

Dietary Lutein Reduces Ultraviolet Radiation-Induced Inflammation and Immunosuppression

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Ultraviolet radiation (UVR) promotes skin cancer development by mutagenic, immunosuppressive, and oxidative-stress-inducing mechanisms; however, certain antioxidants may counteract and prevent UVR-induced photo-damage. Lutein is a xanthophyll carotenoid with potent antioxidant activity. Because reactive oxygen species (ROS) are believed to have a role in UVR-induced skin damage, we investigated whether lutein can modify UVR effects including the tissue swelling response to midrange UVR (280–320 nm, ultraviolet B (UVB) radiation) and UVB suppression of contact hypersensitivity (CHS) in both the local and the systemic models of UV-induced immunosuppression. We found that compared to mice fed the standard laboratory diet, mice fed dietary lutein demonstrated significant inhibition of ear swelling owing to UVB radiation. Mice exposed to 1700 J per m² UVB radiation four times at daily intervals and then sensitized to dinitrofluorobenzene at the site of irradiation showed a decreased CHS response upon challenge. This suppression by UVB radiation was significantly inhibited by lutein feeding. When UVB radiation was given at a single dose of 10,000 J per m² to inhibit the induction of CHS at a distant, nonirradiated site, no effect of lutein was seen. Finally, lutein accumulated in the skin of mice following diet supplementation and was shown to decrease ROS generation following UVR exposure. Thus, lutein modulates the skin's response to UVR and may contribute to the defense against some of the deleterious effects of solar radiation.

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Exposure to ultraviolet radiation (UVR) induces a variety of biologic effects including inflammation, sunburn cell formation, immunologic alterations, and photoaging (Taylor *et al*, 1990; Hruza and Pentland, 1993). Exposure to ultraviolet B (UVB) radiation (290–320 nm) suppresses the immune system (Kripke, 1984) and is the primary cause of nonmelanoma skin cancer in humans and animals (Urbach, 1997). UVB radiation critically damages cellular macromolecules and induces the formation of reactive oxygen species (ROS) (Fuchs, 1992). Ultraviolet A radiation (320–400 nm) contributes up to 95% of total UV exposure and is a significant source of oxidative stress in human skin (Tyrrell, 1991; Parisi and Wong, 2000). UVR-induced ROS include superoxides, singlet oxygen, and hydroxyl radicals and are believed to contribute to skin cancer formation, certain photodermatoses, sunburn, and photoaging (Black, 1987; Darr and Fridovich, 1994). To protect cells from UV-induced damage, the skin has an elaborate antioxidant system consisting of enzymatic and nonenzymatic components to

quench reactive oxygen intermediates. Nevertheless, excessive exposure to UVR overwhelms and depletes the cutaneous antioxidant supply leading to a state of oxidative stress (Fuchs, 1998).

In recent years, the use of supplementary antioxidants as photoprotective agents has been explored. UV-induced erythema in humans is reduced following supplementation with an antioxidant combination that includes β -carotene, vitamin C, and vitamin E (Gruel *et al*, 2002) and following topical application of green tea polyphenol extracts (Elmets *et al*, 2001). The soybean isoflavone genistein inhibits UVB-induced oxidative events in murine skin (Wei *et al*, 2002), and trace minerals such as zinc, with antioxidant properties, are also reported to be photoprotective (Rostan *et al*, 2002). Despite the numerous antioxidants being investigated as potential protective agents, currently only β -carotene has been shown to be effective against visible light sensitivity and is thus recommended for the treatment of erythropoietic protoporphyria (Mathews-Roth, 1998; Rhodes, 1998). Carotenoids are lipophilic micronutrients with the ability to quench ROS and inhibit free radical reactions (Sies and Stahl, 1995). Epidemiologic studies show that a high intake of carotenoid-rich foods is associated with a reduced incidence of many forms of cancer and suggest that this association is due to the antioxidant properties of these compounds (Block *et al*, 1992). Carotenoids are present in the epidermis and dermis and are believed to play an

Abbreviations: CHS, contact hypersensitivity; DHR, dihydrorhodamine; DNFB, dinitrofluorobenzene; ROS, reactive oxygen species; UVB, ultraviolet B; UVR, ultraviolet radiation.

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important part in the skin's antioxidant defense system. Recent evidence demonstrating lower carotenoid concentrations in human basal cell carcinomas, actinic keratoses, and perilesional sites suggest that carotenoids are important in the skin's defense against UVR (Hata *et al*, 2000). The antioxidant potential of carotenoids has thus warranted studies investigating their role in UV-induced skin damage.

UVR reduces plasma carotenoid levels in humans (White *et al*, 1988) and in one mouse model UVB-induced cancer development was prevented or delayed by carotenoids (Mathews-Roth and Krinsky, 1987). β -Carotene is the most extensively studied carotenoid; however, inconsistency between experimental data and clinical studies question the photoprotective effects of β -carotene in normal skin (Green *et al*, 1999; Biesalski and Obermueller-Jevic, 2001). Therefore, studies on the beneficial potential of other carotenoids should be conducted. Lutein is among the most prevalent carotenoids found in normal skin. Distributed among dark green leafy vegetables such as spinach, kale, and broccoli, lutein is the dihydroxy form of α -carotene (Nishino *et al*, 2000). A major carotenoid in the macular pigment of the retina (Bone *et al*, 1985; Handelman *et al*, 1988), lutein effectively filters blue light (400–475 nm) with an absorption maximum of 445 nm and is believed to protect the retina from light-induced oxidative damage (Junghans *et al*, 2001; Landrum and Bone 2001; Krinsky, 2002).

Our purpose was to determine whether dietary lutein could inhibit UVR-induced tissue swelling (as a murine surrogate for sunburn) and UVR-induced immunosuppression. To evaluate UVR-induced immunosuppression *in vivo*, contact hypersensitivity (CHS) responses were assessed in both the low-dose and the high-dose models of UVB-radiation-induced immunosuppression. In the low-dose model, skin was exposed to a dose of UVB radiation too small to cause visible cutaneous changes. Hapten was then applied only to the irradiated site for immunization. This resulted in a suppressed CHS response upon challenge (Beissert and Schwarz, 1999). Immunization at a nonirradiated site leads to a normal CHS response in this model. In the high-dose model, a dose of UVB radiation was given that was large enough to cause visible changes in the skin after a few days. Sensitization was then performed at a distant, nonirradiated skin site. This also resulted in a suppressed CHS response upon challenge (Beissert and Schwarz, 1999). We found that lutein reduces tissue swelling induced by UVB radiation and inhibits the UV-induced immunosuppressive effects in the low-dose model; however, no effect was seen in the high-dose model of UV-induced immunosuppression under the conditions utilized. In accordance with these observations, lutein was found to accumulate in the skin following diet supplementation and to decrease the production of ROS in skin following UVR. Together, these data demonstrate lutein protects the skin from UV-induced damage.

Results

Lutein protects from the sunburn reaction The ear swelling response is a biologic marker of the inflammatory

response *in vivo* (Young *et al*, 1984; Opas *et al*, 1985). To determine whether lutein inhibited ear inflammation following UVB irradiation, the ears of mice were exposed to one dose of 3500 J per m². The ear swelling response was calculated as the difference in ear thickness between the 24- and 48-h postradiation values and the baseline value. Mice in the 0.4% lutein diet group had significantly decreased 24-h ear swelling compared to mice in the standard diet group ($p = 0.01$) as seen in Fig 1. Although the ear swelling of mice in the 0.04% diet group was diminished, this value did not reach statistical significance ($p = 0.07$). We thus conclude that dietary lutein reduces UV-induced inflammation.

Lutein protects from local UVB-radiation-induced immune suppression The role of lutein in UVB-induced immune suppression of CHS is unknown. To determine whether dietary lutein protects from UVB-induced reduction of the CHS response to DNFB in the local model of UV-induced immune suppression, mice received 1700 J per m² UVB to the dorsum for 4 consecutive d. In the standard laboratory diet group, the 24-h CHS response in mice exposed to UVB radiation and sensitized to DNFB was significantly reduced compared to mice only sensitized with DNFB, as expected ($p = 0.002$) (Fig 2). In mice fed either a 0.04% or a 0.4% lutein-supplemented diet, significant suppression of the 24-h CHS response was not observed ($p = 0.162$ and $p = 0.608$, respectively). Similar results were observed after 48 h. Therefore, dietary lutein can inhibit the immunosuppressive effects of UVR on CHS induction in this model.

Lutein has no effect in the systemic model of UVB-radiation-induced immunosuppression To determine whether dietary lutein impairs CHS responses in the

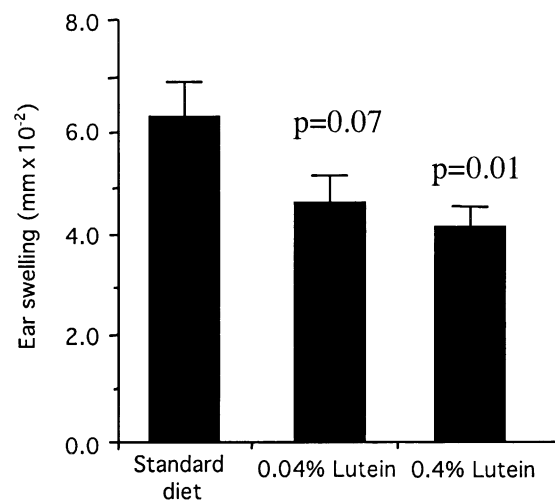


Figure 1
Lutein reduces UVB-induced tissue swelling. The ears of mice in all diet groups ($n = 10$ per group) were exposed to one dose of 3500 J per m². The ear swelling response was calculated as the difference in ear thickness between the 24-h measurement and the baseline value. Mice fed a 0.4% diet for 2 wk had a significantly decreased ear swelling response compared to mice fed a standard diet ($p = 0.01$). Although mice fed a 0.04% lutein diet had diminished ear swelling compared to mice fed a standard diet, this value did not reach statistical significance. Results are represented as the mean \pm SEM.

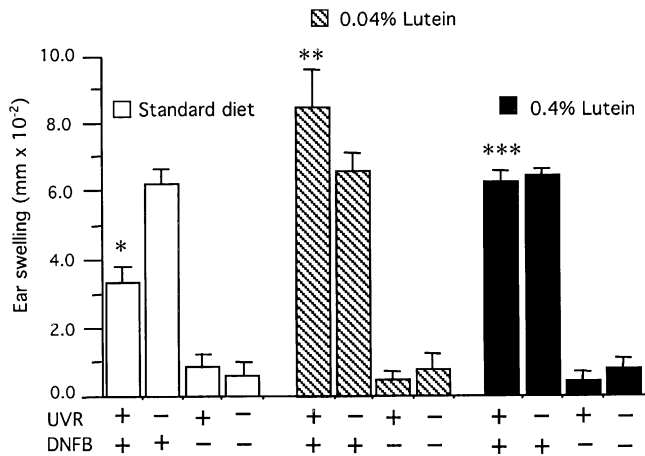


Figure 2
Lutein inhibits UVB-radiation-induced immunosuppression following low-dose UVB exposure. The CHS response as determined by the extent of ear swelling was measured as described under Materials and Methods. Following UVB exposure and DNFB sensitization, the 24-h mean ear swelling response of mice in the standard diet group was significantly less than the positive control, DNFB-sensitized mice (* $p=0.002$). This suppression was no longer seen in mice fed a 0.04% lutein- (** $p=0.162$) or 0.4% (** $p=0.608$) lutein-supplemented diet. Similar results were seen after 48 h. Results are represented as the mean \pm SEM; $n=5$ per group.

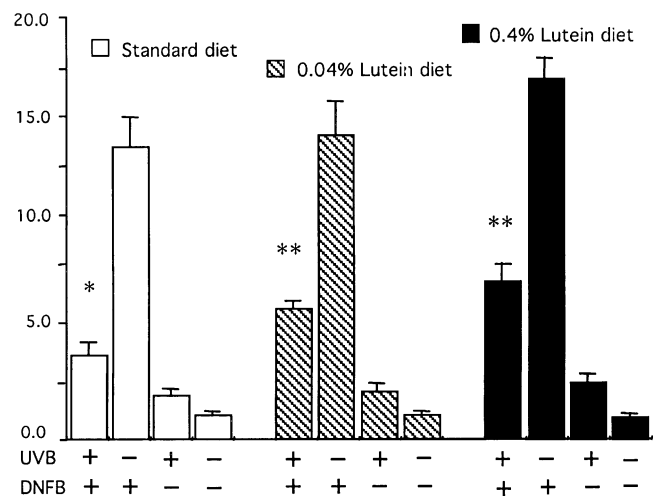


Figure 3
Lutein had no effect in the high-dose model of UV-induced immunosuppression. Following a 3-wk feeding period, the CHS response was determined after exposure to high-dose UVB radiation. Mice in all three diet groups demonstrated a significantly decreased 24-h CHS response following UVB radiation and DNFB sensitization compared to mice only sensitized to DNFB. Similar results were seen after 48 h. Results are represented as the mean \pm SEM. * $p=0.002$ for positive control versus UVB + DNFB in mice fed a standard diet. ** $p=0.0002$ for positive control versus UVB + DNFB in mice fed 0.04% and 0.4% lutein. $n=5$ per group.

systemic model of UV-induced immune suppression, mice received one dose of 10 000 J per m^2 UV radiation to the dorsum. Five days later, 25 μ L of 0.5% DNFB was applied epicutaneously to the abdomen. Ears were challenged 7 d later with 10 μ L of 0.2% DNFB, and ear thickness was measured 24 and 48 h after challenge. In initial experiments, the diets were given for 2 wk before UVR exposure. Because no effect of lutein was observed, we increased the feeding period before irradiation to 3 wk and additional experiments were performed. As seen in Fig 3, in all three diet groups, UVB-irradiated and DNFB-sensitized mice exhibited significantly reduced ear swellings at 24 h compared to the positive control, DNFB-treated mice. This same pattern was seen after 48 h. We thus conclude dietary lutein does not prevent the deleterious effects of UVR on CHS in the systemic model of UV-induced immunosuppression under the conditions utilized.

Dietary lutein increases murine skin content level Dietary lutein is absorbed into the plasma and taken up by the liver and spleen of mice (Park et al, 1998). To determine whether diet supplementation led to lutein accumulation in the skin, the dorsal skin of four nonirradiated and nonsensitized mice from each diet group was removed and analyzed. There were three feeding periods: 14 d (feeding period before irradiation in UV-induced inflammation and local immunosuppression studies), 29 d (total time course of the local UVR-induced immunosuppression studies), and 35 d (total time course of the systemic UVR-induced immunosuppression studies). As shown in Fig 4, for each feeding period a concentration-dependent increase was observed. The highest skin lutein levels were seen in mice fed a 0.4% lutein-supplemented diet, the next highest in mice fed a 0.04% lutein diet and the lowest levels in mice fed a standard laboratory diet. A significant difference was

observed between the regular diet and 0.4% lutein groups ($p<0.05$) in all feeding periods; however, a significant difference between the regular diet group and 0.04% lutein group was only observed after the 35-d feeding period ($p=0.03$). Therefore, lutein accumulates in the skin following diet supplementation. Note that the lutein content of skin from mice fed the control diet is higher in the 14-d experiment compared to the other experiments. Each experiment (i.e., each feeding period) was performed separately and this difference most likely relates to technical differences between experiments. Thus, groups should only be compared within each experiment.

Dietary lutein reduces ROS generation in murine skin To investigate whether lutein can reduce ROS generation in the skin, the dorsal skin of mice ($n=5$ per diet group) was removed after a 28-d feeding period. The skin samples were incubated in the fluorescence probe DHR for 10 min and imaged before and after irradiated with 200 J per m^2 UVR. DHR is nonfluorescent until it reacts with ROS and forms fluorescent rhodamine-123. As shown in Fig 5, a trend toward decreased ROS formation is seen with increased lutein diet content. There was no significant difference observed between the standard diet and 0.04% lutein diet group. Nevertheless, a p value of 0.05 was observed between the standard diet group and 0.4% lutein diet group. We thus conclude lutein decreases ROS generation following UVR.

Discussion

UVR-induced free radicals contribute to inflammation and are implicated in photocarcinogenesis (Black, 1987; Hruza and Pentland, 1993). The epidermal antioxidant defense

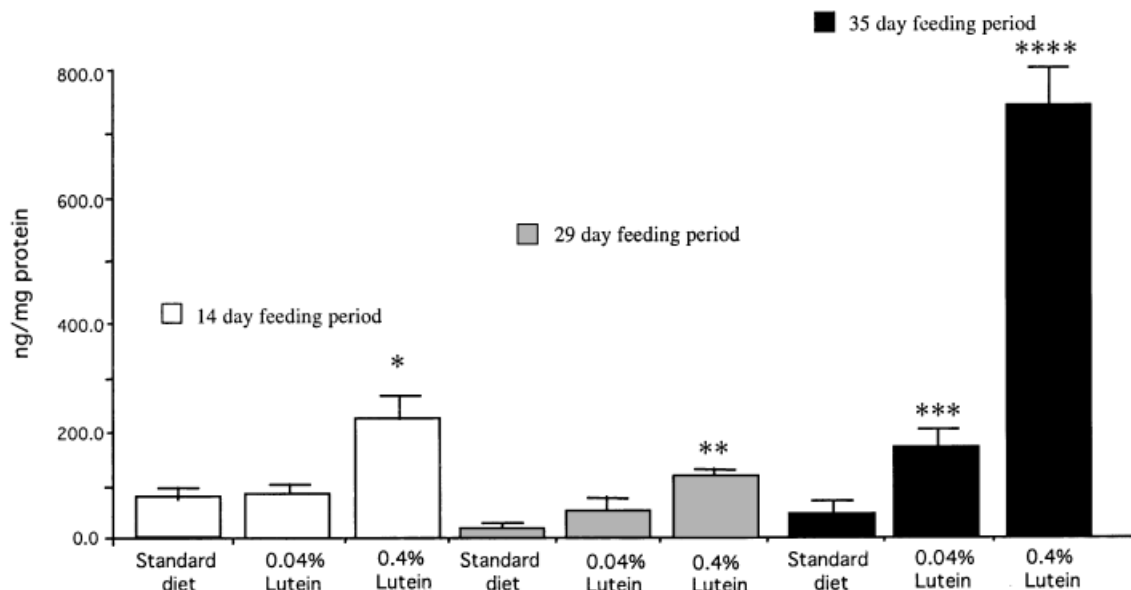


Figure 4

Skin lutein content following diet supplementation. To analyze for skin lutein content, skin samples from four C3H/HeJ mice per diet group (regular diet, 0.04% lutein, and 0.4% lutein) were excised following a 14-, 29-, or 35-d feeding period. All skin samples were rinsed with phosphate-buffered saline, and the subcutaneous fat was mechanically removed. Mice fed a lutein-supplemented diet had increased skin lutein content compared to mice fed a standard diet, with higher levels seen in the 0.4% diet group. Mice fed lutein for 35 d had higher levels than mice fed for 29 d. The results are the mean \pm SEM. * $p=0.02$ for regular diet versus 0.4% lutein. ** $p<0.01$ for regular diet versus 0.4% lutein $p=0.03$ for regular diet versus 0.04% lutein. *** $p<0.01$ for regular versus 0.4% lutein diet.

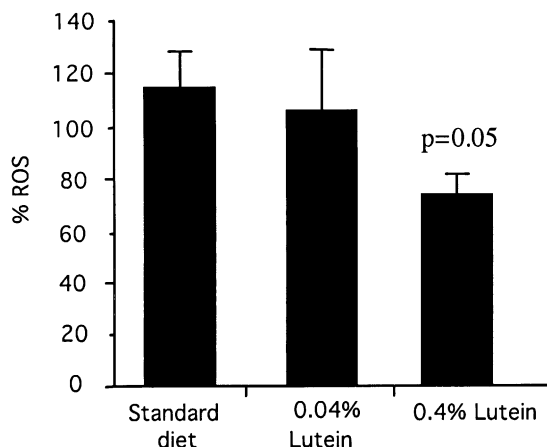


Figure 5

ROS generation (%) in skin following UV radiation. To assess whether lutein can inhibit ROS generation in skin after UVR, skin samples from five C3H/HeJ mice per diet group (regular diet, 0.04% lutein, and 0.4% lutein) were excised following a 28-d feeding duration. The skin samples were imaged with two-photon fluorescence, and the intensity was collected at various depths. Mice fed a lutein-supplemented diet had decreased ROS (%) generated compared to mice fed a standard diet, with the lowest levels seen in the 0.4% diet group. There was no significant difference observed between the standard diet group and 0.04% lutein group. A p value of 0.05 was observed between the standard diet group and the 0.4% lutein diet group. The results are the mean \pm SEM; $n=5$ per group except $n=4$ for the 0.4% lutein group.

system combats ROS-induced oxidative damage; however, antioxidant levels decline following UVR exposure (Shindo *et al*, 1993). An interesting strategy to improve photoprotection is to support the skin's endogenous antioxidant system with exogenous supplementation (Steenvoorden and van Henegouwen, 1997). Within the carotenoid family of anti-

oxidants, β -carotene is the most extensively studied. Nevertheless, conflicting data exist concerning its efficacy in normal skin. Lutein, a carotenoid present in various fruits and vegetables (Mangels *et al*, 1993), is a major carotenoid in human plasma (Parker, 1989) and among the most prevalent carotenoids found in human skin (Hata *et al*, 2000). Lutein is more effective than β -carotene in inhibiting lipid peroxidation (Zhang *et al*, 1991) and is hypothesized to retard the cumulative effects of oxidative damage in the retina (Hammond *et al*, 2001). In addition, lutein is immunomodulatory, stimulating cell-mediated and humoral responses in the canine (Kim *et al*, 2000) and enhancing *in vivo* and *in vitro* antibody production in mice (Jyonouchi *et al*, 1994).

Antioxidants protect keratinocytes from UVB-induced oxidative damage *in vitro* (Stewart *et al*, 1996) and, in humans, modulate the sunburn reaction or UV-induced inflammatory response (Fuchs and Kern, 1998). Lutein scavenges toxic oxygen species *in vitro* (Chopra *et al*, 1993) and, in cultured cells, protects against oxidant-induced damage (Martin *et al*, 1996). A potent antioxidant, lutein is an attractive carotenoid to investigate for possible *in vivo* roles in the skin.

To ensure that the mice were eating the lutein-supplemented diet, weight gain was monitored in the initial experiments. Mice in all three diet groups (standard, 0.04% and 0.4% lutein) were weighed at the start of the feeding period and weekly thereafter until the end of the feeding period. Mice fed a 0.04 or 0.4% lutein-supplemented diet both demonstrated weight gain similar to that of mice fed a standard laboratory diet (data not shown).

We report here that lutein diminished UV-induced inflammation. A decrease in tissue swelling was observed in mice fed both 0.04 and 0.4% lutein diets; however,

a greater decrease was seen in mice fed the 0.4% lutein diet. UV-induced inflammation is partially mediated by oxygen radicals as well as other factors such as histamine, prostanoids, and cytokines (Norris *et al*, 1993; Fuchs, 1998). Carotenoids protect against UV-induced inflammatory erythema in humans (Stahl *et al*, 2000), and quenchers of singlet oxygen and hydrogen peroxide inhibit UV-induced sunburn cell formation, a hallmark feature of UVB-induced damage (Miyachi *et al*, 1983). Lutein's ability to decrease tissue swelling in ears of mice following UVR exposure may be due to its ability to diminish the presence and formation of ROS.

We also demonstrated that a diet of 0.04 and 0.4% lutein prevented suppression of CHS induction following low-dose UVB exposure in mice. Nevertheless, despite elevated lutein levels in the skin, no effect on CHS induction was seen after irradiation with a high, systemic dose of UVB in either lutein diet group. In original experiments, we utilized a dose of 20,000 J per m² UVB radiation in the "high-dose" model. When dietary lutein failed to inhibit systemic UVB-induced immune suppression (data not shown), the protocol was modified to employ 10,000 J per m². Nevertheless, lutein was still ineffective. In the low-dose UV model, lutein may be preventing ROS-induced damage of cell membranes. Cellular targets for free radical reactions include keratinocytes, the major cell to secrete inflammatory and immunosuppressive mediators following irradiation, and Langerhans cells, the major antigen-presenting cell of the epidermis. Lutein may be reducing the damage to cell membranes by ROS and modulating the production of cytokines and prostaglandins. Langerhans cells are critical for CHS induction, and their numbers decrease following UV exposure (Toews *et al*, 1980). ROS are believed to be involved in this decrease in number and in the inhibition of CHS (Yuen and Halliday, 1997). The ROS-induced lipid peroxidation of membrane lipids impairs antigen presentation in the remaining Langerhans cells, further compounding the depletion of cells that follow UV exposure. In addition, ROS have been shown to increase tumor necrosis factor- α secretion (Chaudhri and Clark, 1989), a mediator of CHS suppression in UV-irradiated skin (Streilein *et al*, 1994). It is not entirely clear how UV exposure leads to systemic immune suppression; however, soluble mediators play a role (Beissert and Schwarz, 1999). The inability of lutein to inhibit the systemic suppression of CHS by UVB radiation may be the result of ROS production that is beyond the consumptive capabilities of lutein. This also suggests that the systemic effect may be qualitatively or quantitatively different from the effect in the low-dose model and is not altered by the presence of lutein in the skin.

Diet supplementation with lutein increases skin lutein content. The presence of lutein in the skin of mice fed a standard diet indicates that lutein is present in the standard diet formulation, but in low amounts. Lutein accumulates well in murine skin following supplementation and improves UV photosensitivity as demonstrated by the decrease in UV-induced inflammation. It is important to note that the mice in these experiments were only fed for 2- or 3-wk durations before UV radiation. Whether higher skin levels could exert a greater effect is unknown.

Lutein-supplemented mice demonstrate decreased ROS production in the epidermis following UVR. The 0.4% lutein

diet group had higher skin lutein content and lower ROS production than the 0.04% lutein group, suggesting that the increased presence of lutein in the skin quenches free radicals and inhibits photooxidative damage in murine skin. ROS react with DNA, proteins, and unsaturated fatty acids to induce DNA strand breaks, protein-protein cross-links, and lipid peroxides, all of which can initiate radical chain reactions and enhance oxidative damage (Steenvoorden and van Henegouwen, 1997). The decrease in ROS production presumably limits the extent of cellular damage following UV exposure and is the probable mechanism of action responsible for the observed decrease in UV-induced ear inflammation and inhibition of UV-induced immunosuppression in the low-dose UV model. Lutein, with its antioxidant properties, is thus photoprotective and an interesting candidate for further investigation. The use of a solar simulator for irradiation of skin specimens for this assay rather than a bank of FS-40 sunlamps adds a variable to the interpretation of the results. Both UVB radiation sources and solar simulating radiation, however, result in oxidative events in the skin (McArdle *et al*, 2002; Sander *et al*, 2002) and solar-simulating radiation contains UVB. Thus, we believe that the finding that the lutein diet inhibits ROS formation after exposure to solar-simulating radiation is potentially significant.

Each year, over 1 million new cases of nonmelanoma skin cancer are diagnosed in the United States (<http://www.cancer.org/docroot/home/index.asp>). Supporting the skin's antioxidant defense system is a promising strategy to combat oxidative stress induced by solar radiation. Several studies have assessed the ability of carotenoids to protect the immune system from UV-induced free radical damage. In Caucasian skin, a significant relationship between dietary carotenoid content in the skin and endogenous UV photosensitivity has been suggested (Alaluf *et al*, 2002). Lutein is present in the human diet, and several lines of evidence suggest that it has protective role in chronic disease, immune function, and cancer (Mares-Perlman *et al*, 2002). We provide evidence that lutein may protect the skin from UVR-induced photodamage. Perhaps increasing lutein consumption in the diet could be an effective means of photoprotection alone or in combination with other agents.

Materials and Methods

Animals and diet Female C3H/HeJ mice, 8 to 10 wk of age, were used. There were three diet groups: standard laboratory diet and diets supplemented with 0.04 or 0.4% lutein derived from marigold extract. The animal feed was stock Purina Rodent chow #5001 that was ground and mixed with the FloraGLO lutein 5% beadlets (Kemin Foods, Des Moines, IA) and repelletized (Research Diets, Inc., New Brunswick, NJ). The pellets were vacuum packed in 100-g units that were stored at -80°C. The lutein preparation also contained a small amount (4%) of zeaxanthin, a carotenoid similar in structure to lutein. The food was accessible to the mice at all times and the lutein-supplemented food was replaced every day for the duration of the feeding period. Animal protocols were approved by the Institutional Animal Care and Use Committee.

UVR source A bank of six FS-40 sunlamps (Westinghouse, Bloomfield, NJ) was employed. These bulbs emit a continuous spectrum from 270 to 390 nm with a peak emission at 313 nm;

approximately 65% of the radiation emitted by these lamps is within the UVB range (Rivas and Ullrich, 1992). Measured by an IL-1700 UVR meter (International Light, Newburyport, MA), these bulbs deliver an average flux of 0.4 mW per cm².

UV-induced tissue swelling Mice were fed their respective diets for 2 wk before UVR exposure and for the duration of the experiment. Before UVB irradiation, the thickness of each ear was measured with a spring-loaded micrometer (Mitutoyo, Tokyo, Japan) to determine a baseline thickness. The mice were placed in individual compartments of specially designed cages and their ears were exposed once to 3500 J per m² UVB radiation. Changes in ear thickness were measured at 24 and 48 h after radiation. The ear swelling response was calculated as the difference in ear thickness between the 24- and 48-h measurement and the baseline value, respectively. The values from each ear were averaged together to get the final value for each mouse.

Local, low-dose UVR immunosuppression model Mice were fed their respective diets for 2 wk before UVR exposure, and the diets were continued until the end of the experiment (29 d). Before radiation exposure, the dorsum of each mouse was shaved and the ear surfaces shielded from UVR with electrical tape. The shaved dorsum was exposed to UVB radiation at a dose of 1700 J per m² daily for 4 consecutive d. One hour after the last exposure, 25 μ L of 0.5% dinitrofluorobenzene (DNFB) in olive oil and acetone (1:5) was topically applied to the irradiated site. CHS was elicited 7 d later by challenging the ventral and dorsal surface of both ears of each mouse with 10 μ L of 0.2% DNFB. The 24- and 48-h ear thickness was measured using an engineer's micrometer and then compared with the ear thickness before challenge. The CHS response was calculated as the difference in ear thickness between the baseline value and the 24- and 48-h reading, respectively. The two values were averaged together to get the final value for each mouse. Nonirradiated mice that were ear challenged with prior sensitization served as positive controls, and nonirradiated mice ear challenged without prior sensitization served as negative controls.

Systemic, high-dose UVR immunosuppression model Mice were fed for 2 or 3 wk before UVR exposure, and the diets were continued until the end of the experiment (29 or 35 d). Before radiation exposure, the dorsum of each mouse was shaved and the ears were shielded from UVR exposure with electrical tape. The shaved dorsum was exposed to one dose of 10,000 J per m² UVR. Three days later, the abdomens of the mice were shaved and sensitized with 25 μ L of 0.5% DNFB. CHS was elicited 7 d later by challenging the ventral and dorsal surfaces of both ears of each mouse with 10 μ L of 0.2% DNFB. The 24- and 48-h ear thickness was measured and the CHS response calculated as above.

Skin lutein content determination Nonirradiated, nonsensitized mice ($n=4$ per diet group) were euthanized by carbon dioxide asphyxiation. Hair was removed by plucking and the dorsal skin excised immediately. The underlying subcutaneous fat was mechanically removed in phosphate-buffered saline and the skin samples were frozen at -80°C for future analysis.

Individual carotenoids were measured by HPLC in skin samples previously stored at -80°C . Briefly, samples were thawed and up to 100 mg was weighed into a polypropylene tube. Samples were homogenized with dry ice and 2 mL of methanol:tetrahydrofuran (1:1). Samples were centrifuged for 5 min at 1300 g. A 750- μ L portion of the supernatant was diluted with 750 μ L of phosphate-buffered saline, and 500 μ L of 40% potassium hydroxide was added. Samples were saponified for 30 min at 60°C in a shaking water bath. Two milliliters of saturated NaCl solution was added and the samples were extracted twice by vortex mixing with 4 mL of hexane:ethyl acetate (9:1). A final extraction was performed with hexane:diethyl ether (1:1). The combined organic extract was evaporated under a stream of nitrogen. The residue was dissolved

in 120 μ L of mobile phase facilitated by vortex mixing and ultrasonic agitation. A 45- μ L volume was injected.

The HPLC system consisted of a computer data system, in-line solvent degasser, a quaternary gradient pump, an autosampler maintaining samples at 10°C , a column heater at 31°C , and a programmable ultraviolet-visible detector (ThermoSeparation Products, Fremont, CA). The separation was performed isocratically on a Spherisorb ODS2 column (3 μ m, 4.0 \times 250 mm with titanium frits, ES Industries, West Berlin, NJ) protected by a Javelin guard column containing a similar stationary phase (Keystone Scientific, Bellefonte, PA). The mobile phase consisted of acetonitrile:dioxane:50 methanol/50 isopropanol:triethylamine (80:15:5:0.1) at a flow rate of 1.2 mL per min. The alcohol component contained 100 mM ammonium acetate. The detector wavelength was set at 450 nm to monitor for carotenoids.

Linear calibration curves were prepared consisting of three concentrations of analytes that spanned the physiologic levels. The calibrants included lutein, zeaxanthin, β -cryptoxanthin, lycopene, α -carotene, and β -carotene. Quantitation was performed by external standard calibration using peak areas. Data were normalized to the protein content of each sample.

ROS detection Mice were fed their respective diets for 28 d. Mice ($n=5$ per diet group) were euthanized by carbon dioxide asphyxiation. Hair was removed by plucking and the dorsal skin was excised immediately. The underlying subcutaneous fat was mechanically removed in complete medium and the skin samples were frozen at -80°C for future analysis. Complete medium consists of RPMI 1600 (Cellgro, Herndon, VA) containing 10% fetal calf serum (Life Technologies, Gaithersburg, MD), 100 U per mL penicillin, 100 μ g per mL streptomycin, 0.1 mM nonessential amino acids, 0.1 mM essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES buffer (Life Technologies).

Two-photon fluorescence imaging and ROS detection were performed utilizing the instrument and method previously described (Hanson and Clegg, 2002). ROS were detected through the use of the fluorescence probe dihydrorhodamine (DHR). DHR was nonfluorescent until it reacted with ROS, including H₂O₂, ¹O₂, and NOO[•] and formed fluorescent rhodamine-123 (emission maximum 525 nm). Skin samples ($\sim 0.5 \times 0.5$ cm) were incubated in DHR (100 μ M DHR, 10 min, sample submerged in solution) and imaged before UV irradiation to collect background levels. Images were taken every 5 μ m beginning at the stratum corneum surface (depth $z=0$). The total area of each image was 50 \times 50 μ m. The samples were then removed from the microscope and irradiated by the UV excitation source (200 J/m², equivalent to 12 min of noonday North American summer UV light (Surface Radiation Research Branch, 2001; Hanson and Clegg, 2002)) where the samples are placed such that UV photons enter the skin only through the stratum corneum surface. ROS were generated within the skin following irradiation by solar-simulated UV (Solar Light Co.) (Hanson and Clegg, 2002). The sample was reimaged upon completion of each UV dose. The reduction in ROS at each depth (%ROS at depth (z)) was calculated using the equation

$$\%ROS \text{ at depth } (z) = 100 - 100/[I(z)_{\text{sample}}/I(z)_{\text{control}}].$$

The fluorescence intensity I was collected over each image at each epidermal depth z . At least two unique areas were imaged and the fluorescence was collected per skin sample and at each depth z . The intensity values for these two or more areas were averaged. $I(z)_{\text{sample}}$ was the average fluorescence intensity collected at depth z from a skin sample taken from the animal fed a standard diet or 0.04 or 0.4% lutein. $I(z)_{\text{control}}$ represents the average fluorescence intensity of the skin from a negative control animal. All %ROS (z) values for a given sample were then averaged together. The averaged values for each sample in the same diet group were averaged together and then compared to the other diet groups to determine the effect of 0.04 and 0.4% dietary lutein on UV-induced ROS levels in murine skin.

Statistical analysis The significance of differences in ear swelling, CHS responses, skin lutein content, and %ROS among groups were analyzed using the two-tailed Student's *t* test for unpaired samples. A *p* value of less than 0.05 was considered significant.

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