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DNA damage and susceptibility to oxidative damage in lymphocytes: effects of carotenoids *in vitro* and *in vivo*

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Reports on the effects of carotenoids are conflicting. The present paper examines similarities and differences from contiguous studies *in vitro* and *in vivo*. Single-cell gel electrophoresis was used to measure the frequency of single-strand breaks (SSB) in the cell line MOLT-17 (as a model system) and human peripheral blood lymphocytes (PBL). MOLT-17 cells were supplemented with β-carotene, lutein or lycopene at a range of concentrations (0·00–8·00 μmol/l) using a liposome delivery method. Uptake was dose-dependent. β-Carotene concentration in the media had no effect on SSB in control cells, but incubation with lycopene or lutein (>2·00 μmol/l) increased the numbers of SSB in control cells. MOLT-17 DNA was less susceptible to oxidative damage (100 μmol H_2O_2/l, 5 min, 4 °C) following incubation with carotenoids between 0·50 and 1·00 μmol/l; at >1·00 μmol/l the effects were ambiguous. Apparently healthy male volunteers supplemented their habitual diets with lutein, β-carotene or lycopene (natural isolate capsules, 15 mg/d, 4 weeks) in three independent studies, raising plasma concentrations to different extents. Lycopene and lutein had no effect on SSB in control PBL or following oxidative challenge. However, increased plasma β-carotene was associated with more SSB in control cells whilst PBL DNA resistance to oxidative damage *ex vivo* was unaffected. These results suggest that the carotenoids are capable of exerting two overlapping but distinct effects: antioxidant protection by scavenging DNA-damaging free radicals and modulation of DNA repair mechanisms.

Carotenoids: DNA damage and repair: Single-cell gel electrophoresis: Peripheral blood lymphocytes

There is compelling epidemiological evidence linking a greater consumption of fruits and vegetables with a lower incidence of cancer (World Cancer Research Fund 1997). These foods contain relatively high amounts of components with inherent antioxidant properties, such as vitamin C, carotenoids and flavonoids. This has led to the suggestion that these dietary compounds may augment cellular antioxidant defences and help protect cellular components from oxidative damage. However, in recent years it has been suggested that these compounds may also possess bioactivities, some separate to and others associated with their antioxidant characteristics, which may alter redox signalling and gene expression (Jackson *et al.* 2002). Results confirming the presence and physiological significance of these effects are still required.

The single-cell gel electrophoresis (SCGE) technique has been employed previously to investigate the antioxidant properties of various compounds, including the carotenoids, both *in vivo* (Astley *et al.* 1996; Duthie *et al.* 1996; Astley 1997; Pool-Zobel *et al.* 1997, 1998; Collins *et al.* 1998; Fillion *et al.* 1998; Torbergsen & Collins, 2000) and *in vitro* (Astley, 1994; Astley *et al.* 1997, 2002). Results suggest that lycopene specifically (Porrini & Riso, 2000; Riso *et al.* 1999) and fruits and vegetables in general (Collins *et al.* 1998; Pool-Zobel *et al.* 1997, 1998) reduce the susceptibility of lymphocyte DNA to oxidative damage *in vivo*, at baseline and following oxidative challenge. *In vitro*, however, the results are more inconsistent and there is some evidence that the carotenoids may have a pro-oxidant effect, particularly at higher concentrations (Lowe *et al.* 1999; Woods *et al.* 1999). Few studies have compared directly the effect of individual carotenoids, *in vitro* and *in vivo*, at physiologically relevant concentrations.

The present paper examines results from six studies, three *in vitro* and three *in vivo*. *In vitro*, the human lymphocyte cell line MOLT-17, derived from a 5-year-old female patient with acute lymphocytic leukaemia (Drexler & Minowada, 1989), was supplemented with β-carotene, lutein or lycopene at a range of concentrations (0·00–8·00 μmol/l) using a liposome delivery system (Grolier *et al.* 1992).

Lutein, lycopene or β-carotene, in the form of natural isolate capsules (15 mg/d for 4 weeks), were used to supplement the diet of the same apparently healthy male volunteers, at the same time of year over 3 years, in three separate randomised double-blind studies.

SCGE was employed to measure the frequency of DNA single-strand breaks (SSB) and alkali-labile sites in both MOLT-17 cells and human peripheral blood lymphocytes

Abbreviations: HBSS, Hanks' balanced salt solution; PBL, peripheral blood lymphocytes; RPMI, Roswell Park Memorial Institute; SCGE, single-cell gel electrophoresis; SSB, single-strand break.

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(PBL) at baseline, without the oxidative challenge and following oxidative challenge with H_2O_2. In this way, background levels of DNA SSB and resistance of the DNA to oxidative challenge were assessed simultaneously, before and after supplementation in an *in vitro* model system and *ex vivo*.

Lutein, lycopene and β-carotene were selected because they are three of the most commonly consumed carotenoids in the UK; lutein from green and yellow vegetables, in particular green peas, lycopene from tomatoes and tomato products, and β-carotene from carrots and green vegetables. The range of concentrations used for supplementing the cells *in vitro* was selected on the basis that: (1) plasma levels found typically in northern and southern Europe were included since these regions demonstrate wide differences in both morbidity and mortality rates, which have been attributed to differences in fruits and vegetable consumption; (2) the upper concentration (8·00 μmol/l), which is more than four times the usual plasma concentration found in the UK population (Thurnham, 1988; Olmedilla *et al.* 2001), is at the upper limit of plasma concentrations that might reasonably be achieved by dietary manipulation (i.e. without use of isolate supplements). Although supplements were used in these studies, the doses (15 mg/d) are comparable with the addition of approximately 150 g of an equivalent food source (e.g. 150 g carrots, tomatoes or green peas) per d. Foods were not used in order to avoid possible confounding food-derived effects that could not also be created *in vitro* as well as issues related to personal preference in consumption. Finally, 8·00 μmol/l is twice the concentration (4·00 μmol/l) at which antioxidant protection has been shown to reduce or cease altogether, possibly indicating pro-oxidant behaviour (Lowe *et al.* 1999).

Materials and methods

Liposomes

Carotenoid-free liposomes and liposomes containing lutein, lycopene or β-carotene were prepared as described previously by Astley *et al.* (2002). After production, liposomes were flash frozen and stored under liquid N_2. The carotenoid content of each of the liposome preparations was determined by HPLC (Hart & Scott, 1995).

Cell culture and supplementation

MOLT-17 T-lymphocytes (DSM ACC 36; Deutsche Sammlung von Mikro-organismen und Zelkulturen, Braunschweig, Germany) (Drexler & Minowada, 1989)) were grown in 175 cm^2/800 ml flasks and maintained at a density of 1×10^6 cells/ml Roswell Park Memorial Institute (RPMI) medium 1640 (Life Technologies Ltd, Paisley, Scotland, UK) containing 2 mM glutamine, 100 units penicillin/ml, 100 mg streptomycin/ml and 100 ml fetal calf serum/l (all from Life Technologies Ltd). Cultures were incubated as normal in a humidified incubator (37 °C, 5 % CO_2) and passaged with fresh medium 24 h before supplementation.

Immediately before supplementation, the cells were counted using a haemocytometer (five large squares, minimum of 200 cells) and adjusted to $0{\cdot}55 \times 10^6$ cells/ml with fresh medium. During supplementation fluorescent lights in the sterile hood and culture room were switched off and the flasks were incubated in complete darkness. All subsequent procedures were carried out under non-UV lighting to minimise the risk of degradation of light-sensitive carotenoids.

MOLT-17 cells remained untreated (unsupplemented control) or supplemented with carotenoid-free (non-enriched) liposomes (blanks), which provided a supplementation control, or a single carotenoid at 0·25, 0·50, 1·00, 2·00, 4·00 and 8·00 μmol/l. The volume added to achieve each concentration was calculated from the carotenoid content of the carotenoid-enriched liposomes previously determined by HPLC. Lipid concentrations in the media were maintained at maximum of 100 μl liposomes/ml culture medium, by the addition of carotenoid-free liposomes (i.e. total volume of liposomes added to all cells was 3 ml in a flask containing 30 ml culture medium, made up from a mixture of the relevant carotenoid-enriched and carotenoid-free liposomes).

Cells were incubated with the liposomes for 24 h, as previous analyses had revealed that this period was sufficient for maximum carotenoid incorporation (Astley, 1997). Following incubation with the liposomes, the cells were harvested, washed in Hanks' balanced salt solution (HBSS; Sigma Chemical Co., Poole, Dorset, UK) and counted. Cells (1×10^6, 1 ml) from each treatment were subjected to SCGE analysis (described later). Those cells not used for SCGE were pelleted (400 ***g***, 5 min, 4 °C), suspended in HBSS (1 ml) and stored under liquid N_2 for subsequent carotenoid analysis by HPLC (Hart & Scott, 1995). Six independent supplementation experiments were carried out using MOLT-17 cells, using all carotenoid concentrations on each occasion (i.e. *n* 6).

Cell density and viability

The viability and total cell number of supplemented cells was assessed to demonstrate that supplementation was not having an adverse effect on the lymphocytes, particularly in view of the increased media lipid concentration. A small portion of cells (100 μl) was retained from each flask 24 h after supplementation, and mixed with the staining solution (5 μl/ml; 5 min), which contained fluorescein diacetate (24 μmol/l; Sigma Chemical Co.) and propidium iodide (15 mM; Sigma Chemical Co.) in ethanol. The cells were counted using a haemocytometer and a Nikon Optiphot epifluorescent microscope (Nikon, Kingston-upon-Thames, Surrey, UK) with a B-3A visible filter set in place. Total cell density and the numbers of cells stained red or green were recorded; red cells were categorised as being dead and green cells alive (Ormerod, 1990).

Human intervention study

Following approval from the local Human Research Ethics Committee, written informed consent was obtained from each volunteer. To be eligible, volunteers had to be male non-smokers in the age range 18–60 years with a BMI < 35 kg/m^2. They were requested not to volunteer if they

were taking prescribed medication or vitamin supplements, consuming more than twenty-eight units of alcohol per week or following a special diet, including a vegetarian diet. A screening blood sample (5 ml) was used to exclude individuals who were anaemic or had a high fasting plasma glucose (>6·1 mM). Twenty-eight apparently healthy volunteers were recruited into the study and randomly assigned into two groups.

The study was performed in three parts, each separated by 12 months, using a double-blind crossover format. In each of the parts, the effects of one carotenoid was investigated. Knowledge of the allocated treatment was limited to the study organiser, who took no part in the sample analysis. Anthropometric measurements were recorded, and anticubital venous blood samples (6 ml) were taken from each volunteer after an overnight fast (12 h). Volunteers immediately began taking one capsule per d (group 1, placebo; group 2, carotenoid isolate) to supplement their otherwise unchanged diet. They followed this regimen for 27 d.

A second fasting blood sample (6 ml) was taken on day 28 of the study. Participants switched to the alternative supplement (i.e. group 1; carotenoid isolate; group 2, placebo) for a further 27 d before a final fasting blood sample was taken on day 56. The blood was collected into tubes containing lithium heparin (100 μl in saline (9 g/l NaCl)/10 ml blood) and placed on iced water before isolation of plasma and lymphocytes.

Whole blood (200 μl) was mixed with RPMI medium 1640 containing fetal calf serum (100 ml/l, 1 ml; Life Technologies Ltd), under-layered with LymphoPrep (100–200 μl; Nycomed (UK), Birmingham, UK) and centrifuged (200 ***g***, 3 min, 4 °C). The mononuclear cells were collected from the pink layer at the boundary between the media, and the pellet re-suspended in HBSS (150 μl). These cells were used for SCGE as described later. The remainder of the whole blood (5·8 ml) was centrifuged (1000 ***g***, 20 min, 4 °C) and the plasma layer collected, flash frozen (1 ml aliquots) and stored under liquid N_2. Plasma carotenoid concentrations were determined by HPLC (Hart & Scott, 1995).

β-Carotene capsules were used in the first part. Each contained 15 mg β-carotene as Beta-Tab™ 7·5 % (Hoffman-La Roche Inc., Nutley, NJ, USA), in a base containing microcrystalline cellulose, dibasic calcium phosphate and magnesium sterate. Matching placebo capsules, with an identical base composition, were a gift from Hoffmann-La Roche Inc. (Nutley, NJ, USA). Lycopene, from tomatoes, was provided by Lycored (Beer Sheva, Israel) and lutein extracted from marigolds (79 % *trans*-lutein and 21 % *cis*-lutein) by Quest International (Cork, Republic of Ireland). Both supplements were encapsulated by R. P. Sherer (St Petersburg, FL, USA; 500 mg fill in 15 g maize oil with a matched placebo).

Single-cell gel electrophoresis

Duplicate slides were prepared for each sample *ex vivo* and on each occasion *in vitro* (*n* 6). All procedures were carried out under non-UV fluorescent light, essentially as described previously (Singh *et al.* 1988; McKelvey-Martin *et al.* 1993). Briefly, an equal volume of cell suspensions (100 μl, 1×10^6 MOLT-17 or PBL in HBSS) were mixed with low-melting-point agarose (15·0 g/l; Nusieve, Flowgen Instruments Ltd, Sittingbourne, Kent, UK) and a portion (40 μl) distributed on to microscope slides pre-coated with normal-melting-point agarose (50 μl, 5·0 g/l). Once the mixture had solidified, it was covered with a further layer of low-melting-point agarose (50 μl, 5·7 g/l). The slides were immersed in either H_2O_2 (100 μmol/l, 4 °C) or PBS (2·25 mol/l, 4 °C) for 5 min and then transferred immediately to lysis solution (2·5 mol NaCl/l (Sigma Chemical Co.), 100 mM-Na_2EDTA (BDH, Poole, Dorset, UK), 10 mM-Tris-(hydroxymethyl) aminomethane (Sigma Chemical Co.) pH 10, to which Triton X-100 (10 ml/l) and dimethylsulfoxide (10 ml/l) were added immediately before use) for 60 min at 4 °C. This buffer was decanted off and replaced with electrophoresis buffer (300 mM-NaOH, 1 mM-Na_2-EDTA, pH 13, 4 °C) for 40 min before the slides were placed in an electrophoresis chamber containing fresh electrophoresis buffer (1000 ml). Any empty spaces in the chamber were filled with blank slides. Each slide was placed in the same position for replicate experiments *in vitro* and between rounds of the intervention study. Following electrophoresis (25 V, 300 mA, 4 °C), the slides were washed with two changes of neutralisation buffer (0·4 M-Tris, pH 7·5, 10 min, 4 °C) and the cells stained with ethidium bromide (Sigma Chemical Co.; 1 μg/ml neutralisation buffer, 10 min). Each slide was rinsed in PBS, blotted dry, and the gel section covered with a fresh cover slip before visualisation; this has been described in detail elsewhere (Astley *et al.* 2002).

Descriptive statistics and % DNA in the tail were calculated for each set of images (MOLT-17 cells, 100 images; PBL, fifty images from each duplicate slide). The % DNA in the tail was defined as:

$$\%\ \text{of DNA in tail} = \left((\text{tail intensity} \times \text{tail area}) \Big/ \left((\text{head intensity} \times \text{head area}) + (\text{tail intensity} \times \text{tail area})\right)\right) \times 100.$$

Statistical analysis

Data were analysed using Statistica, version 5.5 (1984–2000; StatSoft Inc., Tulsa, OK, USA). Before this series of experiments, it was demonstrated that the mean value for fifty images (using the in-house program and under the criteria specified earlier for image collection) was not significantly different from those calculated for 100 comets (results not shown; Astley 1997). Thus, fifty images were collected from each slide and the mean % DNA in the tail calculated for both data sets. Data from duplicate slides were compared using the Student's *t* test (Lovell *et al.* 1999; Tice *et al.* 2000): none were demonstrated to be significantly different, and data were combined for all other statistical analyses.

The incidence of SSB and alkali-labile sites in control cells (i.e. no H_2O_2 challenge) and after treatment with H_2O_2 was examined using one-tailed paired *t* test. This was to determine whether: (1) treatment with H_2O_2 increased the incidence of SSB; (2) supplementation *in vitro* or *in vivo* with the carotenoids altered levels of endogenous SSB or increased resistance to oxidative damage.

One-way ANOVA was used to evaluate the effect of medium supplementation on cell carotenoid uptake (i.e. whether there was a change in cell carotenoid uptake with increasing medium concentration). Where the variance ratio (F) was significant, data were compared by a one-tailed Student's t test to determine whether the increase from the lower concentration to the next higher concentrations (e.g. from 0·50 to 1·00 μmol/l) was significant. Significant increases are presented ($P \leq 0{\cdot}05$).

In the intervention study, paired t tests were used to determine whether dietary supplementation increased plasma concentration. Comparisons were made between an individual's baseline plasma concentrations and levels measured at the end of the supplementation period. For half the volunteers, this was week 4; for the others (i.e. those taking the placebo first) it was week 8.

ANOVA was used to examine 'end-of-supplementation' data, to determine if there were any differences in post-supplementation plasma concentrations of lutein, lycopene and β-carotene (i.e. between studies). Where the variance ratio (F) was significant, data were compared by Sheffé *post hoc* test, which is the multiple-analyses equivalent to Student's t test, but unlike Student's t test, this test conserves power.

ANOVA was also used to determine whether increased medium or plasma concentration of the carotenoids had any impact on the incidence of SSB and alkali-labile sites (i.e. % tail DNA). Where the variance ratio (F) was significant, data were compared by Sheffé *post hoc* test. Significantly changes, compared with MOLT-17 cells that remain unsupplemented, are presented. Similarly, for MOLT-17 cells treated with H_2O_2, only significant changes (decreases or increases) compared with cells cultured in the presence of non-enriched (blanks) liposomes are presented. Significant P values were $\leq 0{\cdot}05$. The incidence of SSB in control PBL (no H_2O_2) were compared independently with that observed after H_2O_2 treatment, before and after the period of dietary supplementation. Significant differences (i.e. $P < 0{\cdot}05$) are presented.

Results

Cell density and viability

Addition of carotenoid-free and carotenoid-enriched liposomes had no significant effect on cell viability or total cell number during the 24 h supplementation period (results not shown).

Carotenoid uptake

Uptakes of β-carotene, lutein or lycopene by MOLT-17 cells from media supplemented at a variety of concentrations are shown in Fig. 1. Greatest uptake was observed in MOLT-17 cells incubated with lycopene-enriched liposomes. Supplementation at a typical plasma lycopene concentration (0·25 μmol/l) resulted in no detectable uptake of lycopene by MOLT-17 cells, but there was a significant increase in uptake from 0·50 to 1·00 μmol/l ($P = 0{\cdot}007$) and again from 1·00 to 2·00 μmol/l ($P = 0{\cdot}02$). The increases from 2·00 to 4·00 μmol/l and again from 4·00 to 8·00 μmol/l were not statistically significant. Uptake of

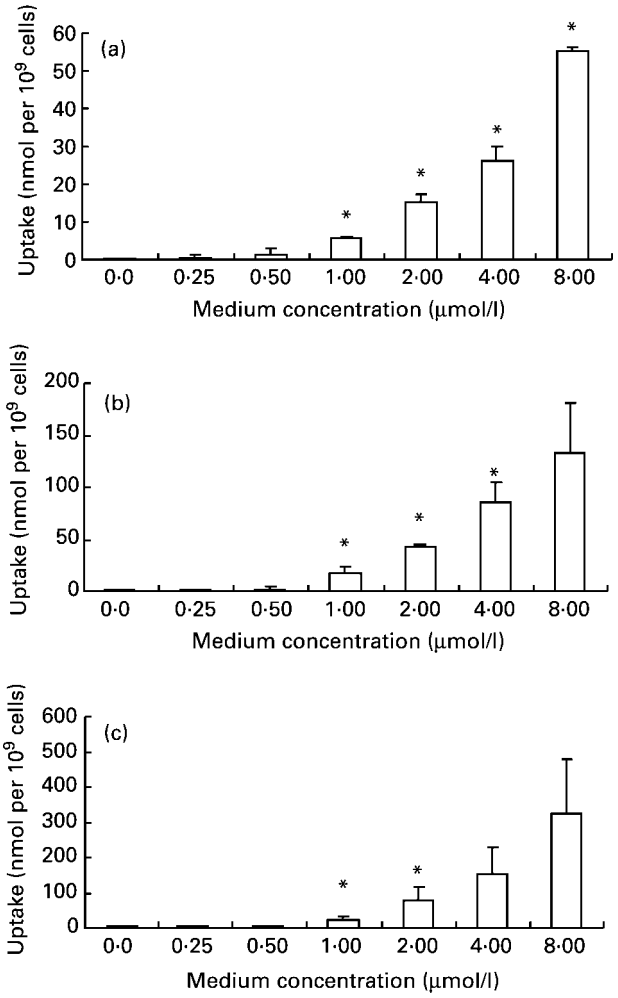

Fig. 1. Uptake of lutein (a), β-carotene (b) and lycopene (c) by MOLT-17 cells after 24 h. Cell uptake was measured by the method of Hart & Scott (1995). Values are means with standard errors shown by vertical bars (n 6). For details of procedures, see pp. 54–55. One-way ANOVA was used to evaluate cell uptake of lutein in response to increasing quantities of lutein-enriched liposomes in the medium. Where the variance ratio (F) was significant, data were compared by a one-tailed Student's t test to determine whether the increase from the lower concentration to the highest concentration to the next highest concentrations (e.g. from 0·50 to 1·00 μmol/l) was significant: $^*P \leq 0{\cdot}01$.

β-carotene and lutein by MOLT-17 cells increased significantly ($P \leq 0{\cdot}05$) at all concentrations between 0·50 and 4·00 μmol/l, and for lutein between 4·00 to 8·00 μmol/l ($P \leq 0{\cdot}05$). At typical plasma concentrations, uptakes of β-carotene (1·00 μmol/l) and lutein (0·50 μmol/l) were 16 nmol per 10^9 cells and 1·5 nmol per 10^9 cells respectively.

The extent of uptake was carotenoid specific: uptake of lutein was about 0·5 %, β-carotene about 2·0 % and lycopene about 4·0 %, regardless of the final concentration of the carotenoid in the medium.

Single-cell gel electrophoresis: in vitro *supplementation*

Mean % tail DNA provides a semi-quantitative measure of the number of SSB and alkali-labile sites present

in a cell. For MOLT-17 cells, with and without H_2O_2 treatment, following incubation with lutein-, β-carotene- or lycopene-enriched liposomes, mean % tail DNA at a series of different carotenoid concentrations are shown in Fig. 2.

Lutein. The number of SSB measured in control cells (i.e. MOLT-17 cells grown in the presence of lutein but not treated with H_2O_2) did not change significantly with increasing media concentrations of lutein, up to 2·00 μmol/l. However, incubation with 4·00 μmol/l lutein resulted in a significant increase in numbers of SSB in these cells (i.e. an increase in control cells, $P \leq 0{\cdot}01$), which persisted, but did not increase further on incubation with 8·00 μmol lutein/l. Between 0·25 and 2·00 μmol/l, there was a gradual but significant decline in the number of SSB produced in MOLT-17 cells following H_2O_2 treatment. However, these did not reach control (unchallenged) values. There was no significant difference between H_2O_2-treated MOLT-17 cells and the control cells following incubation with 4·00 μmol lutein/l. However, this was not because of increased resistance to DNA oxidative damage, but rather the increase in SSB observed in the corresponding control cells. At both 4·00 and 8·00 μmol/l, the number of SSB observed in H_2O_2-treated MOLT-17 cells was elevated compared with cells supplemented with lower concentrations of lutein (i.e. 1·00 to 2·00 μmol/l), but similar to those seen in unsupplemented MOLT-17 cells or MOLT-17 cells supplemented with the lowest levels of lutein (i.e. 0·25 and 0·50 μmol lutein/l).

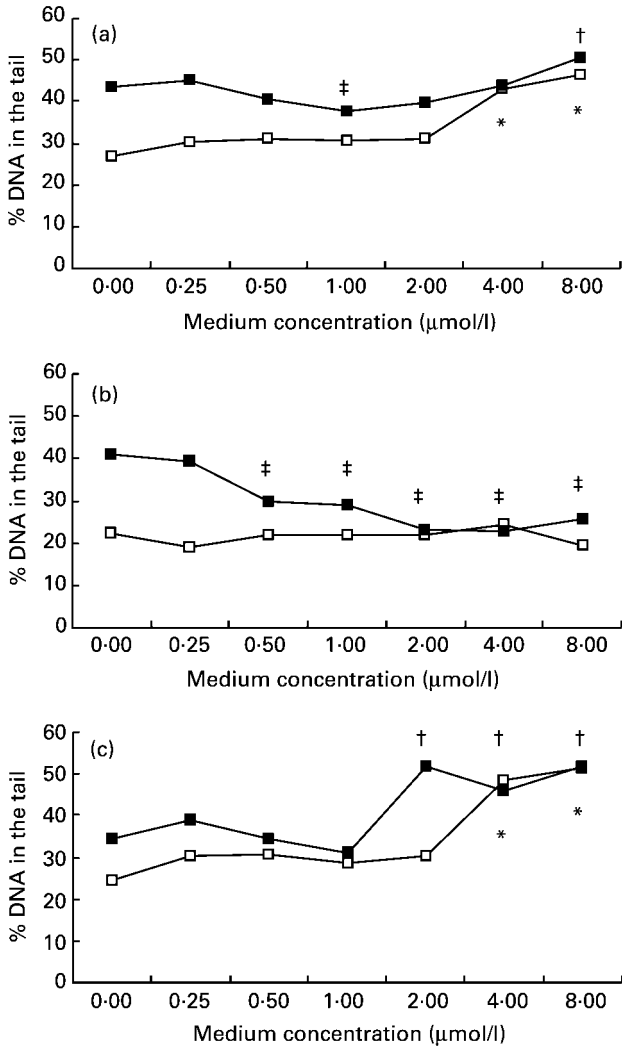

Fig. 2. Single-cell gel electrophoresis analysis of DNA strand breaks in MOLT-17 cells, with (■) or without (□; control) previous H_2O_2 treatment (100 μmol/l, 5 min, 4 °C), following supplementation with lutein- (a), β-carotene- (b) or lycopene- (c) enriched liposomes at a series of concentrations (0·00–8·00 μmol/l). For details of procedures, see pp. 54–55. Values are means (% DNA in the tail: (tail area × fluorescence/((head area × fluorescence) + (tail area × fluorescence))) × 100) (n 6). Standard errors were ≤ 0·05 and therefore not shown. One-way ANOVA was used to determine whether increasing lutein medium concentration has a significant impact on the incidence of DNA damage in control and H_2O_2-treated MOLT-17 cells, independently. Where the variance ratio (F) was significant, data were compared by Sheffé *post hoc* test. Mean values were significantly different following supplementation with enriched liposomes from those of cells cultured in the absence of enriched liposomes: *$P \leq 0{\cdot}05$. Mean values were significantly greater than those cultured without H_2O_2 treatment: †$P \leq 0{\cdot}05$. Mean values were significantly less than those cultured without H_2O_2 treatment: ‡$P \leq 0{\cdot}05$.

β-Carotene. The mean % tail DNA in control cells (i.e. MOLT-17 cells grown in the presence of β-carotene but not treated with H_2O_2) showed no significant change with increasing media concentration of β-carotene. MOLT-17 cells supplemented with β-carotene and subsequently treated with H_2O_2 showed a decline in mean % tail DNA with increasing β-carotene concentrations up to 4·00 μmol/l. More specifically, there was no significant difference in mean % tail DNA, following oxidative challenge, for MOLT-17 cells supplemented with 0·25 μmol β-carotene/l compared with those that remained unsupplemented. Between 0·25 μmol/l β-carotene, and 0·50 and 1·00 μmol β-carotene/l, the number of SSB in oxidatively challenged cells decreased significantly ($P \leq 0{\cdot}01$). There was a further statistically significant decrease on incubation with 2·00 μmol β-carotene/l ($P \leq 0{\cdot}05$), but no significant difference between this and supplementation of the media with 4·00 or 8·00 μmol β-carotene/l.

There was no significant difference in mean % tail DNA between MOLT-17 cells treated with H_2O_2 and their corresponding control cells following supplementation with either 2·00 or 4·00 μmol β-carotene/l. At 8·00 μmol β-carotene/l, there was a significant difference between control and H_2O_2-treated MOLT-17 cells. Unlike lutein-supplemented MOLT-17 cells, the increase in mean % tail DNA was in the H_2O_2-treated MOLT-17 cells not the corresponding control cells. However, the increase was not sufficient to raise the mean % tail DNA above that observed in MOLT-17 cells grown in the absence of β-carotene.

Lycopene. The mean % tail DNA for control cells (i.e. MOLT-17 cells grown in the presence of lycopene not treated with H_2O_2) showed no significant changes with increasing media concentration of lycopene, up to 2·00 μmol/l. As with MOLT-17 cells cultured in the presence of lutein enriched-liposomes, supplementation of MOLT-17 cells with 4·00 μmol lycopene/l resulted in a significant increase in control cell mean % tail DNA ($P \leq 0{\cdot}01$), which persisted but did not increase further at 8·00 μmol/l.

There was no significant difference in mean % tail DNA observed in cells treated with H_2O_2 following incubation with lycopene at 0·25 and 2·00 μmol/l. However, the

difference between control (no H_2O_2) and H_2O_2-treated MOLT-17 cells was significant for unsupplemented cells and cells supplemented with 0·25 and 0·50 μmol lycopene/l, but not for cells supplemented with 1·00 μmol lycopene/l. MOLT-17 cells supplemented with 2 μmol lycopene/l showed a significant increase ($P \leq 0{\cdot}01$) in SSB following treatment with H_2O_2, and the numbers of SSB measured were significantly greater ($P \leq 0{\cdot}01$) than levels observed in unsupplemented MOLT-17 cells or MOLT-17 cells supplemented with lycopene at lower concentrations. There was no further increase in observed numbers of SSB in H_2O_2-treated MOLT-17 cells incubated with 4·00 and 8·00 μmol lycopene/l. There was also no difference between these cells and their corresponding controls because of the increased mean % tail DNA described in the corresponding control MOLT-17 cells.

Human intervention study

Twenty-eight volunteers (*n* 28) took part in a randomised, double blind study. Data for this study were examined at baseline (i.e. week 0) and at the end of the carotenoid-supplementation period, which for half the volunteers was week 4 and for the others week 8 (i.e. week 4/8).

Plasma carotenoid concentrations. In all cases, supplementation with carotenoid-containing capsules significantly increased the relevant plasma carotenoid concentration ($P \leq 0{\cdot}001$; Fig. 3). However, despite supplementation at the same level (15 mg/d) for the same duration (4 weeks), the increase in plasma concentration of β-carotene was markedly greater than that for either lutein or lycopene (ANOVA, week 8, $P \leq 0{\cdot}001$).

Single-cell gel electrophoresis in vivo *supplementation*. Mean % tail DNA for human PBL, with and without H_2O_2-treatment, following supplementation with lutein, lycopene and β-carotene are shown in Fig. 4. Lutein and lycopene supplementation had no effect on mean % tail DNA for PBL at baseline or following treatment with H_2O_2. After supplementation with β-carotene (i.e. week 4/8), there was no significant difference between observed numbers of SSB in PBL without oxidative challenge (control, no H_2O_2) and those that had been subjected to oxidative challenge (H_2O_2 treated; control 42 (SE 2) %, H_2O_2-treated 47 (SE 4) %). There had been a significant difference between control and H_2O_2-treated PBL before supplementation (i.e. week zero; control 24 (SE 2) %, H_2O_2-treated 52 (SE 5) %, $P < 0{\cdot}01$). This was not because of a decrease in numbers of SSB in the challenged PBL post-supplementation (i.e. increased resistance to oxidative damage was absent), however, but rather increased frequencies of SSB in control PBL (i.e. in the unchallenged PBL) from the β-carotene-supplemented individuals.

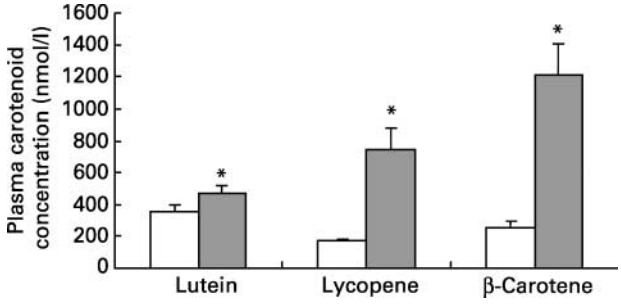

Fig. 3. Plasma carotenoids concentration at baseline (□) and after 4 weeks of supplementation with lutein, lycopene or β-carotene (15 mg/d; ■). Plasma concentrations were measured by the method of Hart & Scott, (1995). For details of procedures, see pp. 54–55. Values are means with their standard errors shown by vertical bars (*n* 28). Paired *t* tests were used to determine whether supplementation increased plasma concentration. Comparisons were made between the individual's baseline plasma concentration and levels measured at the end of the supplementation period. For half of the volunteers, this was week 4; for the others (i.e. those taking the placebo first), it was week 8. Mean values were significantly different from those at baseline: $^*P \leq 0{\cdot}05$.

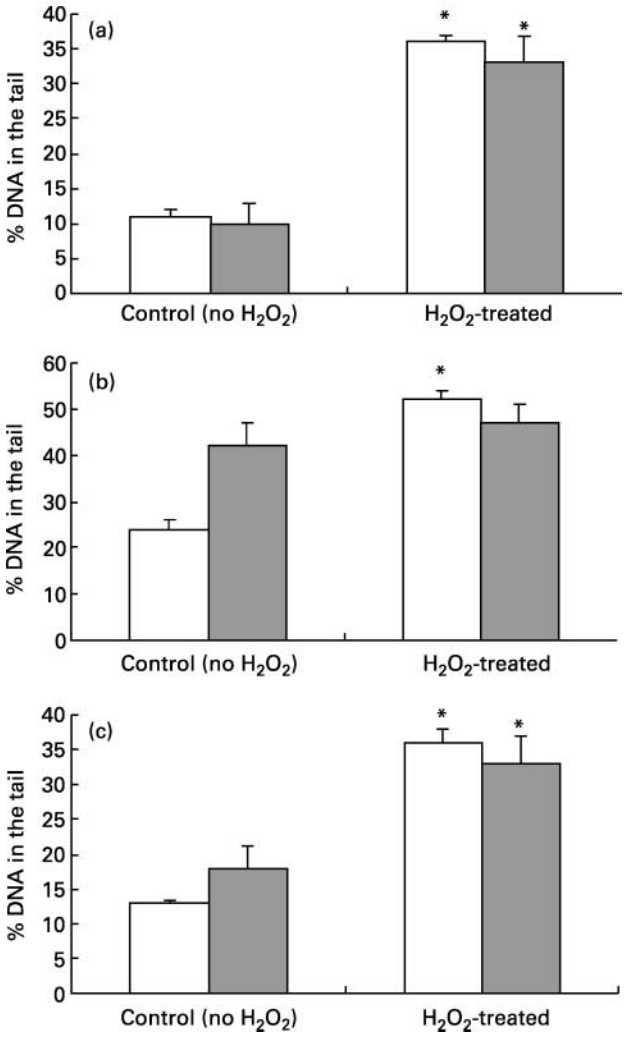

Fig. 4. Single-cell gel analysis of DNA strand breaks in human peripheral blood lymphocytes with or without (control) H_2O_2 treatment (100 μmol/l, 5 min, 4 °C), at baseline (□) and following supplementation (■) with lutein (a), β-carotene (b) or lycopene (c) (natural isolate capsules containing 15 mg/d for 4 weeks). Values are means (% DNA in the tail: (tail area × fluorescence/((head area × fluorescence) + (tail area × fluorescence))) × 100) with their standard errors shown by vertical bars (*n* 28). The incidence of single-strand breaks and alkali-labile sites in control (no H_2O_2) peripheral blood lymphocytes, before and after the period of dietary supplementation, were compared independently using the one-tailed paired *t* test. Mean values were significantly different from those of the corresponding control group: $^*P \leq 0{\cdot}05$.

Discussion

We have demonstrated that uptakes of lutein, β-carotene and lycopene by MOLT-17 lymphocytes are both carotenoid-specific and dose-dependent. Plasma carotenoids are transported exclusively by lipoproteins (Parker, 1996) and cell uptake is probably mediated via surface receptors for LDL or a LDL-receptor-related protein (Norum & Blomhoff, 1992). Intestinal uptake of carotenoids, however, occurs via passive diffusion (Parker, 1996), and in the absence of LDL it is likely that cellular uptake *in vitro* is also a passive process. The incorporation of carotenoids into artificial lipid systems, and the dynamics between such systems and natural membranes, is carotenoid-specific; this process is determined largely by the physical properties of the carotenoid (Landrum & Bone, 2001). In our model system, uptake (as measured by final cellular carotenoid concentrations) was apparently higher than corresponding concentrations measured in lymphocytes *ex vivo* (Astley, 1997; Riso *et al.* 1999). However, as with lymphocytes *ex vivo*, uptake represented only a tiny proportion of available carotenoid (0·1 % *in vivo*, 0·2–5·0 % *in vitro*). Given such specificity, the extent of uptake in these experiments is probably unique, being determined by the liposome mix used, the MOLT-17 cell membranes and the individual carotenoids.

Lowe *et al.* (1999) determined that lycopene and β-carotene increased DNA resistance to oxidative damage (induced by xanthine and/or xanthine oxidase) at relatively low concentrations (1–3 μmol/l), but this protection was rapidly lost with increasing carotenoid concentration (4–10 μmol/l). Similarly, stimulants of oxidative damage (H_2O_2 compared with *tert*-butylhydroperoxide) can elicit different responses from the cells, particular when supplemented with β-carotene at high concentrations (10 μmol/l; Woods *et al.* 1999). Although the consequences of carotenoid uptake, in particular β-carotene, have been shown to be duplicitous in character (Martin *et al.* 1996; Lowe *et al.* 1999; Woods *et al.* 1999; Yeh & Hu, 2000; Young & Lowe, 2001), the point at which enhanced DNA protection is lost may be dependent upon the cells under examination, the oxidant system used and the delivery technique, as well on as the extent of carotenoid uptake.

In the studies described here, oxidative stress, in the form of a single acute challenge (100 μmol H_2O_2/l, 5 min at 4 °C), was used to determine the effect of increased lutein, lycopene or β-carotene, in lymphocytes *in vitro* and *ex vivo*. *In vitro*, the elevation in numbers of SSB in cells not exposed to oxidative challenge could be as a result of increased damage derived from higher lutein and lycopene loading. However, SSB are not indicative of DNA damage alone: particularly in lymphocytes (Collins *et al.* 1995) they can also represent intermediates of repair of damaged bases. In most cells, this repair occurs relatively rapidly. There is evidence, however, that deficiencies in repair enzymes (Kaminskas & Li, 1992), slow removal of 3′ end blocking groups (Izumi *et al.* 2000), low availability of nucleotides (Perrino & Loeb, 1990) or a general reduction in repair capacity characteristic of differentiated or non-dividing cells (van Loon *et al.* 1993) cause SSB to accumulate as indirect indicators of ongoing repair.

MOLT-17 DNA was less susceptible to oxidative damage following incubation with any one of the three carotenoids between 0·50 and 1·00 μmol/l. At concentrations $>1{\cdot}00$ μmol/l, the effects were different for each of the carotenoids. Although there were no differences between supplemented MOLT-17 and their corresponding H_2O_2-treated pair at higher concentrations, this was as a result of increased numbers of SSB at baseline, not enhanced protection from oxidative challenge. These differences may be derived from a combination of anti- and pro-oxidant effects. However, we have produced evidence that suggests carotenoids modulate DNA repair processes (Astley *et al.* 2002). Thus, the effects demonstrated here could also reflect changes in DNA repair capacity.

The nature of carotenoid intra-cellular action will be determined by their chemical composition, and polarity and orientation within the membrane as well as total cellular concentration. For any compound to be effective in protecting DNA directly, it must be associated with or in close proximity to the nucleus and/or DNA itself. Currently, little, if anything, is known about intra-cellular distribution of the carotenoids, but some may be better located to interact with DNA-damaging free radicals than others. This could explain differences in the degree of DNA protection observed even where uptake and loading are similar. Any enhancement of DNA repair is very likely an indirect effect; dietary compounds would need to interact with existing signalling mechanisms and their ability to do this will be dictated by their chemical characteristics, concentration or location within the cell. Thus, the 'antioxidant' capacity of the carotenoids and their ability to influence of DNA repair are very probably two separate and distinct processes, the overlap of which makes interpretation of their effects very difficult. A more important issue is whether the same effects can be observed *in vivo* since neither is of significance if they only occur *in vitro*.

In the intervention studies described here, the same dose of lutein, lycopene and β-carotene was administered to the same volunteers, at the same time of year, over 3 years. This was important because plasma levels of the various carotenoids fluctuate during the year, possibly as a result of changing intake with seasonal availability and consumer choice (Dušinška *et al.* 2002; Cooke *et al.* 2002), and the rise and fall of endogenous levels of DNA damage (Dušinška *et al.* 2002). Although these confounding factors cannot be eliminated, their effect can be marginalised with careful planning. Changes in plasma concentration following supplementation with the three carotenoids were clearly different, but in the absence of pharmacokinetic-type studies it is not possible to conclude why. The amount of dietary fat present in a meal is known to affect the bioavailability and absorption of carotenoids (Jayarajan *et al.* 1980; het Hof *et al.* 1999; Stahl *et al.* 2002), but even in the event of equal absorption, the carotenoids need not raise plasma concentrations to the same extent. Plasma concentrations reflect the degree and rate of absorption, patterns of tissue targeting and tissue loading. This varies from carotenoid to carotenoid (Kaplan *et al.* 1990; Schmitz *et al.* 1991; Stahl *et al.*

1992; Landrum & Bone, 2001) and can be influence by a host of intra- and inter-individual factors including amongst others dose, source, duration of intervention and habitual diet (Astley *et al.* 2002, 2003; Cooke *et al.* 2002).

Lymphocytes are not primary target cells for carotenoids. In the absence of a significant increase in plasma lycopene or lutein concentration, PBL carotenoid concentrations may not increase sufficiently to induce antioxidant protection, pro-oxidant damage or modulate repair. Previous work with β-carotene and carotenoid-rich foods is consistent with the carotenoids providing some degree of protection from oxidative DNA damage. What is not clear, however, is how this protection manifests. Whilst some studies have shown increase cellular resistance to oxidative damage in the absence of any change in endogenous DNA damage, others demonstrate a significant reduction in oxidative damage to DNA bases, at baseline, as well as protection from or enhanced recovery following oxidative damage (Collins *et al.* 1995, 1998; Duthie *et al.* 1996; Pool-Zobel *et al.* 1997, 1998; Fillion *et al.* 1998; Riso *et al.* 1999; Porrini & Riso, 2000; Torbergsen & Collins, 2000; Astley *et al.* 2002).

In the studies described here, DNA resistance to oxidative damage *ex vivo* was unaffected by supplementation with any of the carotenoids. However, supplementation with β-carotene, and the corresponding increase in plasma concentration, was associated with increased numbers of SSB in control PBL (i.e. PBL not subject to H_2O_2 challenge demonstrated more SSB post-supplementation that control (unchallenged) PBL before supplementation). As with MOLT-17 cells, this could be an indication of oxidative damage (i.e. a pro-oxidant effect) or accumulation of repair intermediates (i.e. altered repair capacity and/or rate).

In general, the findings here support the hypothesis that carotenoids and carotenoid-rich foods exert a protective effect *ex vivo* as well as *in vitro*. The suggestion that dietary compounds are able to modulate DNA repair, possibly via redox-sensitive pathways, which ultimately influence nucleotide or base excision repair or transcription-coupled repair, is not new. It has been claimed that β-carotene and vitamin C demonstrate this novel function quite distinct from their direct antioxidant properties (Cooke *et al.* 1998, 2001; Astley *et al.* 2002), the combination of which may be responsible for their anti-cancer properties. Conclusive evidence for the physiological relevance and mechanisms of action will have to be determined using techniques that can discriminate accurately between DNA-damage production and DNA repair.

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