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The effects of retinoids and carotenoids on the *in vitro* function of human monocytes treated with ultraviolet light

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The University of Arizona, 1987

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The Effects of Retinoids and Carotenoids on the in vitro Function of Human Monocytes Treated with Ultraviolet Light

by

David Jay Schoen IV

A Thesis Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

In Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE
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In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 8 7

Statement by the Author

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ABSTRACT

Human peripheral blood monocytes provide a model for the *in vivo* exposure to, and immune functional damage caused by chronic UVB exposure at the skin surface. Retinoids and carotenoids are known immune function enhancers; they can also prevent cellular toxic product formation caused by UVB exposure. Application of these compounds *in vitro* may prevent functional damage to monocytes. Monocytes were exposed *in vitro* to UVB, then assayed for cytotoxic, phagocytic, and antigen presenting abilities. Phagocytic activity was protected from UVB damage by exposure to these compounds; cytotoxic activity was not altered by UVB exposure, but increased by exposure to the compounds. Antigen presentation was not affected by either the UVB or exposure to the compounds. Protection of phagocytic function was not due to release of activating monokines or prostaglandins. Instead, the cell membrane antioxidant properties of these retinoids and carotenoids were the factors that protected the monocyte from phagocytic damage caused by UVB exposure.

Chapter 1

INTRODUCTION

The purpose of the present study is to examine what connections there may be between the functional impairment of monocyte function caused by ultraviolet-B (UVB) exposure, and the immune enhancing and UVB energy quenching properties of the retinoids and carotenoids. Three areas of monocyte function will be investigated: immune phagocytosis, ability to lyse tumor target cells, and the ability to present antigen to autologous T cells, all *in vitro*. Many studies have shown at least some of these immune functions to be decreased by UVB exposure. Perhaps the use of retinoids and carotenoids *in vitro* can mitigate some of the damage that UVB can have on the immune function of the monocyte.

Effects of Carotenoids and Retinoids on Immune Function

Structures and biological functioning

There is increasing evidence that dietary consumption of retinoids and carotenoids can have effects on immune system functions *in vivo*. The parent compound, vitamin A (retinol) has many natural and synthetic analogs, collectively termed retinoids. Among the most biologically active and widely used retinoids are 13 *cis*

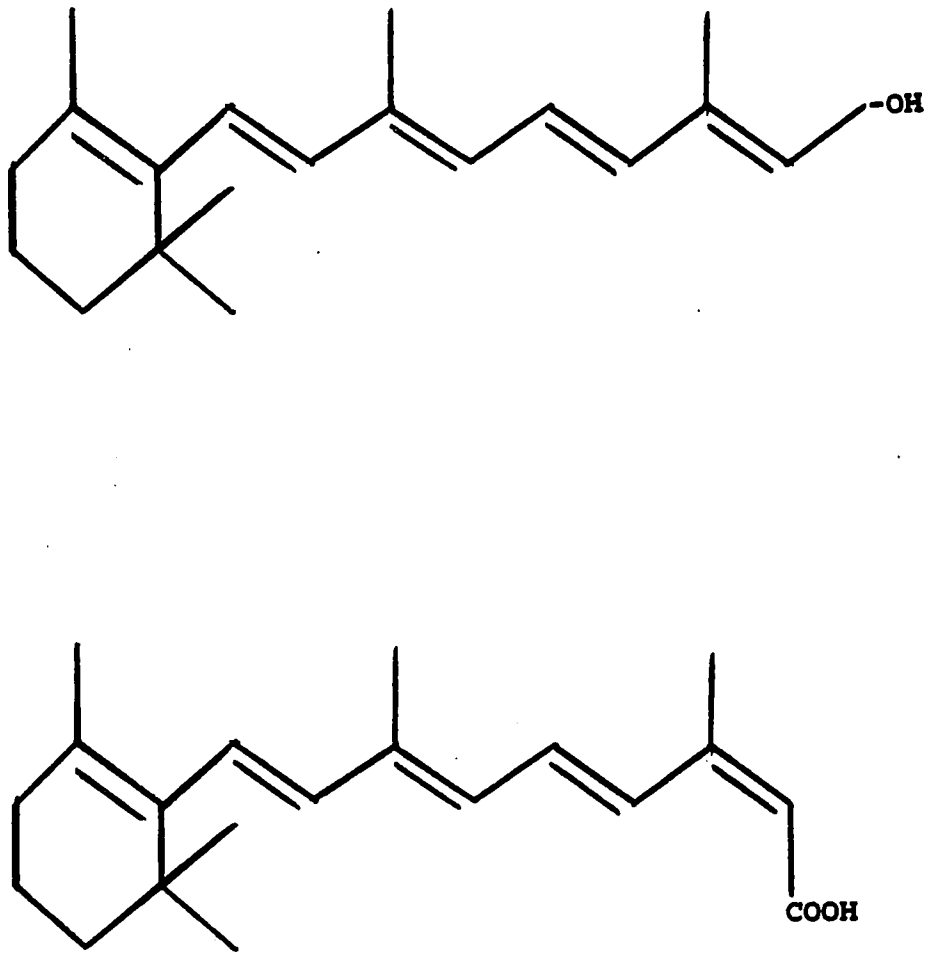

Figure 1. Structures of retinol (Top) and 13 cis Retinoic Acid (bottom).

Modified from Lotan (34).

retinoic acid (13CRA), retinol(ROL), retinal(RAL), retinyl palmitate (RP) and retinoic acid (RA). The biological activity of each of these retinoids depends mainly on the structure of the three main areas of the molecule: these include a cyclic end group, a tetraene chain, and polar end group, as seen in figure 1. Any of these regions can be altered to give literally hundreds of possible new retinoids (34).

The biological activity of any retinoid is very dose dependent, with all retinoids being toxic at higher concentrations in vivo, especially retinol (34). Retinol is the only retinoid that can be stored for long periods in the body; all other types are metabolized at different rates and excreted. The amount of liver storage of retinoids also varies widely between individuals (71).

The carotenoids are pro-vitamin A compounds, which include the carotenes, xanthophylls, lycopenes and their related compounds (59). They are present in all photosynthetic tissues, and in yellow, orange and red plant and animal pigments. These represent the major sources of vitamin A in the human diet (59). The carotenes have an 18 carbon chain of conjugated double bonds that have at each end a 6 carbon ring. Hundreds of variations have been identified; the main therapeutic compounds include beta-carotene (BC), canthaxanthin (CX), and lycopene (LYC).

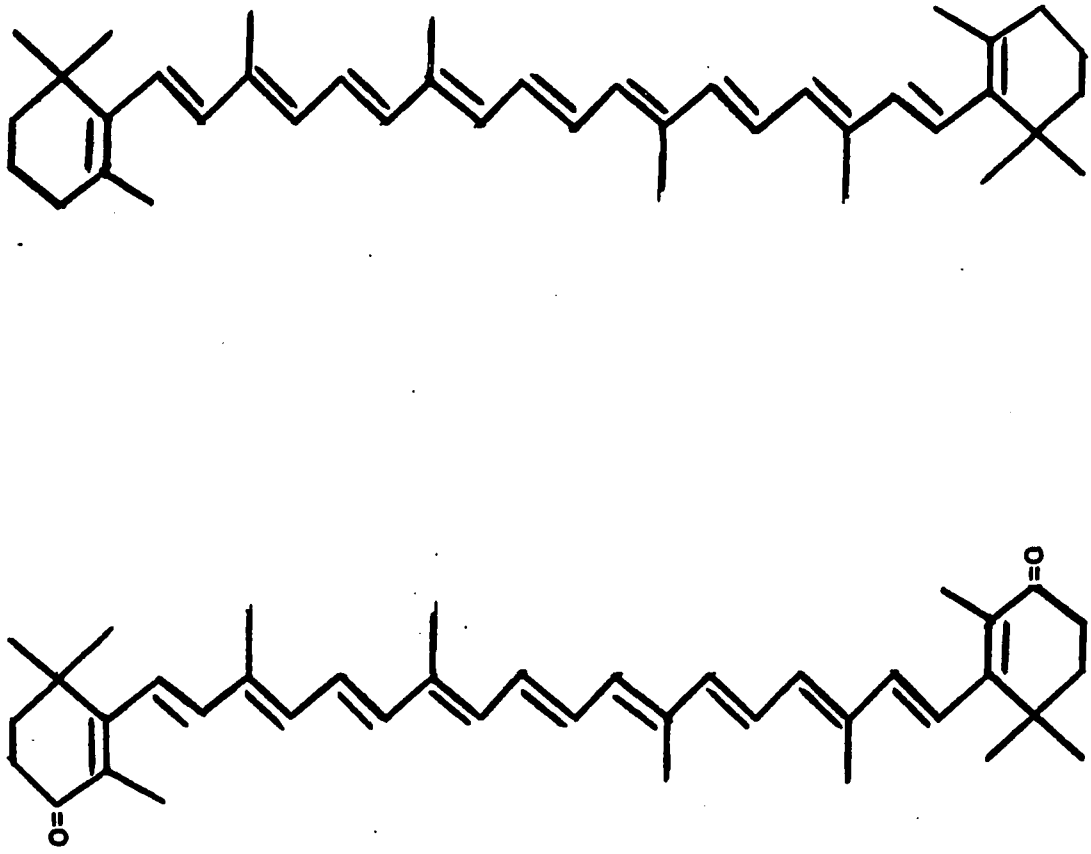

Figure 2. Structure of beta carotene (Top) and canthaxanthin.

Modified from Bendich (3).

As can easily be seen in figure 2, both ring structures can be split in BC to become two molecules of retinol; *in vivo*, only half of this structure is actually converted to retinol; CX has a very similar structure (59). Absorbed carotenoids not cleaved to retinol are circulated in the blood in association with lipid bearing proteins (66).

In vitro actions of retinoids and carotenoids

The *in vivo* mechanisms of action of the retinoids are both varied and complex. Retinoids can function in the maintenance of normal epithelial cell growth (34). They also have a broad and complex interaction with many cells of the immune system, affecting cells involved in both cellular and humoral immune responses, increasing or decreasing cell responses depending on the type of retinoid used and its concentration (71). In monocytic cells, retinoic acid (69) and retinyl palmitate (43,71) were shown to increase interleukin 1 (Il1) production in mice. Other lymphokines, such as IFN-gamma and tumor cytotoxic factor (Abril,submitted) may also be released by retinoid and carotenoid stimulated monocytes.

Retinoid Activity and immune function *in vitro*

Normal monocyte and macrophage function may be stimulated by retinoids *in vivo* and *in vitro* (71). The enzyme Tissue Transgluttaminase is increased by RA *in*

vitro within 90 min of exposure. This increase is associated with a concomitant increase in phagocytic activity as well (6). Increases of in vitro phagocytic activity against opsonized sheep red blood cells (SRBC) by macrophages has been reported when vitamin A and RP were used. Another enzyme to be increased by retinoids(ROL and RA) is arginase, which is important in increased tumoricidal activity in vitro (57). Significant increases in cell mediated cytotoxic effects against tumor cells in mice was seen when 13 cis RA was used in vivo (57). Vitamin A has been shown to increase rat alveolar macrophage tumoricidal activity in vitro (68). Finally, antigen presentation to T cells in vitro was also augmented by application of 13CRA to the monocyte in vitro (71). Therefore, the effects that retinoids have on these cells is definitely positive, augmenting cell function in most cases. The amount of increased cell activity depends on the concentration of retinoid used, and the amount of time the cell is exposed to these compounds.

Carotenoid activity and immune function in vitro

Carotenoid activity and immune function can be different than that of the retinoids, while still sharing some common activities, since these are also vitamin A precursors in most cases. Beta carotene has the greatest

provitamin A actitivity; the molecule can be converted to two retinol molecules in vivo (59). In addition to their function as vitamin A precursors, carotenoids have other important biological roles. The conjugated double bond structure acts as a trap for singlet oxygen formation during photosythesis; and when found in mammalian tissues, it may well have the same function. They dissipate the energy generated by reactions of light energy within the cell. In doing so, they protect the cell from the damaging effects of excited state oxygen species that can be generated by agents such as UV light exposure (28, 59). The capacity to act as free radical traps and antioxidants in vivo is an important protection for the maintenance of normal cell function. This carotenoid activity means intrinsically that carotenoids are unstable, and are degraded rapidly on exposure to light. Recently, it has been found that carotenoids can protect the cell against photosensitization from UV light in human in vivo experiments (38, 39). Mathews-Roth has also found that BC,CX, and phytoene can quench photochemical reactions in the skin that is exposed to UVB light (36).

Little recent research has been done on the direct effects of carotenoids on immune function. The use of BC did potentiate human interferon action on human monocytes in vitro (58). Conversion of the carotenoid to vitamin A in vivo may provide the same immunoenhancing effects as

those of vitamin A itself. Canthaxanthin did enhance T and B cell responses to mitogens (CX cannot be converted to vitamin A in vivo) (3), but few studies have been performed concerning monocyte changes induced by carotenoids. This area of research has become a part of the present study.

Retinoids and Carotenoids near body surfaces

Retinoids and carotenoids do have transport mechanisms allowing them to become part of the skin metabolism. Mathews-Roth (36) demonstrated that oral administration of BC or CX and intraperitoneal administration of phytoene results in considerable amounts of these pigments in the skin. In vivo degradation of these pigments does occur upon exposure to UV light; however, Roe (61) has found that this degradation is maximal in the UVC range, and not in the UVB range. The UVC range does not occur normally in natural light (7).

Relationship Between UVB Exposure and Immune Function

Role of the monocyte in skin immunology

The monocytes and macrophages play an important role in skin immunology. Being Ia+ cells, they, along with the Langerhans cells are responsible for antigen presentation and recognition of foreign or altered Ag. This is especially important in recognition of altered

self skin cells, such as those that are exposed to chronic UV light exposure. If UV damaged epidermal cells are unchecked due to lack of monocyte/macrophage surveillance, they could develop into metastatic cells, such as those seen in melanomas. There has been much recent research in the areas of UV damage to immune function, including the functioning of the UV exposed monocyte and macrophage.

Proximity of the monocyte to areas of UVB exposure

The action spectrum of UV light that induces skin inflammation and irritation, including sunburn, is the UVB range (280-320nm). It has been shown that the monocytes and macrophages do circulate from the regional lymph nodes to the peripheral areas, and come in contact with UVB near the body surface (9,53). Approximately 10 percent of the incident UVB radiation that reaches the skin can penetrate to the level of the surface blood vessels (1). Calculations of blood flow rates indicate that a cell can pass the epidermal-dermal junction in the skin once every 8-10 minutes (51). Of radiation that reaches the skin, it has been found that only 4h of average sun exposure can still deliver a significant quantity of radiation to the blood vessel level, up to 60-70 J/M^2 in some studies (63). Daynes et al.(9) has suggested that UV radiation (UVB) exposure results in an increased rate of entry of monocytes and macrophages into the area of

exposure in the skin, around the area of inflammation. In other words, exogenous inflammatory stimulation of the skin by UV appears to result in a time dependent redistribution of antigen presenting cells from internal to peripheral areas (9).

Changes in monocyte function as a consequence of UVB exposure

The net result of this exposure of circulating monocytes to UVB light is that many immunologic functions are impaired or diminished as a dose-dependent consequence of exposure. Kripke (29) has found these effects to be cumulative, in that chronic exposure to UVB over long periods of time can cause as much damage as large doses of UVB over short periods of time. Therefore, the suggestion that immune responses of the monocyte might be disturbed by environmental radiation exposure is a serious concern.

Cellular levels of UVB damage. The cellular target of UVB damage is nonspecific; i.e., any cellular component can be affected, including the membrane, cytoplasmic, and nuclear components. It is known that proteins absorb UV light at a wavelength of 280 nm, which is in the UVB range (8). Therefore, surface proteins could be damaged on the monocyte, including Ag receptors. Surface Ia Ag found on the monocyte surface are damaged or deleted entirely, which renders them incapable of Ag presentation to

lymphocytes with subsequent decrease of T cell mitogenic responses (7,16,19). Daynes et al. has also suggested that UV may "freeze" the cell membrane, to inhibit cellular interactions required in monocyte differentiation (8). At the molecular level, protein cross-linking may occur, and other changes are possible. These changes include fatty acid and cholesterol oxidations, which can also decrease immune capacities (27).

Intrastrand pyrimidine dimer formation is a known consequence of UV exposure. The cytotoxic T cell is especially susceptible to this action, as active proliferation and DNA replication is necessary for their proper functioning (8). However, accessory cell function, such as that of the monocyte, is not thought to require active DNA replication. Thus, UV-induced pyrimidine dimers may not be expected to affect monocyte function. Damage of cytoplasmic or membrane components can result in cell damage and/or cell death (7,8). This lack of active DNA replication in accessory cells may in effect protect these cells from some of the damaging effects of UVB exposure. This theory has recently been upheld, in that actively dividing lymphocytes were found to be at least 2-3 times more sensitive to UVB compared to the monocyte population from the same subject (8).

At the cytoplasmic level, formation of oxygen radicals and other toxic products is a major concern of

UVB exposure. When the epidermis is exposed to chronic UVB exposures, singlet O_2 and free radical formation do occur and may contribute to the development of skin tumors *in vivo* (4). Monocytes that pass through the epidermis may then be exposed to enough UVB to produce the toxic products that cause much the same damage as seen in the skin epidermal cells.

One of the first and most obvious changes seen is a significant decrease in monocyte viability, which decreased steadily over a 72h period in the experiments of Morison, et al. (44) and Schuller (63). Morison et al. found that after a 73 J/M^2 UVB exposure, monocyte viability was less than fifty percent after 72h incubation. This is a significant change, and may have the effect of rendering the monocyte incapable of responding to antigenic challenge.

Changes in immune functions. There have been many recent studies on how UVB exposure can directly affect monocyte functions *in vitro*. Much of this work concerns impairment of Ag presentation abilities (10,21,51,52, 54,55,67). There is a significant decrease in membrane Ia Ag number and function (23) and also a decrease in IL-1 secretions. Drebin (10) has shown that abrogation of APC function caused by UVB was due to loss of IL-1 production, and that much of this loss could be restored by addition

of exogenous IL-1. However, Ansel, et al., and Elmee et al. (2, 11) did not find a large decrease in IL-1, and suggests that IL-1 may not be the only factor or mechanism that is altered in UVB damage of APC functions. Other investigators, such as Lange-Wantzin, et al. (31) has found that secreted IL-1 was increased by UV, but that membrane IL-1 levels were decreased. Whatever the exact mechanism of action that UVB has on APC function, all studies agree that abrogation of APC function does occur with exposure of cells to UV light. The nature of this defect seems to be a combination of loss or damage to surface Ia and a decrease in IL-1 production (33,62). Roberts et al. (60) believes this response in the monocyte is a normal response, which prevents damage that may be caused by active monocytes at the site of UVB caused inflammation in vivo.

Schuller (63,64) found that a 14 J/M^2 UVB exposure of mouse PEC resulted in a decrease in phagocytic activity by as much as 80 percent. There have been few studies that have dealt with the effects of UVB on monocyte cytotoxic activity. Lynch et al. (35) found that cytotoxic T lymphocytes (CTL) are quite sensitive to the effects of UVB, while the monocyte were not as sensitive. Lynch et al. found that ability of accessory cells (such as monocytes) to induce the CTL was decreased by UVB. No mention is made as to any direct changes in monocyte

cytotoxic activity. As there are not many studies on the effects of UVB on monocyte cytotoxic functions, the exact mechanisms by which this function may be altered is not known.

Studies on UVB and Retinoids and Carotenoids

Almost all of the studies on the effects of UVB and the effects of retinoids and carotenoids on immune function have failed to address the possible relationships between these two areas. Kligman (26), Connor, and Nemoto et al. (6,46) have found that topical application of retinoic acid in mice inhibited increases in epidermal ODC activity that are caused by UVB exposure. This shows that retinoids do control cellular growth and proliferation, but again does not address any changes in immune functions. Mathews-Roth (37) has shown protection against UVB induced tumor production in mice when BC and CX were used in vivo; however, the exact cellular target of this beneficial effect is not discussed. Vahlquist (70) also discusses vitamin A and epidermal effects after UV exposure, but does not discuss immune problems.

Possible Mechanisms of Protection by Retinoids and Carotenoids

Inhibition with prostaglandin (PG) inhibitors

Phagocytosis of latex particles by monocytes does not induce PG synthesis (22). However, phagocytosis of

other inflammatory stimulants such as bacteria (20) and antigenic substances such as SRBC can stimulate PG synthesis (22,72). It will be if interest to determine if PG synthesis is a mechanism by which the retinoids and carotenoids may protect the monocyte immune function after exposure to UVB. Indomethacin (IM) (72) as well as acetylsalicylic acid (ASA) (15) are potent inhibitors of PG synthesis *in vivo*. Addition of these compounds to the monocyte cultures may have effects on immune function after treatment with UVB.

Comparisons with other antioxidants

Like the retinoids and carotenoids (40), other vitamins have antioxidant properties as well. Both vitamin E and vitamin C can act as lipid antioxidants *in vivo*, by acting as free radical scavengers,and by quenching toxic oxygen radical formation in the cell membrane (1,41). The action of the carotenoids and retinoids may function by the same mechanisms, preventing toxic product formation caused by UVB exposure. Use of vitamin E and vitamin C in conjunction with the retinoids should show if the mechanism by which the retinoids and carotenoids exert their protective function is by a possible antioxidant capacity.

Inhibition with monoclonal antibodies

Interactions of monokines in immune function is quite complex. Interleukin 1 (IL-1) and gamma interferon (IFN), are important in monocyte activation (50). Other monokines such as tumor necrosis factor (TNF) is also an important product in monocyte activation, activating cytotoxic functions and other immune functions. Shalaby et al.(65) showed that TNF can increase cell phagocytosis, and others have shown that it can also increase chemotactic activity in vitro (42).

Protection of monocyte phagocytic immune functions under UVB treatment by the retinoids and carotenoids, could be caused by stimulation of monokine release, that could in turn stimulate phagocytic activity after exposure to UVB. Therefore, addition of a MoAb against the monokine of interest (such as anti- Il-1, TNF, or IFN) to the monocyte culture media, would bind and inactivate the monokine if released after exposure to the retinoid or carotenoid. This should provide evidence for the involvement of monokine release in stimulation of monocyte function after exposure to both compounds and UVB.

In summary, chronic exposure of the monocyte to UVB can affect their proper function in many ways, decreasing their effectiveness in preventing tumor promotion caused by UVB. Decreases in monocyte ability to present antigen (29,53) have been reported after in vitro

UVB exposure. UVB can also decrease phagocytic activity (63) and cytotoxic functions may also be affected. Biochemical changes include alteration of IL-1 production and secretion (10,11) and changes in surface Ia antigen structure and function (23). The effects of UVB on monocytes exposed at the body skin surface can be detrimental and these effects can be cumulative over time, seriously impairing monocyte function *in vivo* and *in vitro*.

Mitigating this UVB-induced damage to monocyte function *in vitro* is the purpose of the present study. Because of the stimulatory effects of carotenoids and retinoids on monocyte function (3,26,46,71), these compounds will be applied to the cells *in vitro* to determine if the effects if UVB can be mitigated. If the antioxidant and energy-quenching properties of the compounds tested are effective after UVB exposure, the monocyte function should be protected from significant alterations, depending of the function that is assayed. Proper monocyte function in the skin area, as may be provided by these compounds, would be important in an effective *in vivo* program of carotenoid and retinoid application and possible cancer prevention activities.

Chapter 2

Materials and Methods

Monocyte Preparations

Peripheral blood monocytes were obtained from healthy human subjects ranging from 24-34 years of age. These subjects reported no large dietary or supplementary intakes of carotenoids or retinoids in the past several years. They also reported no excessive exposure to UVB light, as would be found in sunlight. This would amount to less than 30 min of full sunlight daily for each subject. In all, ten subjects were tested at least three times each over the course of approximately 18 months.

Thirty ml of blood was collected in heparinized vacutainer tubes (Vacutainer, Rutherford, NJ). The blood was then layered over 10 ml of lymphocyte separation media (Bionetics, Kensington, MD), and after centrifugation at 500 x g for 20 minutes, the lymphocyte band was aspirated off. The cells were washed twice in 30 ml of clear Hanks' Balanced Salt Solution (HBSS;Gibco, Grand Island, NY). The cells were resuspended in complete Iscoves Modified Dulbeccos Media (IMDM, Gibco),and 15 ml of cells were placed in 75mm petri dishes (Falcon, Oxnard,CA), and incubated for 2 hours at

37·C, as was performed earlier by Fleit, et al. (14), and by Pretell, et al. (53).

The adherent cell population was washed twice in HBSS, and then gently scraped from the plate and resuspended in complete IMDM plus 10% fetal calf serum (Hyclone, Logan,UT). This method of cell isolation was performed by Fleit, et al. (14) and found superior to other methods of purification such as removal of the adherent cells by chemical means. A cytospin preparation of the cell population on glass slides and staining with Modified Wrights Stain (Sigma, St. Louis, MO) was then performed. Differentiation of the cells based on morphology showed that 85 to 95 percent of the adherent cells were monocytes. The purity varied slightly with each subject tested, with an average purity of 91.4 percent. This agrees with results obtained by Pretell, and Lederman, et al. (32,52,53) for cell purity levels. The cell concentration was adjusted to 1 x 10^{6} or 1 x 10^{5} viable monocytes/ml, for use in the assays performed.

Preparation of Retinoids

Each of the retinoids used: 13 cis-retinoic acid (13CRA, Hoffman-La Roche,Fresno,CA),retinol (ROL,Sigma), and each of the carotenoids used: beta-carotene (BC,Sigma) and canthaxanthin (CX,Hoffman-La

Roche) were prepared as follows. A stock solution at 10^{-3} M was made by dissolving the compounds in 100% ethanol. All further dilutions of the compounds was done in complete media with 10% fetal calf serum, with the experimental concentrations used ranging from 10^{-7} to 10^{-9} M. Therefore, the concentration of residual ethanol in the concentrations used was very dilute, too dilute (less than 0.01% in all cases) to be a factor in cell activation.

The concentrations of compounds used in the assays, always between 10^{-7} and 10^{-9} M, were chosen because they represent the actual concentrations of the compounds that could be achieved in an in vivo system. Fresh dilutions were made from the stock solutions every 3-4 weeks. The purity of these retinoids was specified by the manufacturers to be greater than 95 percent purity levels for all the retinoids and carotenoids used.

Source of Ultraviolet Light

The ultraviolet light applied to these cells in vitro was supplied by two Westinghouse FS20 sunlamps (Westinghouse, Pittsburg,PA). The output of these lamps was 80 percent in the UVB range (290-320 nm), as confirmed earlier by Kripke, and Pretell, et al.(29,53).

This wavelength band was selected because Kripke (29) showed that this wavelength was the most damaging to the immune function of living cells, while still maintaining their viability in vitro. The actual output of the sunlamps at the cell level was 3.7 J/M2/sec, at a distance of 12 cm below the bulbs. This value did not vary more than 2 percent over the course of these experiments as measured by radiometer. There was an initial break-in period in which the UV lamps were allowed to run overnight, since variation in output is greatest in the first few hours of use.

The levels of exposure applied to each monocyte preparation was constant, and the same dosages were used in each experiment. The amount of dosage was varied by varying the time of exposure. The range of exposure was from 50-220 J/M2, a dose level that Pretell (52,53,54) has found will cause the greatest effects on the monocyte population while still allowing significant viability.

Cells were prepared for exposure by either centrifuging the 96 well plate at 300 x g, or allowing the cells to adhere to the plate bottom overnight. This allows uniform exposure of all cells in a single layer at the well bottom surface. Just prior to exposure, media was removed and the cells covered in a thin layer of HBSS without phenol red. This allows for the UVB light to

have an unrestricted path to the surface of the monocytes. Exposure was in intervals of 15 sec, ranging from 15 to 60 sec exposure times (55-220 J/M^2) in all cases. After exposure, cells were washed in HBSS and a measurement of cell viability done using Eosin Y. The cells were then ready for either exposure to the test compounds, or for the functional assays to be performed.

Assays of Monocyte Function

Cell preparation prior to assessment of cell function

The following routine was used in preparation of the cells prior to the assays for changes in monocyte functional abilities. First, after the monocytes were plated at a concentration of 1×10^{6} cells/ml (or in the case of assays for Ag presenting ability, 1×10^{5} cells/ml), retinoids or carotenoids were applied for a period of 24h at 37·. After washing the cells at least 2-3 times in clear HBSS the UVB dose was then applied, and again the cells were washed.

The assays for changes in monocyte function were then performed. In the case of antigen presentation and tumor cell cytotoxic assays, the retinoids or carotenoids were again applied for the duration of the test incubation period. This was done to insure that the cells received the maximum amount of exposure to the

compounds in question, as they would in an in vivo situation.

Immune Phagocytosis

In order to determine if the application of retinoids or carotenoids to the monocytes affected their ability to phagocytose opsonized sheep red blood cells (SRBC), the following tests were performed.

Preparation of monocytes

Using 24 well tissue culture plates (Falcon), 0.5 ml of the purified monocytes preparation was added to each well, for a total of 5×10^{5} cells/well. The plate was then centrifuged for 5 min at 300 x g, and the oids and carotenoids applied for 24h at 10^{-7} to 10^{-9} M. After incubation, the cells were washed, the dose of UVB applied, and the cells assayed for phagocytic activity.

Addition of monocyte inhibitors

It was necessary to determine if the addition of the following compounds to the monocyte culture media alters phagocytosis after exposure to the retinoids and carotenoids and UVB. The monocytes were incubated for 24h with these compounds alone, and concomitally with the retinoids or carotenoids. The cells were then washed in

HBSS. These are the following compounds that were tested:

1. The PG synthesis inhibitors indomethacin (IM, Sigma), and acetylsalicylic acid (ASA, Sigma), both at physiological concentrations ranging from 10^{-7} to 10^{-9} M, in complete IMDM.

2. Antioxidants vitamin E (alpha-tocopherol,Sigma), and vitamin C (ascorbic acid, Sigma), also in the same concentration range as the retinoids and carotenoids, 10^{-7} to 10^{-9} M, in complete IMDM.

3. Monoclonal antibodies to monokines, including anti-gamma interferon (Interferon Research, New Brunswick, NJ), anti-tumor necrosis factor (Dr. Philip Scuderi, personal contact), anti-interleukin 1 (Cistron, Pine Brook, NJ). Each of the antibodies was diluted in IMDM without FCS. All have a final concentration of 1 x 10^{3} neutralizing units/ml, and 100 ul was added per well.

Preparation of opsonized sheep red blood cells

The procedure used was similar to that used earlier by Moriguchi (43). Approximately 3 ml of a 10% suspension of sheep red blood cells (SRBC, Gibco) were incubated for 1h at 37· with 0.2 ml of rabbit anti-SRBC Ab, diluted 1/100 (Cappell-Worthington, Malvern, PA). After washing with HBSS, the cells were then exposed to

0.2 ml of fresh ^{51}Cr in PBS (NEN, Boston MA) for 1h at 37·. The specific activity of the ^{51}Cr was 408 mCi/mg. The cells were then resuspended in 10 ml of HBSS, and incubated of 1h to allow for spontaneous release of unbound ^{51}Cr. The SRBC were then resuspended in 10 ml of complete media, for a final SRBC concentration of 1 x 10^6 cells/ml.

Assay of phagocytosis by the monocytes

To each well of the plated monocytes, 0.5 ml of the labelled SRBC suspension was added, and the mixture incubated for 2h at 37·. Activated monocytes should be able to phagocytose SRBC within this 2h period (43). The remaining SRBC were then suctioned off, and the residual non-phagocytosed SRBC lysed with 0.4 M tris-NH_4Cl buffer (Sigma), which will lyse the SRBC but leave the monocytes intact. The monocytes were then washed and lysed with 0.15 ml of a 1% solution of the detergent NP40 (Sigma) which will rapidly and uniformly lyse all cells within 15 minutes. The lysate was collected on cotton swabs, and counted in a gamma counter. Values in cpm were recorded for quadruplicate samples for each UVB level and compound used for each individual assay. Results were recorded as the percent change in total CPM as compared to the unstimulated control values, plus or minus one standard deviation per data point.

Cytotoxicity Against Tumor Cell Lines

Preparation of target cells

In order to examine whether UVB and retinoid treatment affected the monocytes' ability to lyse tumor target cells, the following tests were performed. The human melanoma cell line A375 (18) was used as the target cell for monocyte cytotoxicity experiments. This cell line was chosen because it is a fairly resistant cell line that closely approximates the type of melanoma that could be caused by UVB damage *in vivo* (18). The procedure is from an earlier adaptation by Kleinerman, and others (13,24,25,49), in which approximately 1 x 10^5 viable tumor cells in log growth phase were labelled with 1.0 uCi/ml of ^{125}I-labelled IUDR (NEN). The specific activity of the label is 2000 Ci/mmol. The cells were incubated for 24h to allow for label uptake. The cells were then washed and counted, and their percent viability checked.

Addition of target cells to treated monocytes

Kleinerman, et al., and Norbury (24,25,49) reported that an A375 target cell to monocyte ratio of 1:10 gave the greatest cytotoxic activity in their assay systems. Titrations carried out showed the greatest cytotoxic activity after 72h incubation was with the ratio of 20 monocytes per target cell, as seen in

table 1. This ratio gave a high number of labelled target cell cpm, while still giving significant cell killing by the monocytes. After a 24h incubation, the media in each well was changed to rid the wells of background radiation and dead cell lysate (if present). The cells were then allowed to interact for another 48h. At the end of the incubation period, cells were washed in HBSS, and the remaining tumor cells not killed (lysed) by the monocytes were lysed with 0.1 ml of NP40. Lysate was collected on cotton swabs and quadruplicate samples counted in a gamma counter.

Percent cytotoxicity was recorded as (24,25):

$$100-\left(\frac{\text{CPM A375} \pm \text{CPM test cells}}{\text{CPM A375} + \text{CPM control}}\right) \times 100 = \%\ \text{cytotoxicity},$$

+/- 1 s.d. per data point.

As it is not known whether the effects of the compounds on monocyte function occurs before or after UVB treatment, the cells were exposed to the compounds both before and after UVB treatment. Maximum exposure of the cells to the compounds should allow for any effects that the compounds may have on the cells. It may also approximate the amounts of exposure one might expect in the skin area in vivo. Control cells were also exposed to a proven cell cytotoxic activator, gamma

TABLE 1. DETERMINATION OF OPTIMAL TARGET: MONOCYTE RATIO FOR CYTOTOXICITY

A375:Monocyte Ratio	Aver.Percent Cytotox.[a]
1:10	29.5 +/- 1.6
1:20	34.4 +/- 2.0
1:40	26.6 +/- 1.6
1:60	20.6 +/- 1.3
1:80	21.7 +/- 1.5
1:100	b
1:120	b

[a] Values given are the average of 3 or more trials, +/- 1 s.d.

[b] CPM were too small to be statistically valid.

interferon (IFN, Sigma), at 10U/ml, for the same amount of time as with the retinoids and carotenoids.

Monocyte Antigen Presenting Abilities

Pulsing of monocytes with antigen

In order to determine whether the antigen presenting abilities of the monocytes is altered by treatment with retinoids, carotenoids, and UVB, the following tests for antigen presentation were performed as described by Pretell, et al., and Kripke (29,30,53). Monocytes were plated at a concentration of 1×10^{5} cells/ ml. This cell concentration was used because it closely approximates the natural percentage of monocytes found in the peripheral blood, as compared to a lymphocyte number of 1×10^{6} cells/ml. After plating, the cells were pulsed with tetanus toxin (TT,Calbiochem, La Jolla, CA), an antigen that all T cells can recognize due to prior immunization (5). Some cells were also pulsed with phytohemagglutinin M (PHA, Sigma), or with conconavalin A (ConA, Calbiochem) for 24h. After serial dilutions of TT or mitogens, the optimal concentrations for T cell stimulation were determined. For tetanus toxin, this was found to be 1/400, for PHA 10 ug/ml, and 125 ug/ml for Con A. The retinoids or carotenoids were also applied during this pulsing period, for the same amount of time as the Ag

pulse (24h). After the incubation, the monocytes were washed thoroughly in HBSS. The UVB dose was then applied, the cells again washed, and the cells were prepared for addition to the autologous T cells as described below.

Measurement of T cell mitogenic activity

After exposure to compounds, UVB, and pulsing with Ag or mitogen, the monocytes were then exposed to the nonadherent T cell population, to determine their effects on stimulation of T cell mitogenic activity. These T cells were the fraction of peripheral blood lymphocytes obtained from each subject that did not adhere to plastic for the 2h incubation, or after an overnight incubation. Therefore, each population of T cells was mixed with the monocytes from the same subject in each experiment. These T cells were plated at a concentration of 1×10^{6} cells/ml. In the case of monocytes plated with PHA or ConA, the cells were incubated for 72h at 37·; and in the case of cells incubated with TT, incubation was for 5d. Again, during the entire incubation period, the cells were also exposed to the compound of interest at the same concentrations as those used before UVB exposure. These time intervals were when the maximal T cell mitogenic response to these compounds should occur (5,17,53).

At a period of 18h before the end of the incubation periods, 100 ul of a solution of tritiated thymidine (NEN) in complete media was added to each well. The specific activity of the solution was 10 uCi/ml. After incubation, the cells were harvested on filter paper strips using a cell harvester. The cells ' uptake of the radionuclide was assayed by counting beta emission in liquid scintillation cocktail (Omniflor, NEN). Results are expressed as the stimulation index, which is defined as:

$$\frac{\text{CPM sample}}{\text{CPM control}} \times 100 = \text{S.I.}$$

Viability of Cells During Assay

During the course of each experiment, the viability of the monocytes and lymphocytes used were checked by staining with Eosin Y, as described in other experiments. This dye is similar to an exclusion dye used by Fleit (14), and is superior to viability measurement with trypan blue, which was found not to be accurate in measurement of UV treated cell viability (45). This dye is excluded by viable cells, and is a reliable indicator of cell viability. Results were recorded plus or minus one standard deviation per data point.

Statistical Analysis

Analysis of variance was performed on both the individual experiments performed in each assay, as well as on each experimental group as a whole. The calculations were performed on a Vax-8600 computer using the SPSS statistical program. Results were expressed at a significance level of $p = 0.05$.

Chapter 3

Results

Cell Viability After UVB Treatment

Using exclusion of the dye Eosin Y as a guide for cell viability, the monocytes were checked for viability 24, 48, and 72h after exposure to UVB. The amount of UVB exposure varied from 0-385 J/M^2, in increments of 55 J/M^2. As shown in table 2, there was a significant decrease in viability 48-72h after incubation. The effects were more evident after high doses of UVB (above 220 J/M^2), while at lower doses greater than 70 percent viability was noted, up to the maximum 72h incubation period.

Viability After Retinoid and Carotenoid Treatments

The effects of exposure of monocytes to the retinoids and carotenoids in culture on cell viability was determined. Each compound was incubated with the monocytes for 72h. The compounds ranged in concentration from 10^{-5} to 10^{-10} M.

As shown in table 3, for all of the compounds tested, significant loss of viability was not seen at any concentration lower than 10^{-7} M. Any compound tested that had a concentration of 10^{-7} M or less did not affect cell viability, and therefore proved to be the optimal

TABLE 2. PERCENT VIABILITY OF MONOCYTES AFTER UVB EXPOSURE

UVB dose, J/M^2	Incubation Periods		
	24h	48h	72h
0	98	97	94
55	94	93	90
110	90	90	87
165	91	86	84
220	87	82	80
275	82	76	70
330	75	63	55
385	60	50	41

TABLE 3. PERCENT VIABLE MONOCYTES AFTER CULTURE WITH RETINOIDS AND CAROTENOIDS, AFTER 72H INCUBATION

[Compound,M]	CX	13CRA	BC	ROL	LYC
10-5	69	72	75	66	77
10-6	75	77	80	75	82
10-7	95	90	92	90	93
10-8	98	94	96	92	96
10-9	99	96	98	95	98
10-10	99	99	98	96	99

Control (no compound) had an average of 98 percent viability after 72h.

concentration range to be used in the functional assays that were performed.

Phagocytosis After Treatment with UVB Alone

Using peripheral blood monocytes, the observation that decreases in phagocytosis of opsonized SRBC occur after exposure to UVB (63,64) was confirmed in this study, as seen in figures 3, 4 and 5. At the maximum level of 220 J/M^2 (which is equivalent to a 60 s exposure time), phagocytic activity had decreased to only 34 percent of the unirradiated control value. The decrease in phagocytic activity seemed to be a linear function of the increasing UVB dose.

Phagocytosis After Both UVB and Compound Treatment

Five different compounds were tested for their possible protective abilities: canthaxanthin (CX), beta-carotene (BC), 13 cis- retinoic acid (13CRA), lycopene and retinol. When tested in the physiological range of 10^{-9} to 10^{-7} M, all compounds showed a protective effect. In each case, the percent phagocytosis after UVB was greater when the cells were incubated with the retinoids or carotenoids as compared to incubation with media alone.

Of the five main compounds tested, the carotenoid compounds--BC, CX, as well as 13 CRA, appeared to provide the most protection against UVB damage. As seen in

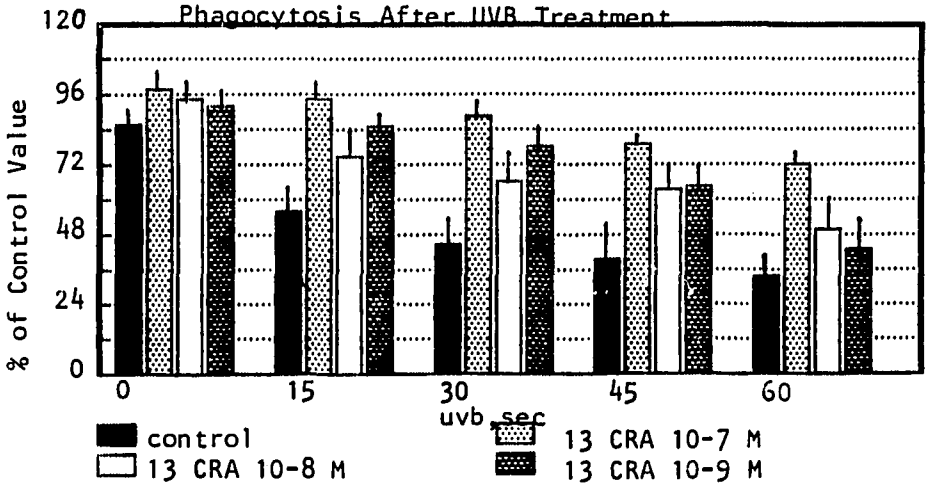

Figure 3. Measurement of phagocytosis after treatment with UVB and 13 cis retinoic acid.

Values are the mean percent of unexposed controls, +/- 1 s.d.

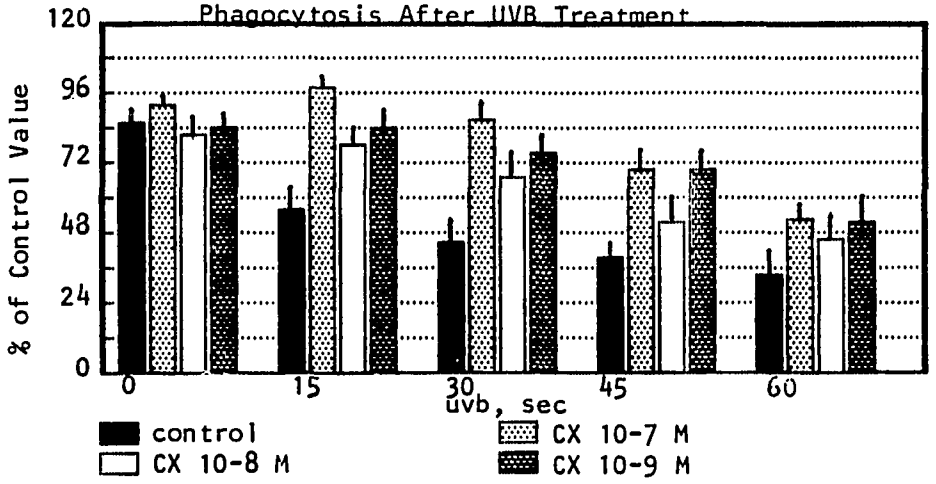

Figure 4. Measurement of percent phagocytosis after treatment with UVB and canthaxanthin.

Values are the mean percent of the unexposed control values, +/- 1 s.d.

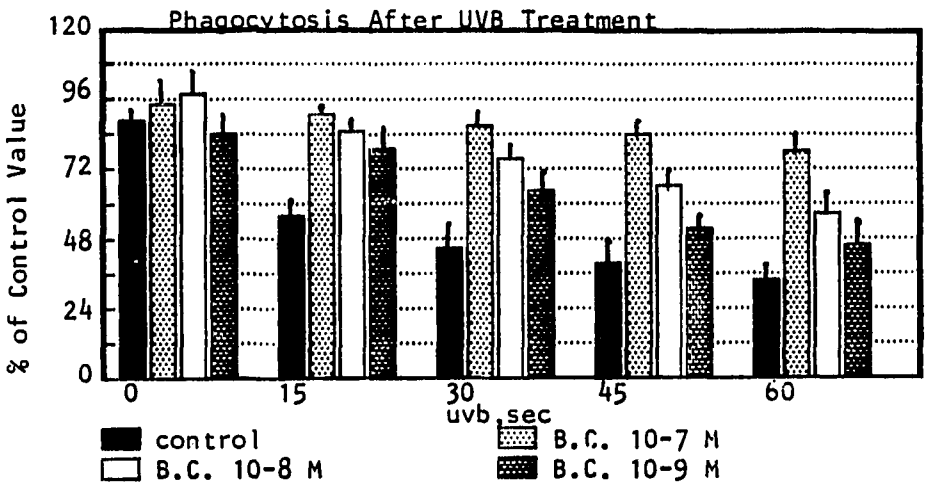

Figure 5. Measurement of percent phagocytosis after treatment with UVB and beta carotene.

Values given are the mean percent of the unexposed control value, +/- 1 s.d.

figure 5, after the maximum 60 sec (220 J/M^2) exposure, BC at 10^{-7} M gave a value for phagocytic activity that was 45 percent higher that of the control. Canthaxanthin gave similar results, with the amount of protection decreasing with decreasing concentration of compound used (figure 4). In figure 3, the results compared to those of CX, especially at the lower doses of UVB. With each of these compounds, the protective effects are evident, even at the maximum UVB exposure level. The other compounds tested, lycopene and retinol, did not show significant protective effects, having phagocytic values at or near those of the controls (data not shown). Subsequent experiments concentrated on the protective effects of CX, BC, and 13 CRA.

It is important to note that the compounds tested did not have a significant stimulatory effect on monocyte phagocytosis; this is also seen in figures 3-5 where no UVB is applied as compared to the control values. The effect that these compounds seem to have on the monocytes is a protective role only, as determined from the statistical analysis. The cells had more phagocytic activity than when no compound is applied. The possible mechanism of this action was investigated, as outline below.

Mechanism of Protection by Retinoids and Carotenoids

Inhibition with Prostaglandin (PG) Inhibitors

The prostaglandin inhibitors acetylsalicylic acid (ASA) or indomethacin (IM) were added to monocytes treated concomitant with retinoids or carotenoids, at the same concentrations as these compounds. As seen in figures 6, 7, and 8, when the highest concentration of carotenoid or retinoid were used (10^{-7} M), protection of phagocytic activity was seen even in the presence of high concentrations (10^{-7} M) of either IM or ASA. The values obtained were very similar to the results obtained in earlier experiments in which only the carotenoids or retinoids were applied, and no prostaglandin inhibitors were used, according to statistical analysis.

Inhibition with monoclonal antibodies

In figures 9, 10, and 11 phagocytic activity was decreased by exposure to UVB, in much the same manner as in previous experiments (figs. 3-5). The addition of monoclonal antibodies to monocytes treated with retinoids or carotenoids did not decrease the retinoid or carotenoid protective capacities, regardless of the type of monoclonal that was used. Each of the compounds tested showed results that were similar to earlier results when the monocytes were incubated only with the compounds and were not incubated with the monoclonals. It appeared that perhaps a small part of the protective

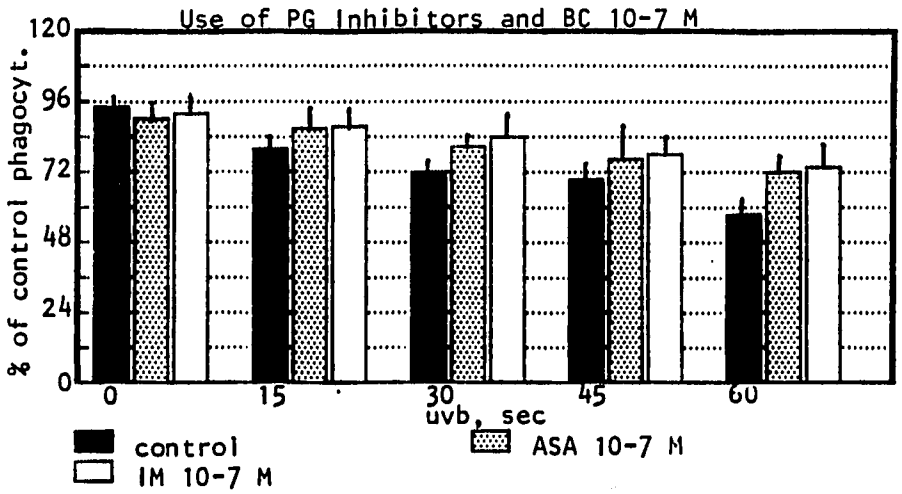

Figure 6. Measurement of percent phagocytosis after treatment with UVB, beta carotene, and indomethacin (IM) or acetylsalylic acid (ASA). . .

Values are the mean percent phagocytosis compared to unexposed control values, +/- 1 s.d.

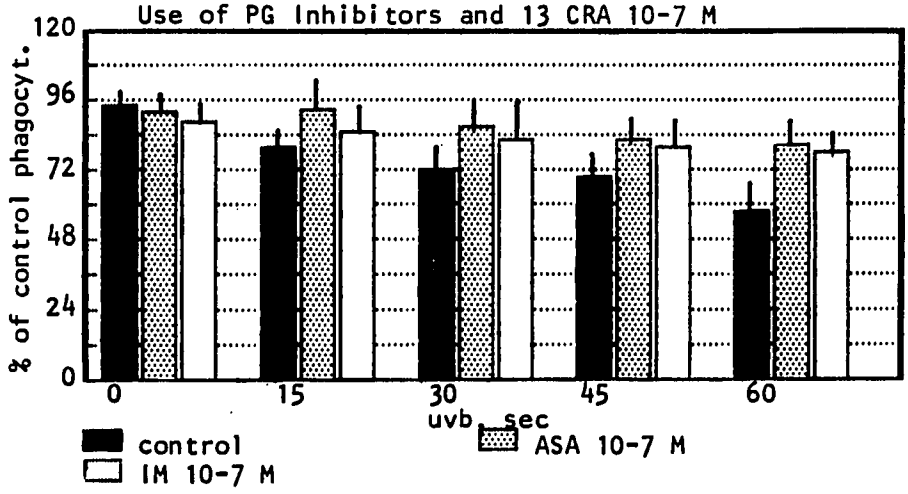

Figure 7. Measurement of phagocytosis after treatment with UVB, 13cis RA, and IM or ASA.

Values are the mean percent phagocytosis as compared to unexposed control values, +/- 1 s.d.

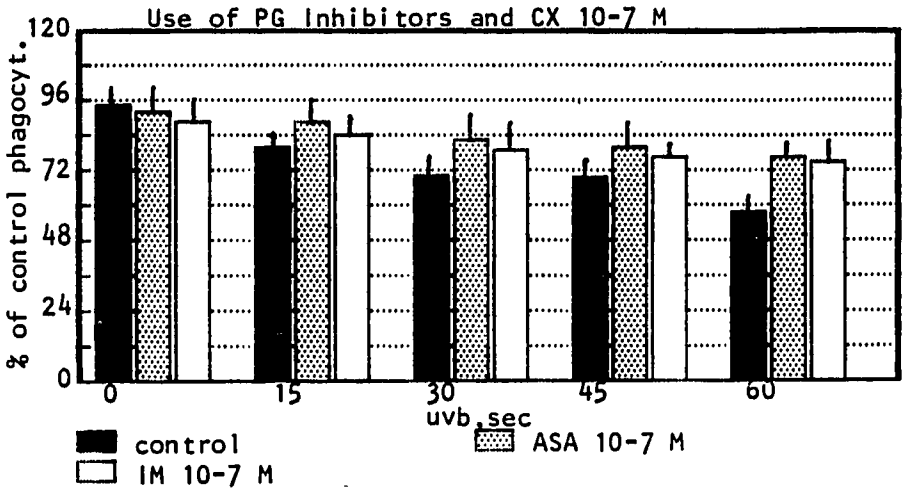

Figure 8. Measurement of phagocytosis after treatment with UVB, canthaxanthin, and IM or ASA.

Values are the mean percent phagocytosis compared to unexposed control values, +/- 1 s.d.

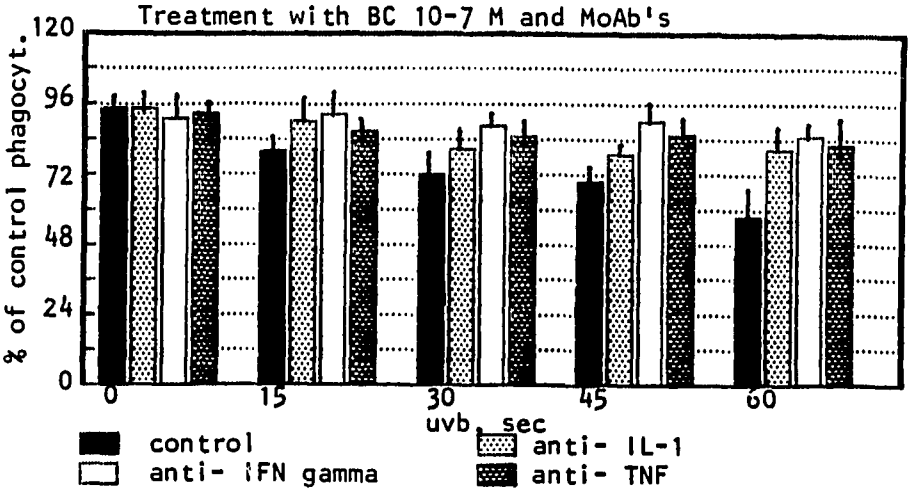

Figure 9. Measurement of phagocytosis after treatment with UVB and BC and monoclonal antibodies.

Values are the percent phagocytosis as compared to unexposed control values, +/- 1 s.d.

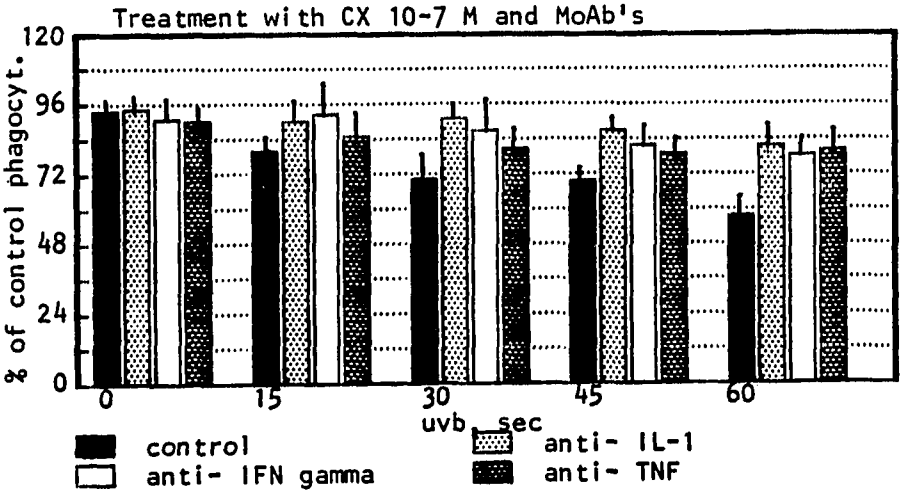

Figure 10. Measurement of percent phagocytosis after treatment with UVB, CX and monoclonal antibodies.

Values are given as percent phagocytosis as compared to unexposed control values, +/- 1 s.d.

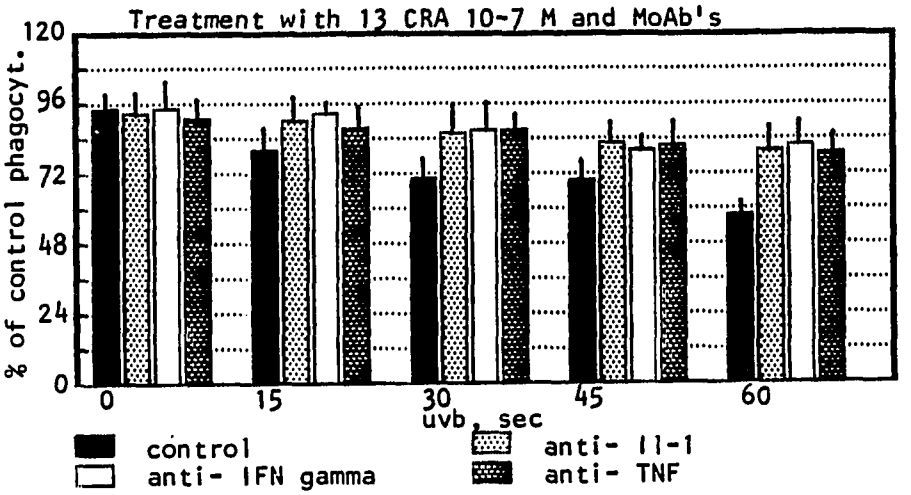

Figure 11. Measurement of phagocytosis after treatment with UVB, 13CRA and monoclonal antibodies.

Values are given as percent phagocytosis as compared to unexposed control values, +/- 1 s.d.

mechanism may be due to release of monokines, but as seen from the results and from statistical analysis the majority of protection was retained when the compounds were added at the maximum stimulatory and protective level, 10^{-7} M.

Comparison with other antioxidants

When vitamin E and vitamin C were added to the monocyte cultures in the same concentrations and times as the carotenoids and retinoids, there was significant protection of monocyte phagocytic function. This protection (figures 12,13) was similar to the results for amounts of protection afforded by the carotenoids and retinoids (figures 3-5), but at an overall lower level of protection as compared to the retinoids and carotenoids. This protection was slightly higher for vitamin E, especially at the highest concentration used (10^{-7} M). Because all of these compounds are potent antioxidants, it is possible that the protection seen for the retinoids and carotenoids was almost entirely due to their antioxidant and UVB energy-quenching capabilities.

Cytotoxicity Against A375 Tumor Cell Line

Spontaneous monocyte cytotoxic activity

Monocytes that were not exposed to either UVB or the compounds were assessed for their spontaneous cytotoxic activity. In each case, the amount of

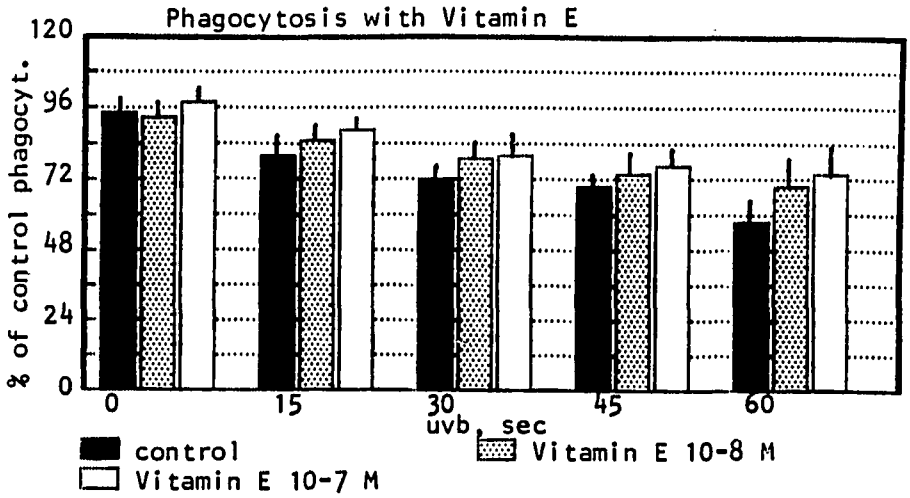

Figure 12. Measurement of percent phagocytosis after treatment with UVB and vitamin E.

Values are the mean percent of the unexposed control values, +/- 1 s.d.

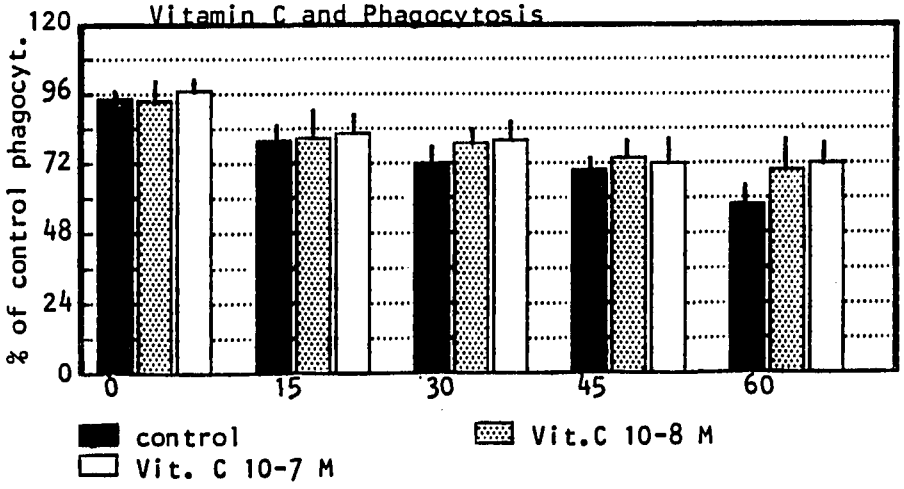

Figure 13. Measurement of percent phagocytosis after treatment with UVB and vitamin C.

Values given are the mean percent of the unexposed control value, +/- 1 s.d.

spontaneous cytotoxicity was less than 10 percent in all cases, as seen in figures 14-16. This spontaneous cytotoxic value was taken into consideration in the calculation of actual treatment cytotoxic values, and the values indicated after treatment with the retinoids or carotenoids.

Cytotoxicity after UVB and retinoid or carotenoid treatment

After 24h exposure to the retinoids or carotenoids, the amount of cytotoxic activity was increased, at each of the UVB dose levels that were used. As seen in figures 14-16, application of BC, CX, and 13 CRA at all concentrations produced a higher percent cytotoxicity level compared to unexposed control values. At the maximum UVB dose, the cytotoxic values were decreased, but were still significantly higher than those of the unexposed controls, at a p value of 0.05. The values at the 0 sec UVB level were in some cases as high as those for addition of gamma interferon to the media, which had an average value of 22.7 percent cytotoxicity. The compounds BC and 13 CRA seemed to have the highest amount of stimulation; their effects decreased with decreasing concentration of these compounds. These results show UVB exposure does not have a significant effect on monocyte cytotoxic activity, and that the retinoids and

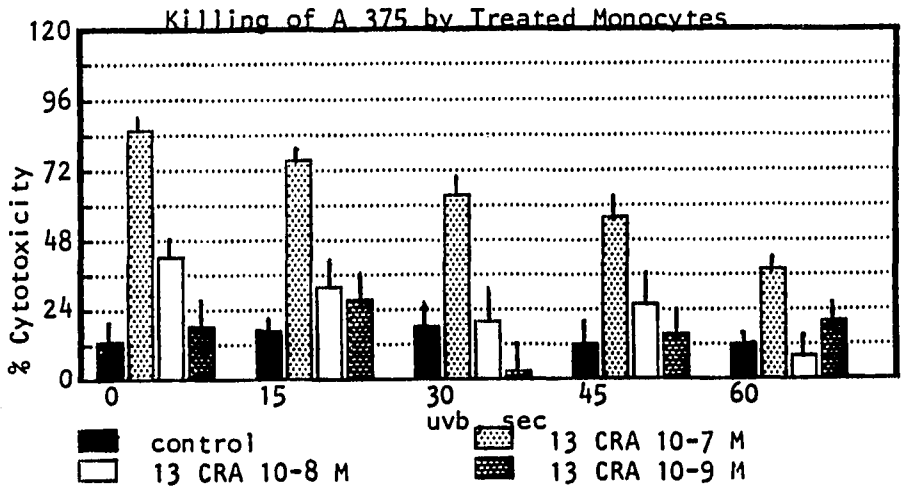

Figure 14. Percent cytotoxicity of A375 by monocytes treated with UVB and 13 cis retinoic acid.

Values given are the mean percent cytotoxicity after a 72h incubation, +/- 1 s.d.

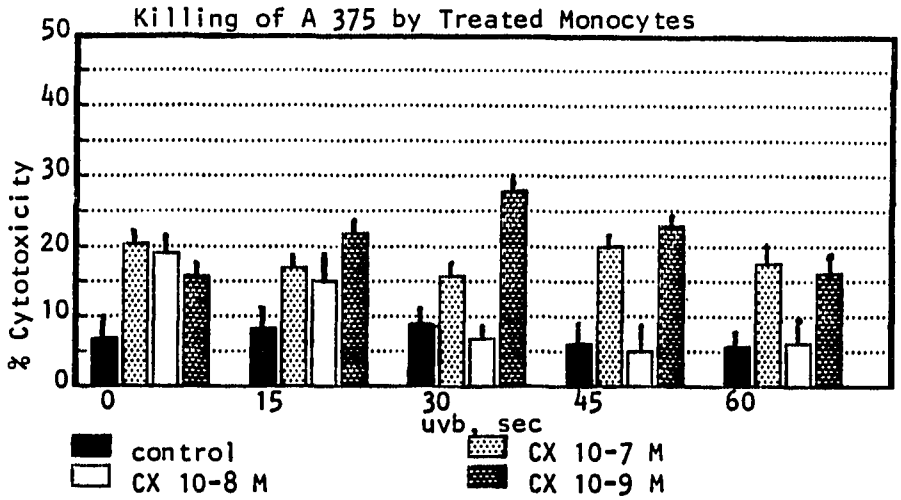

Figure 15. Percent cytotoxicity of A375 by monocytes treated with UVB and canthaxanthin.

Values given are the mean percent cytotoxicity after a 72h incubation, +/- 1 s.d.

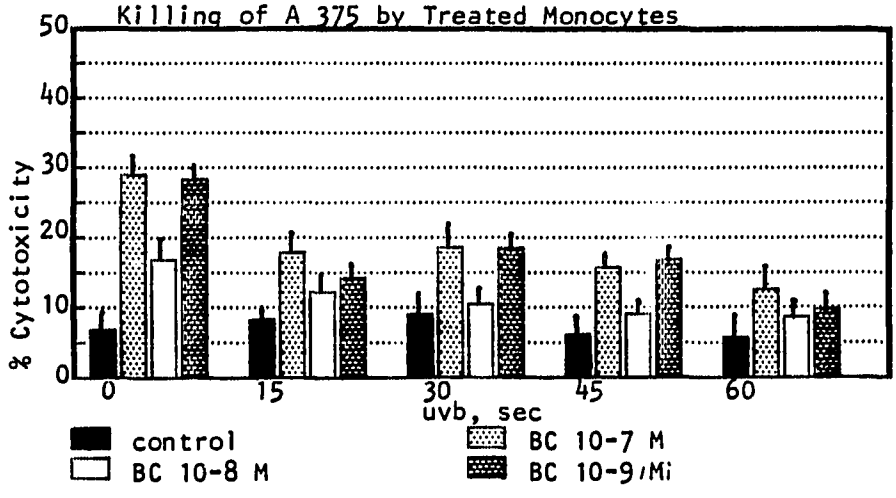

Figure 16. Percent cytotoxicity of A375 by monocytes treated with UVB and beta carotene.

Values are given are the mean percent cytotoxicity after 72h incubation, +/- 1 s.d.

carotenoids had a stimulatory rather than a protective effect on cytotoxic activity.

These results demonstrate an enhancing, rather than a protective property for these compounds in the cytotoxic function of the monocyte. Each of the compounds tested--BC, CX, and 13 CRA, showed enhancement of cytotoxicity that compared closely with the results of the application of gamma interferon to the cells which is a known enhancer of cytotoxic function (50,65). This is evident in figures 14-16, especially at the higher concentrations of the compounds. The mechanism of cytotoxicity did not appear to be greatly affected by treatment with UVB; for example, after 60 sec (220 J/M^2) exposure, cytotoxicity after treatment with BC and 13 CRA at 10^{-7} M had decreased, but not to as great an extent as seen in decreases in phagocytic activity, as determined by statistical analysis. This is positive evidence that the compounds tested did not have a protective, rather than an enhancing effect on monocyte function, at a p value of 0.05.

Mitogenesis After UVB Treatment and Exposure to Retinoids and Carotenoids

Titration of antigen and mitogen

The use of tetanus toxin proved to be ineffective in stimulating T cell mitogenic activity. At any concentration of toxin used, the T cell stimulation index

(SI) after 5 day incubation did not increase significantly above a value of 1.0 (data not shown). The mitogen Con A gave a much greater response, especially after pulsing the monocytes at the 125 ug/ml level. The cells were incubated with the mitogen for 1, 2, 3, 4, or 5 days (data not shown), and the maximal response was seen after 72h incubation (figure 17).

Mitogenic response after UVB and compound treatment

After the maximum level of UVB exposure (60 sec), the T cell stimulation index was not decreased significantly, as determined by the statistical analysis at $p=0.05$. The Con A was taken up by, and presented to the T cells regardless of the dosage of UVB that was applied. UVB exposure did not seem to affect presentation of this mitogen by the monocytes.

When the carotenoid BC at high (10^{-8} M) concentration was applied to the monocytes during mitogen pulse (and before UVB exposure), there was a greater T cell stimulation index as compared to untreated control values. This increase, however, was small; the maximum differences to controls are at the highest UVB levels, as seen in figure 18. The difference between the control values and the treatment values (figure 19) were not significant, as determined by statistical analysis at $p=0.05$. Therefore, application of BC to the cells

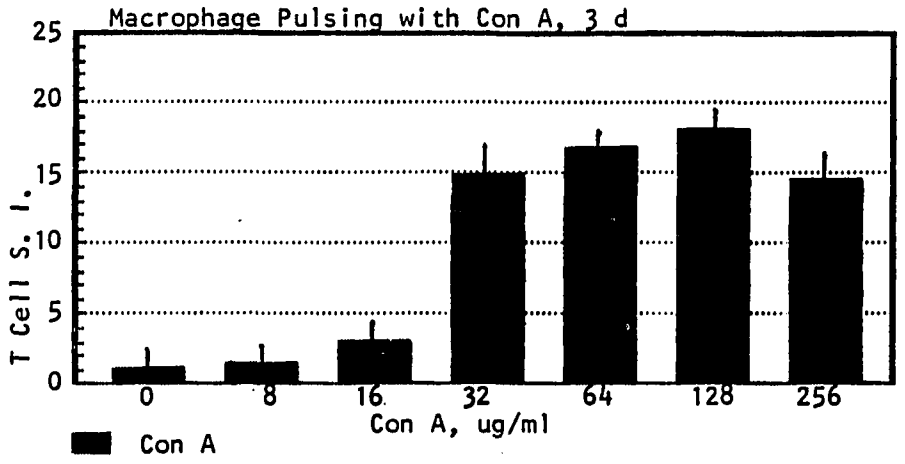

Figure 17. T cell stimulation index after 3 day monocyte pulse with Con A.

Values are given as the mean stimulation index, +/- 1 s.d.

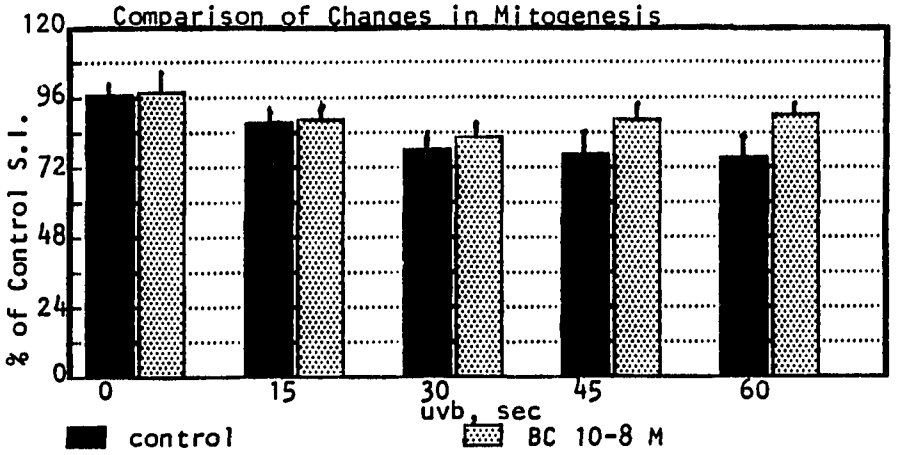

Figure 18. Comparison of changes in antigen presenting ability after UVB and BC exposure.

Values represent the average T cell stimulation index as compared to unexposed control values, +/- 1 s.d.

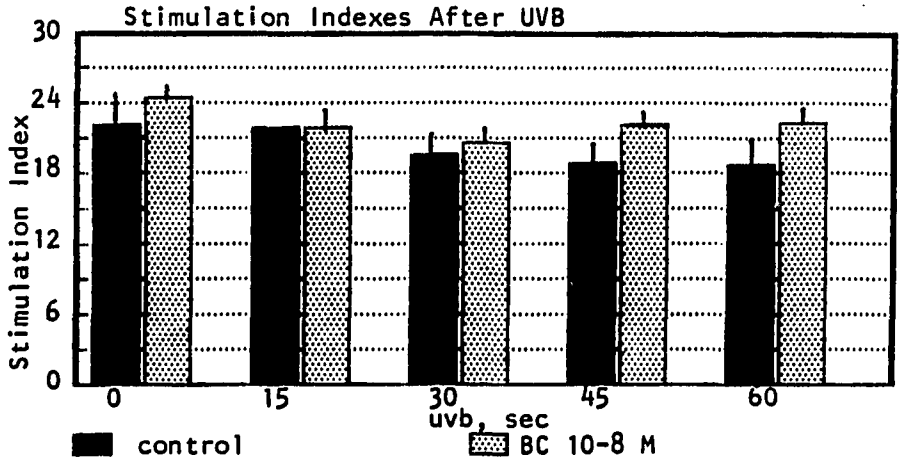

Figure 19. A comparison of T cell stimulation indexes after monocyte exposure to UVB and BC.

Values are the mean S.I. +/- 1 s.d.

concomitant to exposure to the mitogen Con A did not have a significant effect on either UVB protection, or on stimulation of increased antigen presenting activity.

Chapter 4

Discussion

Morison, et al. (44) and Schuller (63) have shown that as the UVB dose increases, the cell viability decreases at a steady rate up to 72h after exposure. This decrease was dependent on the cell type, with some cell types such as T cells being more sensitive to cell death than other cell types (8). They also found that the decrease in viability was not an immediate consequence of UV exposure, but started to become a significant factor 24h after culture. The amounts of UVB applied to the monocytes in culture, allowed enough viable, functional cells to remain at the end of the maximum incubation period, and accurate assays of immune functions could then be investigated.

The findings of Morison, et al. (44) and Schuller (63) that cell viability decreased in culture in a UVB dose-dependent manner were confirmed in the present study. Also confirmed was that after UVB exposure, cell viability decreasaed as time in culture increased. Viability levels consistently decreased as culture times were increased, as seen in table 2. Thus, as Kripke reported (29), the effects of UV are non-reversible and are cumulative over time. Retinoid and carotenoid levels

also affected viability, and all compounds caused a significant decrease in monocyte viability above 10^{-7} M, as seen in table 3. The reason for this decrease is unknown, but may be due simply to the toxicity levels that monocytes can withstand for these compounds is only in the physiological range if 10^{-7} M or less.

In the present studies, the retinoids and carotenoids, particularly 13 cis-retinoic acid, beta carotene and canthaxanthin, proved to be effective in protecting monocytes from some functional damage caused by UVB exposure in vitro. The amount of protection was dose dependent, being maximal at the higher concentrations (10^{-8} to 10^{-7} M) of the compounds that were used. It was found that not all monocyte functions were affected to the same degree by UVB exposure. Also, the retinoids and carotenoids affected various monocyte functions in varying degrees, depending on the type of compound applied and its concentration.

Of all the compounds tested, those with the greatest capacity to act as antioxidants (13CRA, CX, and BC) were the most effective in prevention of UVB damage to the monocyte (figs.3-5). In all of the monocyte functions tested, UVB did decrease cell functions as the level of UVB was increased. When UVB was applied alone, phagocytosis (figs.3-13), and to a lesser extent cytotoxic functions (figs. 14-16) were decreased, while

mitogen presentation to T cells was not greatly affected (figs. 18,19). These results for Ag presentation (figs. 18,19) differ from the results of others, who found UVB decreased Ag presentation by the monocyte (21,47,48,53). These differences may be due to the fact that only a mitogen and not an antigen was presented by the monocyte in the present study. Since presentation of tetanus toxin was not successful, perhaps other antigens such as haptenated-self Ag (55) would show a more pronounced effect of UVB on Ag presentation by the monocyte.

As others have found, the retinoids and carotenoids can increase immune functions of monocytes, including phagocytosis (43), cytotoxicity (57,68) and to some extent antigen presentation (71). It was found that cytotoxic functions were increased by use of the compounds (figs. 14-16) at concentrations as low as 10^{-9} M. Other functions, such as phagocytosis, remained at normal levels, equal to control values (figs.3-11). Ability to present a mitogen (Con A) to T cells also was not significantly changed by application of these compounds without UVB exposure (figs.18,19).

Mathews-Roth and others (38,59) have shown that carotenoids have significant antioxidant properties in vivo, being potent antioxidants and quenchers of singlet oxygen and free radical formation (28,59). Other vitamins have antioxidant properties as well. An

important finding by Anderson (1) was that phagocytic function in other phagocytes (PMN) was enhanced by application of vitamin C, and this change was due to the antioxidant properties of this vitamin. The same would likely be true for vitamin E as well, since it is also a potent antioxidant. In the present study, phagocytosis was protected from UVB damage by pretreatment with BC, CX, and 13CRA at 10^{-7} to 10^{-9} M (figs. 3-11). As these are also potent antioxidants and free radical scavengers (41), the protection of phagocytic function may be due solely to their antioxidant properties. Other antioxidants tested for protective abilities (figs. 12, 13) compared favorably to the results from the retinoids or carotenoids (figs. 3-5). Other monocyte functions, cytotoxicity and Ag presentation, did not seem to be protected by exposure to these compounds (figs.14-16, 18-19).

The retinoids and carotenoids have many complex interactions with monocyte function. Some monokines such as IL-1, IFN and TNF can be released by monocytes stimulated by these compounds (43,71). To determine if the mechanism of protection <u>in vitro</u> was due to release of cell activating monokines or lymphokines, monoclonal antibodies to these compounds were added to the media. Antibodies to these monokines failed to reduce protection of phagocytic function from UVB damage that occured after

application of the compounds (figs.9-11). This was seen as evidence that this protective property may not be due to monocyte stimulation by monokine and other lymphokine release by the monocytes.

Indomethacin and acetylsalicylic acid (IM and ASA) are both inhibitors of cell function, as they inhibit prostaglandin (PG) synthesis (15). The PG's are also known to be important in induction of phagocytosis of some particles (20). Application of these PG inhibitors to monocytes treated with retinoids or carotenoids did not diminish significantly their UVB protective capacity (figs.6-8). Release of cell activating PG's by the monocyte does not seem to be induced by retinoid or carotenoid exposure, and therefore does not appear to be important in protecting phagocytic function from UVB exposure. If PG release is induced by exposure to the compounds, they may induce or otherwise alter functions other than phagocytosis of SRBC in vitro.

The exact cellular target of this protection from UVB damage is still open to question. A likely area for protection would be the cell membrane. An intact, undamaged cell membrane is essential for proper immune function, including phagocytosis. If oxidation of fatty acids and sterols in the membrane is caused UVB exposure (8), then these antioxidants should be able to prevent or mitigate UVB induced membrane damage.

The mechanistic studies of the protective abilities of the retinoids and carotenoids provided many ideas about the nature of this protection. The main area of protection was due mainly to the antioxidant properties of the compounds, which prevent singlet oxygen and other toxic products to form as a consequence of exposure to UVB. At the cell surface, this would be important in leaving the cell membrane intact, which allows for proper immune functioning. Also determined was that different immune functions have much different sensitivities to the effects of UVB. Phagocytosis was quite sensitive to UVB (figs. 3-5) while cytotoxicity and antigen presentation were affected to a lesser extent.

The monocyte is an important cell in immune responsiveness in the skin. Decrease in immune function caused by UVB exposure at the skin surface could result in increased tumor promotion in vivo (6,27). The retinoids and carotenoids have in vivo antitumor activity, and it will be interesting to determine if application of these compounds in vivo could decrease UVB induced tumors due to damage to the monocyte and to other cells of the skin immune system, especially Langerhans cells. Because all people are exposed to some dose of UVB on a daily basis, this protection would be most beneficial over the course of yearly exposure levels.

Other effects on monocyte activity caused by the retinoids and carotenoids, such as increases in prostaglandin release and release of monokines such as IL-1 and IFN, did not seem to be important in protection of the cell from UVB damage (figs. 6-11). This does not mean that they are not important in cell activation; indeed, it is quite probable that cell activation by the compounds does occur in vitro at the concentrations used. Further studies will be needed to determine if there is a link between monokine release and both monocyte activation and UVB protection, at least in vitro.

It is important that retinoids and carotenoids can be delivered in significant quantities to the skin area in vivo (36,37,38). Prevention of certain skin cancers (such as melanomas), and other UVB caused damage could be prevented at the skin surface. Topical application of carotenoids and retinoic acid (6,37,39) has had some success in prevention of some cancers and in reducing sensitivity to UVB. In vivo, mice given oral administration of carotenoids had reductions in UVB induced skin tumors (69). Oral or intravenous administration of these compounds may be a practical way to deliver enough of the compounds to the skin in an effective dose level (36).

In this study it was shown that retinoids and carotenoids can stimulate immune function of peripheral

blood monocyte in humans, as seen in anti-tumor cell cytotoxic functions (figs. 14-16). They also protect monocyte phagocytic function from damage induced by exposure to UVB (figs. 3-5). It was significant that this protection occurred in the concentration range that could be achieved in an in vivo situation, and therefore may be important from a clinical application point of view. There are thousands of possible retinoid and carotenoid combinations, many of which are now becoming commercially available. With all these new compounds, it is probable that a few or several of these could have a positive effect on immune function and UVB protection in vivo. Because toxicity levels can vary greatly from compound to compound, it is also possible that some of these could be applied in vivo in larger doses, with few if many harmful side effects.

The incidence of skin cancers and other UVB induced skin disorders has been growing steadily over the past several years. A change in diet that would include more carotenoids such as beta carotene, and lycopene, as well as retinoids like retinoic acid and vitamin A would be most beneficial in reducing some of the alarming skin cancer statistics. As mentioned above, protection and stimulation of the skin immune system is the most effective preventative measure for prevention of tumor promotion caused by UVB. Retinoids and carotenoids can

accomplish both protection and stimulation, and should be seriously considered as an important part of our diets in the future. This effect on monocyte function does vary from person to person, as most subjects in the present study did show variation in stimulation and protective capabilities that the compounds had on their monocyte function. Overall, however, these effects were always positive, which is important when deciding whether the clinical application of these compounds will be beneficial in the future.

REFERENCES

1. Anderson, R. 1982. Ascorbic acid and immune functions: mechanism of immunostimulation. In: Vitamin C (ascorbic acid) (Counsell, J. ed.), Applied Science Pub., England.

2. Ansel, J., Luger, T., Kock, A., Hochstein, D., Green, I. 1984. The effect of in vitro UV irradiation on the production of Il1 by murine macrophages and P388d1 cells. J. Immunol. 133:1350-1355.

3. Bendich, A., Shapiro, S. 1986. Effect of beta carotene and canthaxanthin on the immune responses of the rat. J. Nutr. 116:2254-2262.

4. Black, H., Chan, J. 1977. Experimental ultraviolet light carcinogenesis. Photochem. Photobiol. 26:183-199.

5. Celada, F., Lanzaveccia, A. 1985. A dramatic representation of immunological cooperative events. Int. Archs. Appl. Dermatol. 76:28-33.

6. Connor, L. 1983. Inhibition of UVB skin carcinogenesis by all trans- retinoic acid regimens that inhibit ODC induction. Cancer Res. 43:171-174.

7. Daynes, R. 1983. The effects of UV exposure on antigen-presenting cell function in the spleen. In: Experimental and Clinical Photoimmunology vol. II(Daynes, R., Krueger, G., eds.) CRC Press, Boca Raton, Fla.

8. Daynes, R., Gurish, M., Lynch, D., Spangrude, G. 1983. Biological consequences of acute and chronic UVR exposure. In: The effect of UVR on the immune system(Parrish,J. ed.) Johnson and Johnson Press, Albuquerque, NM.

9. Daynes, R., Burnham, D., Dewitt, C., Roberts, L., Krueger, G. 1985. The immunobiology of ultraviolet-radiation carcinogenesis. Cancer Surv. 4:51-99.

10. Drebin, J. 1983. Characterization of ultraviolet radiation-induced impairment of antigen-presenting

cell function at the cellular and molecular levels. In: The effect of UVR on the immune system (Parrish,J., ed.) Johnson and Johnson Press, NM.

11. Elmee, C., et.al. 1986. UVB radiation inhibits accessory signals required for mitogenic responses of human T lymphocytes. Clin. Res. 34:748a.

12. Everett, M., Yeargers, E., Sayre, R., Olson, R. 1966. Penetration of the epidermis by ultraviolet rays. Photochem. Photobiol. 5:533-542.

13. Fidler, I., Fogler, W., Kleinerman, E., Saiki, I. 1985. Abrogation of species specificity for activation of tumoricidal properties in macrophages by recombinant mouse or human IFN encapsulated in liposomes. J. Immunol. 135:4289-4296.

14. Fleit, S., Fleit, H., Zolla-Pazner, S. 1984. Culture and recovery of monocytes and cell lines from tissue culture-treated and untreated plastic dishes. J. Immunol. Meth. 68:119-129.

15. Flower, R., et.al. 1974. Drugs which inhibit prostaglandin synthesis. Pharmacol. Rev. 26:33-67.

16. Fox, I., Perry, L., Sy, M., Benacerraf, B., Greene, M. 1980. The influence of ultraviolet light irradiation in the immune system. Clin. Immunol. Immunopath. 17:141-155.

17. Germain, R. 1981. Accessory cell stimulation of T cell proliferation requires active antigen processing, Ia-restricted antigen presentation, and a separate nonspecific second signal. J. Immunol. 127:1964-1966.

18. Giard, D., et.al. 1973. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J.Nat.Acad. Sci. 51:1417-1423.

19. Greene, M., Sy, M., Kripke, M., Benacerraf, B. 1979. Impairment of APC function by UVR. Proc. Nat. Acad. Sci. 76:6591-6595.

20. Grimm, W., et.al. 1978. Prostaglandin synthesis in spleen cell cultures of mice injected with C.parvum. Cell. Immunol. 40:419-426.

21. Gurish, M., Lynch, D., Daynes, R. 1982. Changes in APC function in the spleen and lymph nodes of UV-irradiated mice. Transplan. 33:280-284.

22. Humes, J., et.al. 1977. Macrophages synthesize and release prostaglandins in response to inflammatory stimuli. Nature 269:149-151.

23. Jakway, J., Shevach, E. 1983. Stimulation of T cell activity by UV-treated, Ag-pulsed macrophages: evidence for a requirement for Ag processing and Il-1 secretion. Cell. Immunol. 80:151-162.

24. Kleinerman, E., Schroit, A., Fogler, W., Fidler, I. Fidler,I. 1983. Tumoricidal activity of human monocytes activated in vitro by free and liposome-encapsulated lymphokines. J. Clin. Invest. 72:304-315.

25. Kleinerman, E., Erickson, K., Schroit, A., Fogler, W., Fidler, I. 1983. Activation of tumoricidal properties in human blood monocytes by liposomes containing MTP. Cancer Res. 43:2010-2014.

26. Kligman, L. 1986. Lack of inhibition of ultraviolet radiation-induced ornithine decarboxylase activity by retinoic acid. Photochem. Photobiol. 43:449-453.

27. Kochevar, I. 1983. Photobiologic mechanisms in photoimmunology: membrane alteration and photoallergy. In: The effect of UVR on the immune system (Parrish, J., ed.) Johnson and Johnson Press, Albuquerque, NM.

28. Krinsky, N., Deneke, S. 1982. Interaction of oxygen and oxy-radicals with carotenoids. J. Nat. Cancer Inst. 69:205-209.

29. Kripke, M. 1984. Immunological unresponsiveness induced by ultraviolet radiation. Immunol. Rev. 80:87-102.

30. Kripke, M., McClendon, E. 1986. Studies on the role of APC's in the systemic suppression of contact hypersensitivity by UVB radiation. J. Immunol. 137:443-447.

31. Lange-Wantzin, G., Rothlein, R., Kahn, J., Faanes, R. 1987. Effect of UV irradiation on expression of membrane Il1 by rat macrophages. J. Immunol. 138: 3803-3807.

32. Lederman, M., Schacter, B., LeVine, M., Ellner, J. 1986. Inhibition of human lymphocyte proliferation by UVR: effects if UVB on T cell and macrophage accessory function and induction of suppression mechanisms. J. Lab. Clin. Med. 107:66-72.

33. Letvin, N., Fox, I., Greene, M., Benacerraf, B., Germain, R. 1980. Immunologic effects of whole body ultraviolet(UV) irradiation. II. Defect in splenic adherent cell antigen presentation for stimulation of T cell proliferation. J. Immunol. 125:1402-1404.

34. Lotan, R. 1980. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. Biochim. Biophys. Acta 605:33-91.

35. Lynch, D., Gurish, M., Daynes, R. 1981. The effects of ultraviolet radiation on the generation of anti-tumor cytotoxic effector cell responses in vitro. J. Immunol. 127:1163-1168.

36. Mathews-Roth, M. 1982. Antitumor activity of beta-carotene, canthaxanthin, and phytoene. Oncology 39:33-37.

37. Mathews-Roth, M. 1985. Carotenoid dose level and protection against UVB induced skin tumors. Photochem. Photobiol. 42:35-38.

38. Mathews-Roth, M. 1986. Carotenoids quench evolution of excited species in epidermis exposed to UVB light. Photochem. Photobiol. 43:91-93.

39. Mathews-Roth, M. 1986. Beta carotene therapy of erythropoetic protoporphyria and other photosensitivity diseases. Biochimie 68:875-884.

40. Mathews-Roth, M. 1987. Photoprotection by carotenoids. Feder. Proc. 46:1890-1893.

41. McCay, P., King, M. 1980. Vitamin E: its role as a free radical scavenger and its relationship to the microsomal mixed function oxidase system. In: Vitamin A: a comprehensive treatise(Machlin, L., ed.) Marcel Dekker, Pub., New York.

42. Ming, W., Bersani, L., Mantovani, A. 1987. Tumor necrosis factor is chemotactic for monocytes and PMN. J. Immunol. 138:1469-1474.

43. Moriguchi, S., Sone, S., Kishino, Y. 1983. Changes in alveolar macrophages in protein deficient rats. J. Nutrition 113:40-46.

44. Morison, W., Parrish, J., Anderson, R., Bloch, K. 1979. Sensitivity of mononuclear cells to UV radiation. Photochem. Photobiol. 29:1045-1047.

45. Morison, W., Parrish, J., McCauliffe, D., Bloch, K. 1980. Sensitivity of mononuclear cells to UVR: effect of subsequent stimulation with PHA. Photochem. Photobiol. 32:99-101.

46. Nemoto, O., Koizumi, H., Aoyagi, T. 1986. A novel synthetic vitamin A-like compound inhibits UVB-stimulated epidermal ODC activity. Arch. Dermatol. Res. 278:407-409.

47. Noma, T. 1985. Characterization of the accessory cells involved in Ts induction. J. Immunol. 134:1374-1380.

48. Noonan, F., Kripke, M., Pedersen, G., Greene, M. 1981. Suppression of contact hypersensitivity in mice by UV irradiation is associated with defective antigen presentation. Immunol. 43:527-533.

49. Norbury, K., Fidler, I. 1975. In vitro tumor cell destruction by syngeneic mouse macrophages: methods for assaying cytotoxicity. J. Immun. Meth. 7:109-122.

50. Oppenheimer, J., Ruscetti, F., Steeg, P. 1984. Interleukins and interferons. In: Basic and clinical immunology (Stites, D., Stobo, S., Fudenberg, H., Wells, J., eds.) Lange Medical Publications, Los Altos, CA.

51. Parrish, J., Kripke, M., Morison, W. 1983. Effects of UV radiation on animals. In: Photoimmunology (Parrish, J. ed.) Plenum Press, New York.

52. Pretell, J. 1983. Effects of UVB radiation on peripheral blood mononuclear cells in the in vitro response to soluble antigen. In: The effect of ultraviolet radiation on the immune system (Parrish, J., ed.) Johnson and Johnson Press, Albuquerque, NM.

53. Pretell, J., Wimberly, J., Parrish, J. 1984. UVR-induced defects in accessory cell function in the human proliferative response to tetanus. Clin. Exper. Immunol. 56:105-113.

54. Pretell, J., Wimberly, J., McCauliffe, D., Parrish, J. 1984. Comparison of effects of UVA, UVB, and UVC on growth and viability of mitogen-stimulated human peripheral blood cells. Photochem. Photobiol. 39:369-374.

55. Pretell, J., McCauliffe, D., Parrish, J. 1984. UVR inhibits both accessory cell and responder T function in the proliferative response to haptenated self. Photochem. Photobiol. 40:337-342.

56. Raz, A., Fogler, W., Fidler, I. 1979. The effects of experimental conditions on the expression of in vitro-mediated tumor cytotoxicity by murine macrophages. Cancer Immunol. Immunother. 7:157-163.

57. Rhodes, J., Oliver, S. 1980. Retinoids as regulators of macrophage function. Immunol. 40:467-472.

58. Rhodes, J., Stokes, P., Abrams, P. 1984. Human tumor-induced inhibition of IFN action in vitro: reversal of inhibition by beta-carotene(provitamin A). Cancer Immunol. Immunother. 16:189-192.

59. Ritenbaugh, C. 1987. Carotenoids and cancer. Nutr. Today 22:14-19.

60. Roberts, L., Samlowski, W., Daynes, R. 1986. Immunological consequences of ultraviolet radiation exposure. Photoderm. 3:284-298.

61. Roe, D. 1987. Photodegradation of carotenoids in human subjects. Feder. Proc. 46:1886-1889.

62. Sauder, D. 1983. Effect of UV radiation on alloantigen presentation and on epidermal cell derived thymocyte activating factor(ETAF). In: The effect of ultraviolet light on the immune system (Parrish, J. ed.) Johnson and Johnson Press, Albuquerque, NM.

63. Schuller, G., Hellman, K. 1981. UVR induced changes in macrophage activity. Photochem. Photobiol. 34:741-743.

64. Schuller-Levis, G., Hellman, K. 1983. Modulation of macrophage function by ultraviolet radiation. In: The effect of ultraviolet radiation on the immune system (Parrish, J. ed.) Johnson and Johnson Press, Albuquerque, NM.

65. Shalaby, M., et.al. 1985. Activation of human polymorphonuclear neutrophil functions by interferon gamma and tumor necrosis factors. J. Immunol. 135: 2069-2073.

66. Simpson, K., Chichester, C. 1981. Metabolism and nutritional significance of carotenoids. Ann. Rev. Nutr. 1:351-374.

67. Spellman, C., Tomasi, T. 1983. Ultraviolet light-induced suppression of antigen presentation. J. Clin. Immunol. 3:105-110.

68. Tachibana, K., et. al. 1984. Stimulatory effect of vitamin A on tumoricidal activity of rat alveolar macrophages. Brit. J. Cancer 49:343-348.

69. Trechsel, U., Evequoz, V., Fleisch, H. 1985. Carotenoid dose level and protection against UVB induced skin tumors. Biochem. J. 230:339-344.

70. Vahlquist, A., Berne, B. 1984. Sunlight, vitamin A and the skin. Photoderm. 3:203-205.

71. Watson, R. 1984. Immunoregulation by retinoids and carotenoids. In: Nutrition, disease resistance and immune function (Watson, R., ed.) Marcel Dekker, Pub., New York.

72. Webb, D. 1982. In: Immunopharmacology and the regulation of leukocyte function (Webb, D. ed.) Marcel Dekker Press, New York.