Eur J Nutr (2005) 44 : 114–120
DOI 10.1007/s00394-004-0501-3

ORIGINAL CONTRIBUTION

In vivo and in vitro evidences that carotenoids could modulate the neutrophil respiratory burst during dietary manipulation

Stéphane Walrand
Marie-Chantal Farges
Olivier Dehaese
Nicolas Cardinault
Régine Minet-Quinard
Pascal Grolier
Corinne Bouteloup-Demange
Josep Ribalta
Brigitte M. Winklhofer-Roob
Edmond Rock
Marie-Paule Vasson

Summary *Background* The primary role of polymorphonuclear neutrophils (PMNs) is to destroy pathogenic microorganisms after phagocytosis by producing reactive oxygen species (ROS) and toxic molecules. However, PMNs produce sufficient amounts of ROS during an oxidative burst to be autotoxic and detrimental to their own functions and to possibly cause DNA damage, protein and lipid oxidation and cell membrane destructuration. *Objective* The aim of this study was to investigate *in vivo* the role of the antioxidant capacities of carotenoids in modulating ROS content in PMNs during oxidative burst. Moreover to investigate the direct or indirect effect of carotenoids, the modification of PMN ROS content was explored after *in vitro* supplementation with β-carotene or lycopene, chosen taking account of their vitamin A and no vitamin A precursor effect, respectively. *Design In vivo study*: Venous blood was collected from 10 healthy male volunteers and ROS production from phorbol myristate acetate (PMA)-stimulated PMNs was determined, by flow cytometry using the fluorescent dye dihydrorhodamine 123, at baseline, after 3 weeks of carotenoid depletion (carotenoid intake limited to 25 % of usual intake) and after 5 weeks of carotenoid repletion (30 mg β-carotene, 15 mg lycopene and 9 mg lutein per day). *In vitro study*: ROS content in PMA-stimulated PMNs isolated from carotenoid depleted subjects and controls was quantified after an *in vitro* enrichment with β-carotene (1 μmol/L) or lycopene (0.3 μmol/L). *Results In vivo* carotenoid depletion increased PMN H_2O_2 content after PMA activation by 38 % ($p < 0.05$ *vs* baseline), while supplementation for 5 weeks restored basal H_2O_2 generation ($p < 0.05$ *vs* depletion). Although H_2O_2 measurement in PMNs from non-depleted subjects was not affected by an *in vitro* supply with β-carotene or lycopene, a significant decrease in H_2O_2 content by 78.9 % and 81.2 %, respectively, was observed in PMNs from carotenoid depleted subjects ($p < 0.01$ *vs* depleted control subjects). *Conclusions* The carotenoid ROS quenching capacities control both *in vivo* and *in vitro* the PMNs ROS generation and probably protect these cells against DNA, membrane lipid and protein damages during oxidative burst. Moreover, these effects appear independent from the metabolic conversion of carotenoids to vitamin A.

Key words carotenoids – beta-carotene – lycopene – lutein – depletion – oxidative burst – reactive oxygen species – humans – polymorphonuclear neutrophils – flow cytometry – dihydrorhodamine

Received: 16 June 2003
Accepted: 27 February 2004
Published online: 6 May 2004

S. Walrand · M.-C. Farges · O. Dehaese · R. Minet-Quinard · M.-P. Vasson (✉)
Laboratoire de Biochimie, Biologie Moléculaire et Nutrition, EA 2416
Faculté de Pharmacie
Centre de Recherche en Nutrition Humaine d'Auvergne
28 place Henri-Dunant, BP 38
63001 Clermont-Ferrand cedex, France
Tel.: +33-4/7317-8048
Fax: +33-4/7327-4942
E-Mail: m-paule.vasson@u-clermont1.fr

N. Cardinault · P. Grolier · E. Rock
Equipe Vitamines, Unité des Maladies Métaboliques et Micronutriments
Centre de Recherche en Nutrition Humaine d'Auvergne, INRA Theix
Saint Genès Champanelle, France

C. Bouteloup-Demange
Laboratoire de Nutrition Humaine
Centre de Recherche en Nutrition Humaine d'Auvergne
Clermont-Ferrand, France

J. Ribalta
Unitat de Recerca de Lípids i Arteriosclerosi
Facultat de Medicina
Universitat Rovira i Virgili
Reus, Spain

B. M. Winklhofer-Roob
Institute of Molecular Biology, Biochemistry and Microbiology
Karl-Franzens University
Graz, Austria

EJN 501

Introduction

Polymorphonuclear neutrophils (PMNs) migrate into tissues in response to invading pathogens. Their primary role is to destroy pathogenic microorganisms after phagocytosis by producing lethal reactive oxygen species (ROS) during a complex process known as the respiratory burst [1]. Superoxide ($O_2^{\cdot -}$) is formed initially after the reduction of molecular oxygen by single electrons. The latter originate from nicotinamide adenine dinucleotide phosphate (NADPH) generated *via* the oxidative segment of the pentose phosphate pathway. This process is catalyzed by the NADPH oxidase system which is an enzyme complex present in PMNs and other immune cells. In resting cells, NADPH oxidase is dissociated and thus inactive consisting in one membrane bound and four components stored in cytosolic granules [1, 2]. Upon PMN activation, the cytosolic proteins translocate to the membrane component and a functional electron-transfer system is formed leading to $O_2^{\cdot -}$ generation. The $O_2^{\cdot -}$ is thereafter rapidly transformed into other ROS which include hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and hypochlorous acid (HOCl) [3]. The importance of $O_2^{\cdot -}$ and H_2O_2 production for PMN bactericidal activity is well known since a lack of production of these molecules in patients with chronic granulomatous disease results in an increased susceptibility to bacterial infection [4].

The respiratory burst products are not only able to kill invading microorganisms, but in excess may also damage host cells and, consequently, may be responsible for some of the tissue destructuration associated with many inflammatory reactions. In this context, the ROS quenching ability of PMNs is important since free radicals produced in biological systems, i. e. during oxidative burst, cause DNA damage, protein and lipid oxidation and cell membrane destructuration. Baehner et al. [5] observed that PMNs elaborate sufficient amounts of H_2O_2 during oxidative burst to be autotoxic and to retard cell movement during chemotaxis and phagocytosis.

Previous studies [6, 7] have shown that carotenoids such as β-carotene, lycopene or canthaxantin are potent quenchers of free radical species. These micronutrients are present in significant amounts in human plasma and tissues [8, 9] and may have specific protective function related to their high antioxidant capacity [10]. For example, the consumption of tomato products, which contain large amounts of lycopene, has a significant reducing effect on oxidative damage to lymphocyte DNA caused by H_2O_2 in *ex vivo* experiments [11]. In the same way, low carotenoid status in cystic fibrosis patients increased ROS concentration and normalisation of their carotenoid status reduced pulmonary inflammation [12].

This study aimed to investigate from healthy volunteer population the effects of *in vivo* depletion and repletion of carotenoids on H_2O_2 content in stimulated PMNs. Moreover, in order to determine whether conversion to vitamin A is involved in this effect [13], we measured the modification of PMN ROS content after *in vitro* supplementations with β-carotene or lycopene, representing a provitamin A and non-provitamin A carotenoid, respectively.

Subjects and methods

Characteristics of the subjects

Ten male healthy non-smokers (56 ± 4 years) were enrolled from the French volunteer population recruited for the European VITAGE project examining the fat soluble vitamins status and functions during healthy ageing (FP5-RTD, QLK1-CT-99–00830).

None of the volunteers was suffering from any relevant acute or chronic disease, had any signs of infection or inflammation nor was taking any drugs known to affect the immune system (anti-inflammatory drugs, hormones, analgesics, recent vaccination). They were all lean with a body mass index within the normal ranges (20 to 25 kg/m^2) and did not follow a specific dietary regimen nor take antioxidant supplement. The study was carried out after approval by the Auvergne County Ethics Committee and was performed in accordance with the ethical standards of the Declaration of Helsinki. All subjects gave informed written consent prior to their inclusion in the study.

Experimental design

Blood sample treatment: Fasting peripheral blood samples were collected into vacutainer tubes containing EDTA at baseline (12 ml) and after carotenoid depletion (12 ml) and repletion (6 ml) periods. One part (6 ml) of the blood was used for the determination of ROS concentration in stimulated PMNs and the other part (6 ml) was employed to isolate PMNs and to study the *ex vivo* effects of carotenoid enrichment, using baseline and depleted samples only. All samples were immediately used for the oxidative burst assay.

For in vivo carotenoid manipulation in healthy humans, 6 ml of blood were initially used for baseline determinations of ROS production by stimulated PMNs. Thereafter, the subjects were on a carotenoid-depleted diet for 3 weeks. In order to limit the carotenoid intake to 25 % of the usual intakes, a nutritional training was organized to explain to the volunteers how to compose their meals to have low intakes of fruits and vegetables containing carotenoids. Another blood sample was taken after this 3 week-depletion period. Afterwards participants immediately started the 5 week-repletion

European Journal of Nutrition (2005) Vol. 44, Number 2

period taking 3 capsules per day containing a total of 30 mg/d β-carotene, 15 mg/d lycopene and 9 mg/d lutein (corresponding to a total of 54 mg/d all-trans carotenoids), together with the diet usually consumed before the study. At the end of this period, a final blood sample was collected. These amounts of carotenoids are like those usually used in large carotenoid supplementation studies [14].

For in vitro PMN β-carotene or lycopene enrichment assay, 6 ml of blood were collected both from non-depleted subjects and from carotenoid-depleted subjects to isolate PMNs.

PMN preparations

From the blood obtained after *in vivo* carotenoid treatments, red blood cells were haemolyzed using ammonium chloride solution. Leukocytes were thereafter washed twice in RPMI-1640 medium (Sigma-Aldrich, Saint Quentin Fallavier, France). Cells were tested for viability (> 95 %) using trypan blue dye exclusion test. The population of PMNs was recognized using the flow cytometer gating (see below).

Concerning the *in vitro* supplementation assay, 6 ml of whole blood were stratified on a discontinuous Ficoll-Hypaque density gradient (Histopaque® 1077 and 1119, Sigma-Aldrich) and then centrifuged (700 g, 30 min, 20 °C) to obtain PMNs. PMNs were then collected on the corresponding layer (1.077 < density < 1.119) and washed in RPMI-1640 medium (Sigma-Aldrich). Cells were tested for purity (> 95 %) and viability (> 95 %) using May-Grunwald-Giemsa staining and trypan blue dye exclusion test, respectively. The final cell suspension was adjusted in RPMI-1640 medium to the cell density of 1.10^6 PMN/ml after counting in a Malassez chamber. PMNs were thereafter suspended in RPMI medium without carotenoids or enriched with all-trans carotenoids: 1 µmol/L β-carotene or 0.3 µmol/L lycopene. β-carotene and lycopene were first dissolved in tetrahydrofuran (THF, Sigma-Aldrich) and added to RPMI-1640 PMN suspension (10^6/ml) in appropriate volumes to obtain chosen carotenoid enrichment and to limit the THF concentration at 0.25 %. For each PMN suspension, 4 assays were performed as follows: 1) PMNs with RPMI (control), 2) PMNs with RPMI + THF 0.25 % (control THF), 3) PMNs with β-carotene (1 µmol/L in THF 0.25 %) and 4) PMNs in lycopene (0.3 µmol/L in THF 0.25 %). The final concentrations of β-carotene and lycopene were chosen within physiological concentrations found in healthy non-depleted human serum. The H_2O_2 concentration in PMNs was quantified by flow cytometry immediately after the addition of the THF or carotenoids as indicated below.

Flow cytometry analysis of H_2O_2 content in stimulated PMNs

PMNs (10^6 cells/ml) were preincubated for 15 min at 37 °C under permanent shaking with dihydrorhodamine 123 (DHR, 1 µmol/L). DHR is freely permeable, and after oxidation by ROS into rhodamine 123 (RHO) it emits a bright fluorescence. Since RHO is known to bind to cellular membranes, the fluorescent signal is exclusively localized inside the cell [15]. PMNs were then stimulated with phorbol 12-myristate 13-acetate (PMA: 10 µmol/L, Sigma-Aldrich) for 10 min at 37 °C. This latter agent activates NADPH oxidase by enhancing protein kinase C and, thus, stimulates production of ROS. After activation, PMNs were kept on ice to stop reactions until flow cytometry analysis.

Analyses were carried out on a Coulter Epics XL (Beckman-Coulter, Villepinte, France) equipped with an argon laser (488 nm emission) and interfaced to the System II software (Beckman-Coulter). PMNs were recognized on the basis of the forward-angle light scatter (FS) and the side-angle light scatter (SS) allowing to identify PMNs and to exclude other cell types (a few contaminating lymphocytes and erythrocytes), debris and aggregates from the analysis. A total of 10^4 events were collected in the PMN gate. A sodium chloride/potassium chloride solution (Beckman Coulter) was used as sheath fluid. The fluorescence of cells was recorded under 488 nm excitation. Green fluorescence from DHR was measured in the FL1 channel through a 525 nm band pass filter (BP) in combination with a 550 nm dichroic long pass (DL) mirror. As previously shown, DHR is specifically oxidized by H_2O_2 [15], allowing the quantification of H_2O_2 production by estimating DHR fluorescence intensities.

Plasma carotenoid analysis

Extraction of carotenoids was performed from 400 µL plasma samples using ethanol containing echinenone (0.2 µg/ml) as an external standard and 800 µL of hexane. After vortex mixing for 10 min at 446 g, the supernatant fraction was decanted twice and the combined extract evaporated to dryness under N_2 and redissolved in the mobile phase (acetonitrile/dichloromethane (1:1 vol/vol)). Analysis by reverse-phase high-performance liquid chromatography was performed according to a method previously described [16]. The apparatus consisted of a two columns set in series, a 3 µm Nucleosil C18 (150 × 4.6 mm, inner diameter, Interchim, Montluçon, France) and a 5 µm Vydac TP54 C18 (250 × 4.6 mm, Hesperia, CA, USA). The columns were coupled to a Waters system (Waters SA, Saint Quentin en Yvelines, France) with an UV-VIS detector (Waters 996) set at 450 nm. An isocratic mobile phase of acetonitrile/

dichloromethane/methanol containing 50 mM ammonium acetate/water (70/10/15/5: v/v/v/v) with a flow rate of 2 ml/min was used. Carotenoid concentrations were calculated using their respective standards (Hoffmann-La Roche, Basel, Switzerland) in the all-trans form for the β-carotene and lutein, and in both cis and all-trans forms for lycopene. A photodiode array detector supported by the Millemium 32 Chromatography Manager computing system (Waters) was used to confirm the spectrum identity of carotenoids. Furthermore, the carotenoid concentrations in the sample were corrected by the recovery of the external standard echinenone. The recovery rates were between 80 and 100 %. The coefficient of variation was always lower than 10 %.

Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed on PCSM software (Deltasoft, Grenoble, France). Comparison of means was carried out using the repeated-measure analysis of variance (ANOVA) for the *in vivo* experiment and by one-way ANOVA for the *in vitro* experiment. In both cases the ANOVA was followed by the Newman-Keüls post hoc test. Values of $p < 0.05$ were considered as significant.

Results

Carotenoid intakes and plasma concentrations at baseline and after carotenoid-depletion and repletion periods

Baseline plasma carotenoid concentrations are in agreement with the literature (Table 1) [17]. As documented by the food consumption records, 3-weeks carotenoid depletion period induced a reduction of 75.8 ± 14.1 % and 99.4 ± 0.4 % of the usual β-carotene and lycopene intakes, respectively. Under these conditions, plasma β-carotene, lycopene and lutein levels were reduced by 38 %, 44 % and 24 %, respectively (Table 1). After 5 weeks of carotenoid supplementation, plasma β-carotene, lycopene and lutein concentrations were approximatively 6, 6 and 3 times, respectively, higher compared to the depleted status ($p < 0.01$ *vs* basal and depleted periods). In addition, plasma retinol concentrations were not modified throughout the study (data not shown).

Table 1 Carotenoid concentrations in plasma at baseline and after carotenoid-depletion and repletion periods

µmol/L	β-carotene (all trans)	Lycopene (all trans)	Lutein (cis-trans)
Baseline	1.017 ± 0.170[a]	0.372 ± 0.112[a]	0.394 ± 0.056[a]
Depletion	0.631 ± 0.091[a]	0.208 ± 0.052[a]	0.300 ± 0.036[a]
Repletion	3.677 ± 0.906[b]	1.266 ± 0.143[b]	0.758 ± 0.076[b]

Values are means ± SEM. Repeated-measure analysis of variance (ANOVA) + Newman-Keüls test: a≠b with $p < 0.01$ for the same carotenoid studied

H_2O_2 content in stimulated PMNs at baseline and after in vivo carotenoid depletion and repletion periods

At baseline, the content of H_2O_2 in PMA-activated PMNs reached values common for healthy adults [18], while carotenoid depletion induced a significant increase (38 %, $p < 0.05$ *vs* basal state, Fig. 1). In addition, supplementation with β-carotene, lycopene and lutein for 5 weeks restored basal H_2O_2 content by PMA-stimulated PMNs ($p < 0.05$ *vs* depletion period).

H_2O_2 content in stimulated PMNs from carotenoid depleted subjects after in vitro β-carotene or lycopene treatment

As already shown [19, 20], THF used at low concentration (0.25 % final) for carotenoid solubilization was not toxic for PMNs and did not significantly modify their ROS production (NS: control THF *versus* Control, Fig. 2a and 2b). H_2O_2 measurement in PMNs from non-depleted subjects was not affected by the *in vitro* β-carotene or lycopene treatment (Fig. 2a). On the other hand, *in vitro* addition of β-carotene and lycopene to PMNs from carotenoid depleted subjects resulted in an important decrease in H_2O_2 content by 79 % and 81 %,

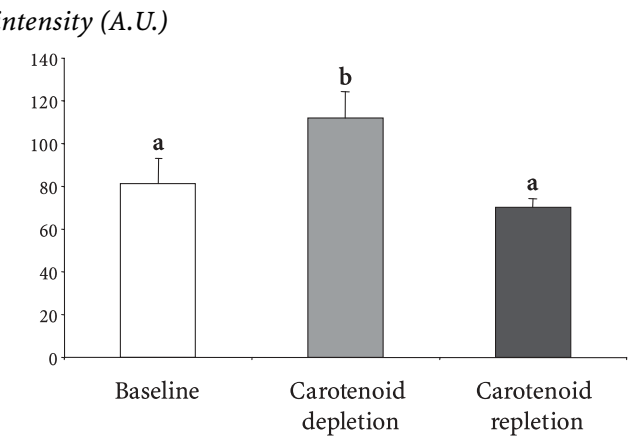

Fig. 1 Hydrogen peroxide content in PMA-stimulated PMNs at baseline conditions and after carotenoid depletion and repletion periods. Hydrogen peroxyde content was quantified at baseline, after 3 weeks of carotenoid depletion (diet avoiding carotenoid-containing food) and after 5 weeks of carotenoid repletion (3 pills/d containing a total of 30 mg β-carotene, 15 mg lycopene or 9 mg lutein). Results are expressed as fluorescence intensity (Arbitrary unit, A.U.). Values are means ± SEM. Repeated-measure analysis of variance (ANOVA) + Newman-Keüls test: a≠b with $p < 0.05$

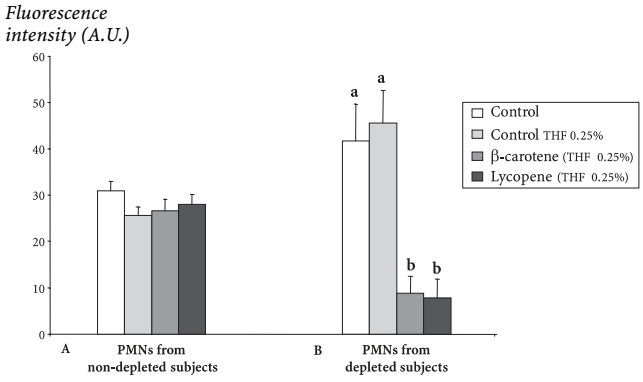

Fig. 2 In vitro enrichment with β-carotene or lycopene and its effects on hydrogen peroxide content in PMA-stimulated PMNs from non depleted subjects (**A**) and 3 weeks carotenoid depleted subjects (**B**). Hydrogen peroxyde content was quantified after in vitro PMN treatment with 0.6 µmol/L β-carotene or 0.3 µmol/L lycopene (respectively dissolved in THF 0.25 %) or without carotenoid (control, THF (0.25 %)). Results are expressed in fluorescence intensity (Arbitrary unit, A.U.). Values are means ± SEM. Analysis of variance (ANOVA) + Newman-Keüls test ($a \neq b$ with $p < 0.05$). *THF* tetrahydrofuran

respectively ($p < 0.01$ *versus* Control, Fig. 2b). Moreover, we confirmed that carotenoid depletion enhanced the H_2O_2 content of PMA stimulated PMNs (35 % depleted *vs* non-depleted subjects, Fig. 2b), as previously shown in the *in vivo* assay (38 % depleted *versus* basal status).

Discussion

It has been suggested that carotenoid consumption should be increased because of their antioxidant properties [21]. However, despite some observations in animal models, there are few studies in humans describing the *in vivo* and *in vitro* effects of carotenoid supplementation on ROS content in PMNs of carotenoid-depleted subjects. In this study, we showed that carotenoid depletion and repletion affect plasma carotenoid levels in healthy male humans. However, dietary manipulations used, i. e. a decrease in vegetable and fruit intakes, may probably induce a plasma reduction in other micronutrients, such as vitamins or phenolic compounds which were not determined in the present investigation. In addition, the dietary restriction used results in a reduction of the plasma concentrations of the three carotenoids studied but without significance probably because of the low number of subjects and the short depletion period. However, all recruited subjects had good response to carotenoid supplementation as evidenced by a significant rise in serum carotenoid concentrations.

In vivo and *in vitro* changes in carotenoid exposure modulated the H_2O_2 content in PMNs. DHR fluorescence intensities obtained during the *in vitro* carotenoid enrichment were lower as compared to those measured from PMNs isolated after the *in vivo* investigation. This may be linked to the longer cell preparative procedure used for the *in vitro* assay.

Previous studies [22, 23] have shown that plasma carotenoid concentrations were strongly related to lymphocyte and PMN carotenoid status: an increase in plasma carotenoid content led to an enrichment in leukocyte carotenoid concentrations. In our study, *in vivo* carotenoid depletion might strongly enhance the ability of PMA-stimulated PMNs to produce reactive oxygen species *in vitro*. However, the high H_2O_2 content in PMNs might also be explained by an imbalance between the production of ROS and the reduced antioxidant molecules in the cell. In addition, supplemental *in vitro* or *in vivo* carotenoids normalized ROS content in PMNs. This observation clearly demonstrated that carotenoids modulated the amount of ROS in PMNs during oxidative burst, probably by a quenching effect. Such an antioxidative effect has been expressed by other studies focusing on the quenching effects of vitamin E on PMN oxidative burst [24–26]. However, some authors [27] observed that repletion with β-carotene (15 mg/d during 4 weeks) of previously carotenoid-depleted healthy male adults did not affect superoxide production by PMA-stimulated PMNs. In this latter study, in spite of no variation in ROS production, β-carotene supplementation reduced serum lipid peroxide concentrations with an inverse relationship between serum β-carotene and lipid peroxide levels [27].

In addition to their quenching properties, carotenoids might act by modulating the activity of enzyms implicated in the ROS biochemical pathway. Dixon et al. [28] evaluated, in adult women, the effect of carotenoid depletion (68 days) and repletion (15 mg/d during 28 days) periods on the erythrocyte superoxide dismutase (SOD) activity. This activity was depressed in carotene-depleted women and recovered with repletion. On the other hand, Girodon et al. [29] failed to show any modification of the SOD activity in humans receiving β-carotene supplementation (6 mg/d) during 2 years. The biological effects of carotenoids on the ROS biochemical pathway might be dependent on the cellular pool of other antioxidants and ROS precursors. These latter points need to be elucidated by additional studies.

Cell culture experiments [30, 31] demonstrated that high concentrations of β-carotene (10^{-5} mol/L) or lycopene (3.10^{-6} mol/L) increase the level of DNA strand breaks in HT29 or HepG2 cells exposed to H_2O_2. These high β-carotene and lycopene concentrations cannot be achieved in human plasma, even by consuming a diet very rich in carotenoids and supplements, probably due to a control mechanism regulating plasma carotenoid levels [32]. That is also the reason why we used physiological amounts of carotenoids in the present *in vitro* study (β-carotene 10^{-6} mol/L or lycopene 3.10^{-7} mol/L). Thus, we demonstrated the effect of small quantities of

carotenoids which can be achieved by following the recommended intake of carotenoid-rich food.

Studies using animal models [33] also showed that β-carotene or canthaxanthin supplementation decreased the oxidative damage generated in immune cells during inflammatory responses. These two carotenoids inhibited the loss of macrophage receptors following exposure to ROS. The protective effects of carotenoids on membrane structure may explain the immune-enhancing action of these micronutrients. This action may include the quenching of ROS which exert immune-suppressive properties [34]. Peroxidation of cell membrane lipids can decrease membrane fluidity and, therefore, depress phagocytosis. In addition, membrane receptors, required for endothelium adhesion and antigen recognition by immune cells, can also be damaged by peroxidation of membrane lipids [33, 34].

We also confirmed *in vitro* the antioxidant capacities of carotenoids on H_2O_2 generated during oxidative burst in PMA-stimulated PMNs. *In vitro* carotenoid treatment of the cells isolated from carotenoid-depleted subjects allowed us to separate the direct and vitamin A-conversion independent effects of carotenoids on H_2O_2 content in PMNs. It was not possible to determine whether the effects observed in our study after the *in vivo* carotenoid supplementation were due to carotenoids themselves or to vitamin A which can itself decrease ROS content in immune cells [13, 35–38]. We therefore exposed *in vitro* carotenoid-depleted PMNs to β-carotene or lycopene at physiological concentrations. Lycopene is a carotenoid without provitamin A function but with antioxidant activity, whereas β-carotene possesses both antioxidant and provitamin A properties, through the β-carotene-monooxygenase activity. The profiles of PMN respiratory burst response to PMA are almost superimposable whatever the carotenoid used. Therefore, the modulating effects of carotenoids on PMN ROS production *in vivo* seem to be independent of their conversion to vitamin A. This latter observation remains to be confirmed by the *ex vivo* treatment of PMN with retinol.

In conclusion, our data demonstrated a potential role of carotenoids at physiological levels on the modulation of PMN oxidative burst. However, because of the experimental design, i. e. dietary manipulation, used in the present study, the *in vivo* effects observed concerning ROS may be related not only to carotenoids but probably to other micronutrient fluctuations. However, we clearly showed that the quenching capacities of carotenoids modulated *in vitro* ROS content in PMNs. This may protect PMNs against DNA, membrane lipid and protein damages during oxidative burst, and also may avoid collateral tissue damages by diffusible ROS during inflammation. In addition, the anti-oxydant capacity of carotenoids may contribute to their immune-enhancer effect by avoiding the destructuration of membranes and receptors. This effect appeared independent from the metabolic conversion of carotenoids to vitamin A. However, more studies are required to evaluate the importance of the consumption of carotenoid-rich foods in improving the nonspecific immune response and reducing the risk of the development of diseases related to oxidative stress.

Acknowledgments This study has been carried out partly with financial support from the Commission of the European Communities, specific RTD programme "Quality of Life and Management of Living Resources", QLK1-CT-1999–00830, VITAGE. It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

References

1. Dahlgren C, Karlsson A (1999) Respiratory burst in human neutrophils. J Immunol Methods 232:3–14
2. Babior BM (1984) The respiratory burst of phagocytes. J Clin Invest 73:599–601
3. Casimir CM, Teahan CG (1994) The respiratory burst of neutrophils and its deficiency. In: Hellewell PG, Williams TJ (Eds) Immunopharmacology of neutrophils. Academic Press, London, pp 27–54
4. Smith RM, Curnutte JT (1991) Molecular basis of chronic granulomatous disease. Blood 77:673–686
5. Baehner RL, Boxer LA, Allen JM, Davis J (1977) Autooxidation as a basis for altered function by polymorphonuclear leukocytes. Blood 50:327–335
6. Jandacek RJ (2000) The canary in the cell: a sentinel role for β-carotene. J Nutr 130:648–651
7. Mortensen A, Skibsted LH, Truscott TG (2001) The interaction of dietary carotenoids with radical species. Arch Biochem Biophys 385:13–19
8. Su LC, Bui M, Kardinaal A, Gomez-Aracena J, Martin-Moreno J, Martin B, Thamm M, Simonsen N, van't Veer P, Kok F, Strain S, Kohlmeier L (1998) Differences between plasma and adipose tissue biomarkers of carotenoids and tocopherols. Cancer Epidemiol Biomarkers Prev 7:1043–1048
9. Wallstrom P, Wirfalt E, Lahmann PH, Gullberg B, Janzon L, Berglund G (2001) Serum concentrations of beta-carotene and alpha-tocopherol are associated with diet, smoking, and general and central adiposity. Am J Clin Nutr 73: 777–785
10. Azais-Braesco V, Winklhofer-Roob B, Ribalta J, Hanley B, Vasson MP, Brtko J, Brigeluis-Flohe R, Bronner A (2000) Vitamin A, vitamin E and carotenoid status and metabolism during ageing: functional and nutritional consequences. Endocr Regul 34:97–98
11. Porrini M, Riso P (2000) Lymphocyte lycopene concentration and DNA protection from oxidative damage is increased in women after a short period of tomato consumption. J Nutr 130: 189–192
12. Winklhofer-Roob BM, Schelgel-Hauter SE, Khoschsorur G, van't Hof MA, Suter S, Shmerling DH, (1996) Neutrophil elastase/α1-proteinase inhibitor complex levels decrease in plasma of cystic fibrosis patients during long-term oral β-carotene supplementation. Pediatr Res 40:130–134

13. Duszka C, Grolier P, Azim EM, Alexandre-Gouabau MC, Borel P, Azais-Braesco V (1996) Rat intestinal beta-carotene dioxygenase activity is located primarily in the cytosol of mature jejunal enterocytes. J Nutr 126:2550–2556
14. Cook NR, Le IM, Manson JE, Buring JE, Hennekens CH (2000) Effects of beta-carotene supplementation on cancer incidence by baseline characteristics in the Physicians' Health Study (United States). Cancer Causes Control 11: 617–626
15. Walrand S, Valeix S, Rodriguez C, Ligot P, Chassagne J, Vasson MP (2003) Flow cytometry study of polymorphonuclear neutrophil oxidative burst: a comparison of three fluorescent probes. Clin Chim Acta 331:103–110
16. Lyan B, Azais-Braesco V, Cardinault N, Tyssandier V, Borel P, Alexandre-Gouabeau MC, Grolier P (2001) Simple method for clinical determination of 13 carotenoids in human plasma using an isocatric high-performance liquid chromatographic method. J Chromatogr B Biomed Sci Appl 751:297–303
17. Olmedilla B, Granado F, Southon S, Wright AJA, Blanco I, GilMartnez E, vanden Berg H, Corridan B, Roussel AM, Chopra M, Thurnham DI (2001) Serum concentrations of carotenoids and vitamins A, E and C in control subjects from five European countries. Brit J Nutr 80:227–238
18. Walrand S, Moreau K, Caldefie F, Chassagne J, Portefaix G, Cynober LA, Beaufrère B, Vasson MP, Boirie Y (2001) Specific and nonspecific immune response to fasting and refeeding is differentially affected in healthy young and elderly humans. Am J Clin Nutr 74: 670–678
19. Peram V, Iftikhar S, Lietz H, Mobarhan S, Frommel TO (1996) Cytotoxic effect of beta-carotene in vitro is dependent on serum concentration and source. Cancer Lett 116:133–118
20. Pfitzner I, Francz I, Biesalski K (2000) Carotenoid: methyl-β-cyclodextrin formulations: an improved method for supplementation of cultured cells. Biochim Biophys Acta 1474:163–168
21. Rock E, Winklhofer-Roob BM, Ribalta J, Scotter M, Vasson MP, Brtko J, Brigelius-Flohe R, Bronner A, Azais-Breasco V (2001) Vitamin A, vitamin E and carotenoid status and metabolism during ageing: functional and nutritional consequences (VITAGE PROJECT). Nutr Metab Cardiovasc Dis 11:70–73
22. Chew BP, Park JS, Weng BC, Wong TS, Hayek MG, Reinhart GA (2000) Dietary beta-carotene absorption by blood plasma and leukocytes in domestic cats. J Nutr 130:2322–2325
23. Chew BP, Park JS, Wong TS, Kim HW, Weng BB, Byrne KM, Hayek MG, Reinhart GA (2000) Dietary beta-carotene stimulates cell-mediated and humoral immune response in dogs. J Nutr 130: 1910–1913
24. Engle WA, Yoder MC, Baurley JL, Yu PL (1988) Vitamin E decreases superoxide anion production by polymorphonuclear leukocytes. Pediatr Res 23: 245–248
25. Meydani SN, Beharka AA (1996) Recent developments in vitamin E and immune response. Nutr Rev 56:S49-S58
26. Lee CY, Man-Fan Wan J (2000) Vitamin E supplementation improves cell-mediated immunity and oxidative stress of Asian men and women. J Nutr 130: 2932–2927
27. Mobarhan S, Bowen P, Andersen B, Evans M, Stacewicz-Sapuntzakis M, Sugerman S, Simms P, Lucchesi D, Friedman H (1990) Effects of β-carotene repletion on β-carotene absorption, lipid peroxidation, and neutrophil superoxide formation in young men. Nutr Cancer 14:195–206
28. Dixon ZR, Burri BJ, Clifford A, Frankel EN, Schneeman BO, Parks E, Keim NL, Barbieri T, Wu MM, Fong AK (1994) Effects of a carotene-deficient diet on measures of oxidative susceptibility and superoxide dismutase activity in adult women. Free Radic Biol Med 17: 537–544
29. Girodon F, Blache D, Monget AL, Lombart M, Brunet-Lecompte P, Arnaud J, Richard M-J, Galan P (1997) Effect of two year supplementation with low doses of antioxidant vitamins and/or minerals in elderly subjects on levels of nutrients and antioxidant defense parameters. J Am Coll Nutr 16:357–365
30. Lowe GM, Booth LA, Young AJ, Bilton RF (1999) Lycopene and beta-carotene protect against oxidative damage in HT29 cells at low concentrations but rapidly lose this capacity at higher doses. Free Radic Res 30:141–151
31. Woods JA, Bilton RF, Young AJ (1999) β-carotene enhances hydrogen peroxide-induced DNA damage in human hepatocellular HepG2 cells. FEBS Letters 449:255–258
32. Porrini M, Riso P, Testolin G (1998) Absorption of lycopene from single or daily portion of raw and processed tomato. Br J Nutr 80:353–361
33. Bendich A (1989) Carotenoids and the immune response. J Nutr 119:112–115
34. Bendich A (1991) β-carotene and the immune response. Proc Nutr Soc 50: 263–274
35. Kensler TW, Trush MA (1981) Inhibition of phorbol ester-stimulated chemiluminescence in human polymorphonuclear leukocytes by retinoic acid and 5,6-epoxyretinoic acid. Cancer Res 41:216–222
36. Wolfson M, Shinwell ES, Zvillich M, Rager-Zisman B (1988) Inhibitory effect of retinoic acid on the respiratory burst of adult and cordon blood neutrophils and macrophages: potential implication to bronchopulmonary dysplasia. Clin Exp Immunol 72:505–509
37. Twining SS, Schulte DP, Wilson PM, Fish BL, Moulder JE (1997) Vitamin A deficiency alters rat neutrophil function. J Nutr 127:558–565
38. Ahlemeyer B, Bauerbach E, Plath M, Steuber M, Heers C, Tegtmeier F, Krieglstein J (2001) Retinoic acid reduces apoptosis and oxidative stress by preservation of SOD protein level. Free Radic Biol Med 30:1067–1077