of the prostaglandin PGE2, which is a major prostaglandin synthesized by monocytes and macrophages, and is immunosuppressive. Therefore, carotenoids may alter the arachidonic acid metabolism cascade to suppress PGE2 production.

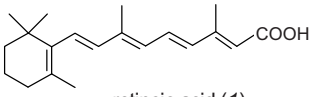
The anticancer action of carotenoids can be mediated through the induction of phase II detoxication enzymes, expression of which is regulated by the antioxidant response element (ARE) and the transcription factor Nrf2 (Nuclear factor E2-related factor 2). Indeed, lycopene, and to a lesser extent β-carotene and astaxanthin, are potent activators of ARE [109]. Induction of phase II enzymes by carotenoids and their metabolites is discussed more fully in *Chapters 11* and *18*.

Evidence has accumulated to show that carotenoids can exhibit pro-oxidant activity, especially at high concentrations and depending on the biological environment in which they act [110,111] (*Chapter 12*). The relevance of this to effects on signalling pathways and apoptosis, and to effects of carotenoids and their oxidative breakdown products on cancer, especially lung cancer in smokers, is discussed in *Chapters 11* and *18*.

Many mechanisms have been proposed by which carotenoids could modulate immune responses. The action of the provitamin A carotenoids could be mediated through their prior conversion to vitamin A and especially retinoic acid (*1*) (see *Chapter 8* and *Volume 4, Chapter 16*). The action of retinoic acid on the immune system is well studied; it can modulate immune cell differentiation and proliferation, apoptosis, and gene regulation. β-Carotene also can be cleaved excentrically to products such as 10′-apo-β-caroten-10′-al (**499**). The biological actions of the apocarotenoids are discussed in *Chapter 18*; it is not known if they have any effects on the immune system.



10′-apo-β-caroten-10′-al (**499**)



retinoic acid (*1*)

E. Summary and Conclusions

Carotenoids in general have been shown to improve cell-mediated and humoural immune response in healthy individuals. Improvements in immune responses following supplementation with carotenoids are observed more consistently when the immune system is compromised or is antigenically-challenged, conditions associated with age-related immune suppression, inflammation and disease states, and exposure to environmental pollutants. Carotenoids modulate many facets of the immune system, notably lymphocyte proliferation

and cytotoxic activity, cytokine and Ig production, cutaneous DTH response, and phagocytic cell activity. The actions of carotenoids are mediated through their ability to regulate ROS in the immediate cellular environment, ultimately modulating shifts in immune cell sub-populations, and to regulate the expression of genes and gene products that are associated with cell signalling, cell-cycle progression and apoptosis. Interpretation of results is challenging; the interrelationships between pathways, and the effects of carotenoids, are complex (see *Chapter 18*, Fig. 2).

Animal studies have produced more consistent results than have human nutrition intervention studies, perhaps because of the greater ability to control dietary manipulations and the lower genetic variations in animals. Inconsistent results among studies have largely been due first to differences in the particular carotenoids used, with different carotenoids exerting somewhat different but overlapping immune modulation action and, second, to the carotenoid dose and duration of supplementation used, with high carotenoid amounts exerting effects opposite to those of an optimal dose. The source/matrix of the carotenoids (pure form *versus* whole food) which is related to the interaction of one carotenoid with another or with other food components, and the different uptake efficiency of a particular carotenoid into blood and tissue in different species are also significant factors.

Interpretation of an immune response must consider the physiological state of the individuals, *i.e*. healthy *versus* disease state, or young *versus* aged. Stimulation of certain aspects of immune function is generally considered desirable, but over-stimulation can be harmful. A strategy for alleviating immune suppression and inflammation, and slowing progression of the associated diseases, would involve limiting the over-production of ROS.

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