

β -Carotene and the immune response

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There have been consistent findings in over thirty epidemiological studies that high intakes of carotenoid-containing foods and high serum β -carotene levels are associated with lower incidences of certain cancers, especially lung cancer. One possible mechanism for the chemoprevention may be enhancement of immune responses (Bendich & Olson, 1989). The immuno-enhancement may be due to an increased vitamin A status from provitamin A carotenoids, or involve the antioxidant and singlet oxygen-quenching capacities of carotenoids having nine or more conjugated double bonds or involve other mechanisms as yet undetermined.

The present review examines the effects of carotenoids on many aspects of immune function in normal animals as well as in tumour-bearing animal models with the objective of elucidating the non-vitamin A effects of carotenoids. In addition, the recent findings of direct effects of carotenoids on decreasing malignant transformations are discussed.

HISTORICAL PERSPECTIVE

Moore (1930) reported that β -carotene was a precursor of vitamin A in animals. In the same year, Green & Mellanby (1930), using vitamin A-deficient rats, found that dietary carotenoids could overcome bacterial infections. The level of carotenoids needed to keep the rats healthy and free of infections was greater than that required to stimulate growth in the vitamin A-deficient rats (Green & Mellanby, 1930). Clausen (1931), examining children with respiratory infections, found that supplementing the diet with carotenoids reduced the number and severity of these infections.

The immediate explanation for these findings was that the carotenoids were sources of vitamin A and that vitamin A was actually the immuno-enhancing, anti-infective agent. However, in the 1950s, investigators at the Karolinska Institute, in an attempt to extract an anti-bacterial substance from non-pathogenic bacteria, discovered that the tomato juice used in the culture media contained an effective anti-bacterial agent (Lingen, 1958). Further characterization of the lipophilic fraction resulted in the discovery that the lycopene in the tomato juice was the active agent (Lingen *et al.* 1959). Lycopene, a carotenoid which lacks vitamin A activity, yet is a potent antioxidant and singlet O₂ quencher, was extensively tested for its ability to prolong survival of mice following bacterial infection. Pure, synthetic crystalline *all-trans* lycopene was compared with that extracted from tomatoes and both preparations, when injected intraperitoneally, increased the resistance of mice to bacterial infections with *Klebsiella pneumoniae*. A single injection of 1 mg lycopene/ml 24 h before infection allowed mice to survive a dose of 10⁻⁴ of the lethal (LD 50) dose of bacteria for 76 h whereas the control mice only survived for 76 h when the dose was 10⁻⁸ of the LD 50 dose; effectively 1000-fold increase in resistance in the lycopene-treated group (Table 1).

Vitamin A was ineffective and the *cis*-isomer of lycopene was approximately 50% as effective as the *trans*-isomer. Oral administration of the lycopene suspension was also not

Table 1. *Effect of lycopene on survival of bacterial infections*
(adapted from Lingen *et al.* 1959)

	Dilutions of LD 50 of <i>Klebsiella pneumoniae</i>			
	10 ⁻⁴		10 ⁻⁸	
	Period post injection (h)	Survival	Period post injection (h)	Survival
Control	32	0/6	76	1/6
Lycopene (1 mg/ml, i.p.)	76	2/6	76	6/6

LD, lethal dose; i.p., intraperitoneally.

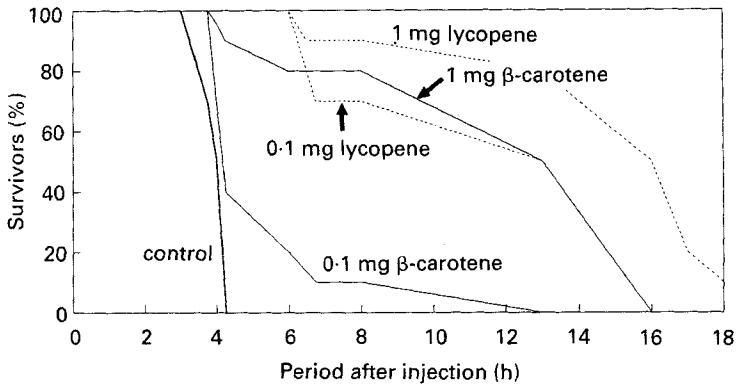


Fig. 1. Effect of carotenoids on survival of mice following an injection of *Klebsiella pneumoniae*. Adapted from Lingen *et al.* (1959).

effective (Lingen, 1958). β -Carotene was also protective, but not as potent as lycopene (Fig. 1). Crocetin, bixin and crocin (other non-vitamin A carotenoids) showed protection.

Lycopene administration before an initial infection also increased resistance to subsequent infections even though lycopene was not given at the time of re-infection. Lycopene administration before X-irradiation protected mice from lethal bacterial infections which killed irradiated, infected control mice (Forssberg *et al.* 1959). Thus, the first suspicion of a non-vitamin A effect of carotenoids on enhancement of immune functions was reported in 1958.

The pioneering work of the Karolinska group also included a thorough examination of the effects of lycopene administration on the growth of Ehrlich ascites tumour cells in mice (Lingen, 1958; Lingen *et al.* 1959). Pretreatment with lycopene increased the time to tumour development and survival time (Fig. 2). Injection of lycopene following the administration of low concentrations of tumour cells also retarded the growth of the tumours. The authors state that other ascites tumours, but not all such tumours, were affected similarly and suggest that tumours with greater antigenicity were more susceptible to growth retardation by lycopene.

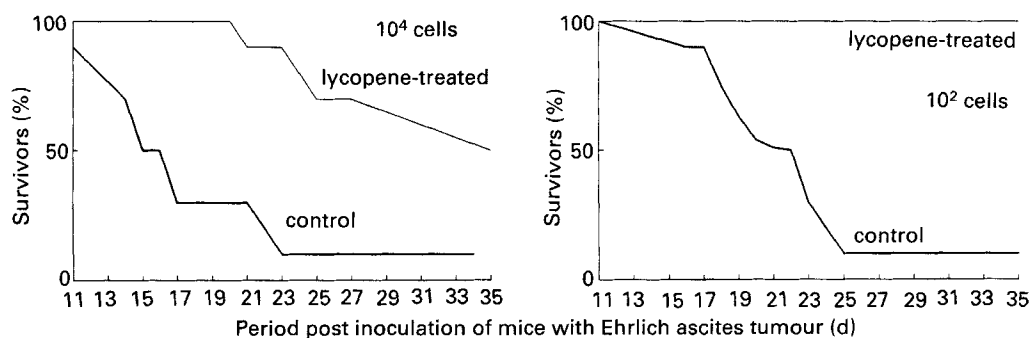


Fig. 2. Effect of lycopene on survival of mice injected with Ehrlich ascites tumour cells at different dosage levels. Adapted from Lingen *et al.* (1959).

The research with carotenoids was abandoned because of an inability to determine a mechanism of action at that time (L. Ernster, personal communication). It was not until 30 years later that investigators knowledgeable of the antioxidant and singlet O_2 -quenching capacities of carotenoids re-examined the same phenomena described by these early investigators (Krinsky, 1989). The newest research, discussed later, tends to suggest that carotenoids not only affect the activity of immune cells, but act directly on tumour cells to alter their metabolic activities. These effects may or may not involve the antioxidant activities of carotenoids. There may be other mechanisms of action.

SIMILAR EFFECTS OF β -CAROTENE AND NON-VITAMIN A CAROTENOIDS ON IMMUNE RESPONSES

The similar effects of β -carotene and vitamin A on the organs and functions of the immune system have been previously described (Bendich, 1988, 1989, 1990). Since vitamin A is an essential nutrient and a well-recognized immuno-enhancing agent, experiments to determine the potential non-vitamin A-associated, immuno-enhancing effects of β -carotene required a vitamin A-replete animal. β -Carotene immuno-enhancement, therefore, would have to be measured as activity above and beyond that seen with vitamin A.

If carotenoids enhance immune response separate from any vitamin A activity, then β -carotene, the most efficient precursor of vitamin A, and a carotenoid-lacking vitamin A activity should show the same profile of immuno-enhancement. Canthaxanthin is very similar to β -carotene in chemical structure; however, due to the 4,4'-oxo configuration, canthaxanthin does not serve as a precursor for vitamin A in mammals. The chemical structure of canthaxanthin which includes nine conjugated double bonds, allows it to act as an antioxidant and singlet O_2 quencher (Fig. 3).

T- AND B-LYMPHOCYTE PROLIFERATION

In our research, animals were fed on nutritionally complete diets which were then supplemented with either the Roche® water-soluble beadlets containing β -carotene, canthaxanthin or placebo beadlets (2 g/kg diet; Bendich & Shapiro, 1986). T- and

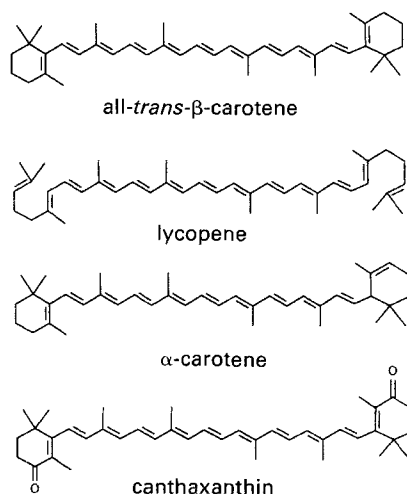


Fig. 3. Chemical structures of various carotenoids. β -Carotene has the potential to form two molecules of retinal; α -carotene can form one molecule of retinal; lycopene and canthaxanthin are non-vitamin A carotenoids.

B-lymphocyte proliferative activity was measured following stimulation of these cells with mitogenic agents. This assay mimics the cell-mediated immune response which is elicited when the immune system is challenged by a pathogenic organism.

Both T- and B-lymphocyte proliferative responses were enhanced when the diets contained either of the carotenoids (Fig. 4). The profile of the immuno-enhancement was similar to the T-cell mitogens and to the B-cell mitogen tested.

Two important points must be made concerning the degree of immuno-enhancement seen in these experiments. First, the diet was nutritionally complete and, therefore, further enhancement of immune responses following the carotenoid supplementation would not be expected to be as great as that seen when a nutritionally deficient and consequently immunosuppressed animal is given a nutritionally complete diet. Second, the animals used in these experiments were healthy and growing and presumably immunocompetent. Therefore, one would not expect carotenoid supplementation to result in a much higher level of immuno-enhancement. However, significant immuno-enhancement was observed for T- and B-lymphocyte responses at numerous time-points with both carotenoids.

Results from serum and tissue analyses clearly showed that canthaxanthin was not converted to vitamin A. It was, therefore, concluded that the immuno-enhancement seen in these experiments by both carotenoids was due to a carotenoid effect separate from any provitamin A activity.

T-HELPER CELL LEVELS

T-helper cell numbers were significantly increased in individuals given β -carotene supplementation (180 mg/d; normal intake is approximately 1–3 mg/d) for 2 weeks (Alexander *et al.* 1985). The authors suggest that this enhancement may be beneficial to those suffering from AIDS since this population of T-cells is the target for AIDS virus infection and destruction.

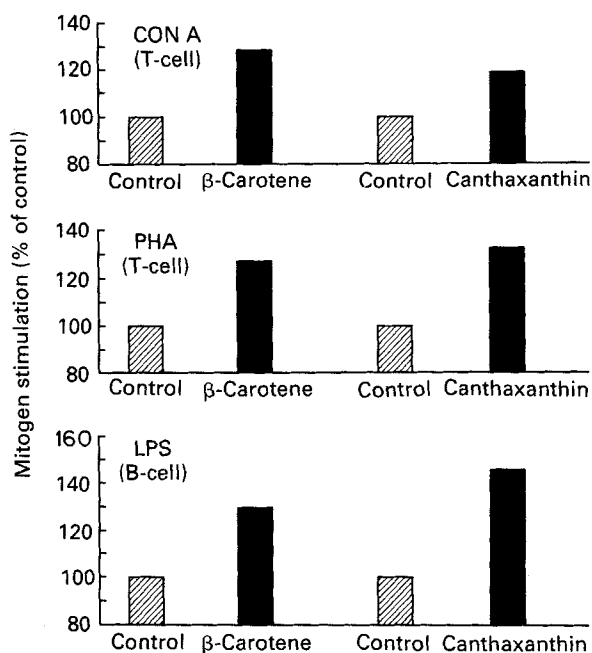


Fig. 4. Effect of dietary intake of carotenoids (2 g/kg diet, in form of beadlets) on the proliferative responses of rat splenocytes to T- and B-cell mitogens. Adapted from Bendich & Shapiro (1986). Con A, concanavalin A; PHA, phytohaemagglutinin; LPS, lipopolysaccharide.

MACROPHAGE FUNCTIONS

Macrophages are responsible for antigen presentation to lymphocytes required for specific cell-mediated immune responses. Oxidative damage caused by free radicals or other reactive O_2 intermediates generated during inflammatory responses have been shown to decrease the macrophage membrane receptors. β -Carotene and canthaxanthin together inhibited the loss of macrophage receptors following exposure to reactive O_2 intermediates (Gruner *et al.* 1986).

Macrophages also recognize the presence of tumour cells and present tumour antigens to lymphocytes. Subsequent production of macrophage cytokines such as tumour necrosis factor- α and the enhancement of tumour cell cytotoxicity have been shown in hamsters pretreated or given β -carotene or canthaxanthin following tumour induction, or both (Shklar & Schwartz, 1988; Schwartz & Shklar, 1989; see also discussion on p. 269). Abril *et al.* (1989) have reported the secretion of a novel cytokine with cytotoxic capacity when human peripheral blood mononuclear cells are cultured with β -carotene. Retinol and retinoic acid can inhibit macrophage activities which are stimulated by interferon. β -Carotene overcomes the inhibitory activity of retinol and retinoic acid on macrophage functions (Rhodes, 1983; Rhodes *et al.* 1984).

NATURAL KILLER CELL CYTOTOXICITY

Natural killer cells from human peripheral blood killed significantly more tumour cells when incubated with β -carotene than human cells not exposed to β -carotene.

Table 2. *Carotenoids and human natural killer (NK) cells*

Effects	References
In vitro exposure	
Increased markers of NK cells	Prabhala <i>et al.</i> (1989)
Increased killing of tumour cells	Leslie & Dubey (1982)
In vivo following supplementation	
Increased markers of NK cells in elderly	Watson <i>et al.</i> (1990)
Increased markers of NK cells in individuals with precancerous lesions that regressed	Garewal & Shamdas (1991)
Increased killing of tumour cells by NK cells from vegetarians	Malter <i>et al.</i> (1989)

α -Carotene also enhanced tumour killing (Leslie & Dubey, 1982). Both these carotenoids have vitamin A activity. Vegetarians, whose vitamin A, vitamin E and vitamin C serum levels were equivalent to non-vegetarians, but whose β -carotene serum levels were twice as high as non-vegetarians, had twice the natural killer cell activity as the non-vegetarians (Malter *et al.* 1989).

Peripheral leucocyte cells from eleven adults, when cultured with 1 nM- β -carotene or 1nM-canthaxanthin for 72 h showed enhanced levels of cell surface markers indicative of natural killer cells, such as increases in surface markers for human leukocyte antigen-Dr, transferrin and interleukin 2 receptor (Prabhala *et al.* 1989). Similar enhancement in these markers was also seen when elderly individuals were given β -carotene (30 mg/d for 2 months) (Watson *et al.* 1991). Increases in circulating natural killer cells from 4 to 28% were also noted when smokers with precancerous oral lesions were given 30 mg β -carotene/d for 3–6 months. The precancerous lesions regressed during the supplementation period (Garewal & Shamdas, 1991).

The effects of carotenoids on human natural killer cells discussed previously are summarized in Table 2.

SKIN TUMOURS

β -Carotene supplementation has been shown to protect against photosensitivity associated with genetically inherited skin diseases such as erythropoietic protoporphyria (Mathews-Roth, 1989). Carotenoids are present in human skin and their concentration is decreased following exposure to u.v.-A and u.v.-B (White *et al.* 1988). Exposure to u.v. light also depresses certain immune functions, including delayed hypersensitivity (Kim *et al.* 1990). Studies in laboratory animals suggest that β -carotene as well as carotenoids lacking vitamin A activity can protect against skin cancer (Mathews-Roth, 1989). Skin tumours are more antigenic than many other types of cancers and individuals who are immunosuppressed, such as renal-transplant patients, have an increased risk of skin cancer (Greenwald & Greenwald, 1983).

Recent laboratory studies utilizing skin tumour cells have indicated that carotenoids are capable of enhancing tumour immunity. A subcutaneous injection of squamous carcinoma cells was administered to the cheek pouch of hamsters. Local injection of β -carotene to the same cheek pouch given the tumour cells significantly decreased the

incidence and lowered the number of tumours which developed per cheek pouch. Macrophages taken from tumour-bearing hamsters treated with β -carotene killed more of the tumour cells *in vitro* than macrophages from tumour-bearing controls. In addition, the ability of the macrophages to generate tumour necrosis factor (TNF) was enhanced significantly in the β -carotene-treated group. Administration of canthaxanthin resulted in similar decreases in tumour burden and increases in TNF whereas 13-*cis*-retinoic acid, a metabolite of vitamin A, increased tumour growth. These investigators have also shown that oral administration of carotenoids can protect against the formation of tumours in the hamster cheek pouch induced by several cancer-causing agents (Schwartz *et al.* 1990*a,b*).

OTHER TUMOURS

Fibrosarcoma cells were subcutaneously injected into mice which were then orally administered β -carotene (Roche® beadlets) or placebo for 9 d (Tomita *et al.* 1987*a*). The primary tumour was excised and a second injection of the same tumour or a different tumour was given. Secondary challenge of the immune response to the initial tumour was enhanced significantly in the β -carotene group. The tumours were half the size of those in the placebo group. The groups given β -carotene and injected with a different tumour the second time had tumours which were the same size as those found in the placebo group.

The mechanism proposed for the decreased tumour size in the β -carotene group challenged with the same tumour involves the generation of tumour-specific cytotoxic T-lymphocytes and the subsequent recognition and lysis of the tumour cells by tumour-specific cytotoxic T-lymphocytes. Mice given β -carotene and then challenged with a different tumour following excision of the primary tumour would, presumably, also have developed cytotoxic T-lymphocytes. However, these would kill only tumour cells with the same antigenic profile as the primary tumour. Therefore, the specificity of the cytotoxic response precludes the lysis of tumour cells with different antigenicity.

In an extension of this protocol, tumour-bearing mice, either given β -carotene or placebo, were killed. Lymph node cells were removed and then mixed with fresh tumour cells. The cell mixture was then inoculated into healthy mice. Those mice receiving lymph node cells from β -carotene-treated mice mixed with tumour cells had one-seventh the tumour burden of mice given lymph node cells from placebo-treated mice mixed with tumour cells. If the lymph node cells from the β -carotene groups were exposed to a substance that destroyed cytotoxic T-lymphocyte activity, and then were injected with tumour cells into healthy mice, the tumour growth was no longer curtailed.

These studies were repeated with carotenoids lacking provitamin A activity. Both canthaxanthin and astaxanthin significantly reduced the size of tumours formed following the challenge dose and decreased tumour growth following transplant of lymph node cells from carotenoid-treated mice (Tomita *et al.* 1987*b*).

In another protocol utilizing the transfer of immune cells (from the spleen rather than lymph nodes), Gensler (1989) examined the effects of vitamin A in combination with canthaxanthin on the immune responses of tumour-bearing mice. Four groups of mice were given either a canthaxanthin-free, low-dose vitamin A diet (control); canthaxanthin-free, high-dose vitamin A diet; high-dose canthaxanthin, low-dose vitamin A diet or high levels of both compounds for 18 weeks before u.v.-B irradiation

which continued for 27 weeks. Exposure to u.v.-B for this extended period has been shown to elicit suppressor cells in the spleen. Spleen cell suspensions from mice in the four groups were transferred to mice simultaneously inoculated with tumour cells. The tumours in the mice given spleen cells from donors fed on the high-dose canthaxanthin and vitamin A diet were half the size of those seen in control mice. Either compound alone lowered the tumour burden, but not significantly. The effect of the combination seemed to be additive, suggesting that vitamin A and the non-vitamin A carotenoid act to enhance different aspects of tumour immunity.

AUTOIMMUNITY

Tomita *et al.* (1990) report that administration of β -carotene or astaxanthin to autoimmune-prone MRL mice resulted in the reduction in the lymphadenopathy and prolonged the lifespan.

POTENTIAL MECHANISMS OF ACTION OF CAROTENOIDS ON IMMUNE FUNCTIONS

β -Carotene and a number of non-vitamin A carotenoids enhance many aspects of immune function including proliferation, induction of specific effector cells as well as the secretion of cytokines required for the communication between immunologically competent cells. The mechanisms for immuno-enhancement may include the quenching of reactive O_2 intermediates which are immunosuppressive and can in fact be immunotoxic. When O_2 intermediates react with lipids, the lipid peroxides generated are also immunosuppressive. Peroxidation of cell membrane lipids can decrease membrane fluidity and, thus, depress proliferation. Membrane receptors, required for antigen recognition, can also be damaged by peroxidation of membrane lipids. Reactive O_2 intermediates can generate and are products of the arachidonic acid cascade. Certain prostaglandins and leukotrienes depress immune responses. Carotenoids may affect immune function because of their antioxidant and singlet O_2 -quenching capacities (Bendich, 1989, 1990).

DIRECT ANTI-MUTAGENIC EFFECTS OF CAROTENOIDS

Renner (1985) showed decreased chromosomal abnormalities in bone-marrow cells from hamsters given β -carotene (250 mg/kg body-weight) and direct-acting mutagens compared with hamsters given the mutagens alone. Vitamin A did not protect bone-marrow cells from the mutagens. The author suggests that β -carotene could protect against mutagens that do not require metabolic activation, since β -carotene did not protect against cyclophosphamide-induced mutagenicity. However, Belisario *et al.* (1985) using diets containing 500 mg β -carotene/kg body-weight, saw a significant decrease in urinary mutagens following exposure to cyclophosphamide. Similar decreases in the mutagenicity of this agent were also seen *in vitro*, suggesting that β -carotene may block the oxidation of cyclophosphamide to its active, mutagenic metabolite. Raj & Katz (1985) found that mice given diets supplemented with β -carotene had less chromosomal breaks in bone-marrow cells following exposure to either benzo(a)pyrene or mitomycin C compared with controls. Both these mutagens require metabolic activation.

Table 3. *In vivo anti-mutagenic actions of carotenoids*

Carotenoid	Activity	Reference
β -Carotene	Decreased chromosome abnormalities	Renner (1985)
Vitamin A	No effect	
β -Carotene	Decreased urinary mutagens	Belisario <i>et al.</i> (1985)
β -Carotene	Decreased chromosome breaks	Raj & Katz (1985)
β -Carotene	Decreased micronuclei	Stich <i>et al.</i> (1985)

Stich *et al.* (1985) found that the number of abnormal micronucleated cells in the oral mucosa of individuals with precancerous lesions was significantly reduced following administration of 180 mg β -carotene/week for 10 weeks in vitamin A-sufficient subjects, but no effects of β -carotene in presumably vitamin A-deficient smokers (Stich *et al.* 1984).

Pung *et al.* (1988), using labelled β -carotene or canthaxanthin, found that both compounds inhibited chemically- and radiogenically-induced transformation of tumour cells (10T1/2 cells) in vitro without being metabolized to vitamin A. The carotenoids were non-toxic and when washed from the cultures, the tumour cells reverted to the neoplastic morphology.

He & Campbell (1990) examined the effects of β -carotene and cryptoxanthin, which have provitamin A activity, and lycopene and canthaxanthin, which lack provitamin A activity, on aflatoxin B₁ activation to a mutagenic agent. The carotenoids, other than lycopene, significantly inhibited mutagenic activity. β -Carotene and canthaxanthin inhibited the activation of aflatoxin to the mutagenic metabolite whereas cryptoxanthin inhibited the phenotypic expression phase of mutagenesis; there was no evidence of conversion of the carotenoids to vitamin A.

Hazuka *et al.* (1990) hypothesize that β -carotene may reduce transformation by lowering the adenylate cyclase (EC 4.6.1.1) activity of cancer cells. In vitro addition of β -carotene to melanoma cells in culture decreased their transformation via induction of differentiation while decreasing the activities of enzymes associated with adenylate cyclase activity. Adenylate cyclase is involved in the regulation of cellular differentiation.

An alternate mechanism of action has been suggested by the recent findings of Schwartz *et al.* (1990b) that carcinoma cells are inhibited from proliferation and synthesize a specific heat-shock protein when cultured with β -carotene or canthaxanthin. Disruption in mitochondrial respiratory activities has previously been linked to the synthesis of heat-shock proteins (Pelham, 1986); Schwartz *et al.* (1990b) suggest that β -carotene alters the metabolic state of tumour cells resulting in the synthesis of a regulatory molecule that curtails proliferation.

The in vitro and in vivo findings of the previously described studies are summarized in Tables 3 and 4.

CONCLUSIONS

The carotenoids are important constituents of diets that include brightly coloured fruits and vegetables. Until recently, the major function attributed to carotenoids was that of precursor of vitamin A. Early findings of enhanced resistance to bacterial infections in

Table 4. *In vitro* anti-mutagenic activity of carotenoids

Carotenoid	Activity	Reference
β -Carotene Canthaxanthin	Inhibit transformation 10 T 1/2 cells	Pung <i>et al.</i> (1988)
β -Carotene Cryptoxanthin Canthaxanthin	Inhibit activation and mutagenesis of aflatoxin B ₁	He & Campbell (1990)
Lycopene	No effect	
β -Carotene	Inhibits transformation of melanoma cells	Hazuka <i>et al.</i> (1990)
β -Carotene Canthaxanthin	Inhibit transformation of carcinoma cells	Schwartz <i>et al.</i> (1990a)

children given β -carotene-rich foods were attributed to the vitamin A potential rather than any separate carotenoid immuno-enhancement (Clausen, 1931). However, the findings of Lingen (1958) and Lingen *et al.* (1959) provide strong evidence that carotenoids enhance immune responses independent of any vitamin A activity. More research into the resistance-enhancing effects of carotenoids is strongly recommended.

In the interim, epidemiological studies have linked diets rich in carotenoids with significantly lower risk of many cancers. The importance of the immune system in cancer prevention and the association of carotenoid chemoprevention with carotenoid immuno-enhancement is a very recent occurrence. Thus, research in the 1980s has sought to define which immune effector cells and activities are affected by carotenoids. To date, this list includes lymphocytes, macrophages, natural killer cells and others. Synthesis and secretion of tumour cytotoxic factor(s) by immune cells is also enhanced by carotenoids.

Research to demonstrate whether carotenoid immuno-enhancement involves the quenching of free radicals or the lowering of lipid peroxide levels, and alterations in membrane fluidity or changes in the activation of the arachidonic acid cascade, or both, are strongly encouraged. β -Carotene has been shown to suppress the generation of arachidonic acid products *in vitro* from non-lymphoid tissues (Halevy & Sklan, 1987).

There is also growing evidence that carotenoids can directly affect cancer cells. Mechanisms of action may include free radical and singlet O₂ quenching which would result in lowered damage to DNA; decreased adenylate cyclase activity, which would depress proliferation; generation of regulatory protein(s) that could alter cell cycles and metabolism, and other mechanisms as yet unknown. Carotenoids have been shown to protect cells from mutagens; however, the exact mechanisms have not been completely determined. With the increased awareness of their importance, there is certain to be further research on the protective effects of the carotenoids against cancer.

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