

β -Carotene concentration in buccal mucosal cells with and without dysplastic oral leukoplakia after long-term β -carotene supplementation in male smokers

KE Liede¹, G Alfthan², JHP Hietanen¹, JK Haukka², LM Saxen¹ and OP Heinonen³

¹University of Helsinki, Institute of Dentistry, Helsinki; ²National Public Health Institute, Department of Nutrition, Helsinki; and

³University of Helsinki, Department of Public Health, Helsinki, Finland

Objective: To measure the β -carotene concentration in buccal mucosal cells in smoking men who had received long-term β -carotene (BC) supplementation in a controlled trial. To assess the association of cellular BC on the prevalence of dysplasia in oral leukoplakia.

Design: An end-of-trial examination of a part of subjects in the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study.

Subjects and methods: 343 men who for 5–7 years had received BC (20 mg/d) or α -tocopherol (AT) (50 mg/d), or both of these or placebo. BC concentration of buccal mucosal cells was compared in the subjects with BC supplementation ($n=173$) to that of those without it ($n=170$). Oral mucosae were examined clinically and lesions showing leukoplakia histopathologically.

Results: Mean (s.d.) BC concentration in buccal mucosal cells was 7.7 (10.3) mg/kg protein in the subjects who received BC compared to 1.1 (1.7) mg/kg protein in those who did not. The BC concentration in the cells of supplemented subjects correlated with their serum BC levels ($P < 0.001$). AT supplementation had no effect on BC concentration nor was daily amount of smoking statistically significantly associated with the BC concentration in buccal cells. Altogether 17 subjects showed oral leukoplakia, 7 had dysplasia. In these 7 subjects, the BC concentration in buccal mucosal cells did not differ statistically significantly compared to subjects with only hyperkeratosis ($n=10$) (F -test, $P=0.74$).

Conclusions: After long-term BC supplementation, BC concentration in oral mucosal cells was 7-fold greater than without supplementation. There was no evidence to support an association between cellular BC concentration and precancerous lesions among the few subjects having them in their oral mucosae.

Descriptors: β -carotene; oral mucosa; dysplasia; long-term supplementation

Introduction

The kinetics of β -carotene is not known in detail. Considerable inter-individual variation is known to exist in its absorption and the time to peak concentration in plasma, irrespective of whether BC containing food or vitamin supplementation is used (Brown *et al*, 1989; Albanes *et al*, 1992; Johnson & Russell, 1992; Micozzi *et al*, 1992). Nierenberg and Nann (1992) have developed a method for determining BC in tissues, and Peng *et al* (1995) have measured BC levels in dermal and buccal mucosal cells. However, there are no reports on intracellular measurements after long-term use of this antioxidant supplement, although intracellular concentrations have been assessed after BC supplementation for short periods, e.g. three days to one week (Gilbert *et al*, 1990; Cooney *et al*, 1991; Murata *et al*, 1992) or four months (Stich *et al*, 1986). The findings have been somewhat conflicting. Gilbert *et al* (1990) detected differences among various populations and Stich *et al* (1986) found considerable inter-individual

differences in BC content of buccal mucosal cells, whereas Cooney *et al* (1991) observed no such differences. Smokers show a lower BC concentration both in plasma (Witter *et al*, 1982; Davis *et al*, 1983; Russel-Briefel *et al*, 1985; Chow *et al*, 1986) and in buccal mucosal cells (Peng *et al*, 1995), compared to that of nonsmokers.

Several clinical studies have suggested that β -carotene (BC) supplementation reduces oral leukoplakia, considered to be a precancerous lesion (Stich *et al*, 1988; Garewal *et al*, 1990; Toma *et al*, 1992). On the basis both of epidemiological studies and of animal trials, BC has been presumed to prevent both oral and pulmonary carcinomas (Peto *et al*, 1981; Byers *et al*, 1987). However, two extensive controlled clinical trials on prevention of cancer, the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study (ATBC study) and the Beta Carotene and Retinol Efficacy Trial (CARET), have yielded unexpected results concerning cancer prevention (ATBC Study Group, 1994a; Omenn *et al*, 1996a). In contrast to the study hypothesis in both studies, the risk of lung cancer was increased in subjects with BC supplementation (Albanes *et al*, 1996) and in subjects with a combination of BC plus retinyl palmitate (Omenn *et al*, 1996a,b). Long-term BC supplementation seemed not to have significant effect on the prevalence of oral leukoplakia (Liede *et al*, 1998).

In the present study, the BC concentration in buccal mucosal cells was measured in male smokers after 5–7 years' supplementation. In addition, the possible association of BC concentration with oral precancerous lesions was tested, and the concentration in buccal cells of subjects with prevalent dysplasia in keratotic lesions of the oral mucosa was compared to the concentration in subjects without dysplasia. All subjects had taken part in the extensive ATBC cancer prevention trial and were studied near the end of the trial.

Subjects and methods

In 1992–1993, 427 male cigarette smokers were randomly selected from participants in the ATBC study, all residents of Helsinki. Eighteen individuals declined to participate, and those 409 who agreed to participate were aged 55–74 (mean 59.7) years. Details of the controlled ATBC trial have been reported elsewhere (ATBC Study Group, 1994b). Briefly, the trial, conducted in southwestern Finland, was devised to explore any possible lung cancer-preventive effect of α -tocopherol (AT) and β -carotene (BC) in smoking men (ATBC Study Group, 1994a). The subjects were identified by the population register in 1985–1988, on the basis of residence area, age (50–69 years), and smoking habits: at least 5 cigarettes per day. Exclusion criteria were cancer, severe coronary disease, chronic renal failure, hepatic cirrhosis, use of anticoagulant medication or vitamin A and E supplements, or alcohol abuse. The participants were randomly allocated to four groups (ATBC Study Group, 1994b). One group received a placebo capsule; in the other groups the capsule had been supplemented with either 20 mg of β -carotene, 50 mg of α -tocopherol (vitamin E), or both. Compliance, assessed by counts of remaining capsules at thrice-yearly follow-up visits, by measurement of serum BC levels after 3 years of supplementation, and by measurements in random serum samples throughout the study, showed that 4 out of 5 participants took more than 95% of their capsules (ATBC Study Group, 1994a). Daily smoking habits were recorded at each visit. The serum BC concentration of untreated subjects in the ATBC study were comparable to baseline BC levels in nonsupplemented individuals in other studies (Comstock *et al*, 1987; Stryker *et al*, 1988; ATBC Study Group, 1994b).

Oral study

The subjects of the oral study were randomly chosen from among the four ATBC study groups on the basis of residence (Helsinki) and having ≥ 15 remaining teeth (teeth were necessary for the oral study because dental health was also examined).

The oral study included a total of 409 participants. Cell samples of 66 subjects were lost, however, because of a freezer breakdown, and the measurements of the present study were made in 343 subjects (Figure 1). The oral examination was carried out in the fall of 1992 and spring of 1993, at the end of the ATBC trial. Informed consent was obtained from each participant, and the oral study plan was approved by the Ethics Committee of the Institute of Dentistry, University of Helsinki.

Cell collection

All the cells for the present study were collected from the buccal epithelium, irrespective of the location of possible leukoplakia. Cells were collected by gently brushing the

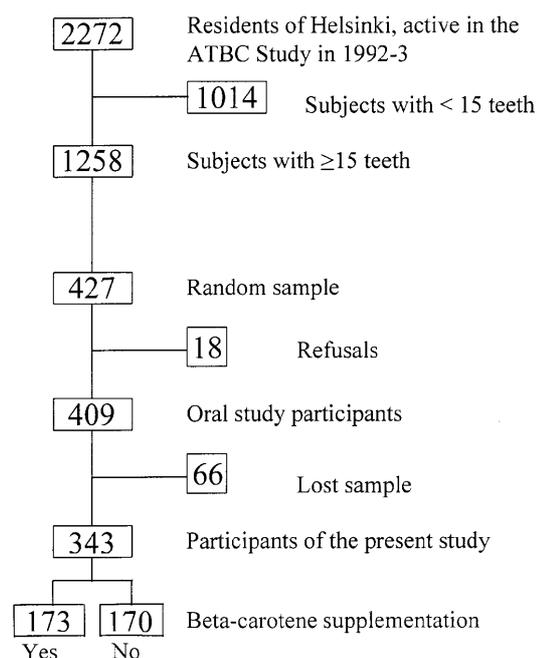


Figure 1 Recruitment of participants in the study.

buccal mucosa of both cheeks eight times with a toothbrush (Pepsodent Professional, extra soft), after which the subjects rinsed their mouths with 20 ml of distilled water, in which the toothbrush was also washed. The samples were centrifuged at 2700 g for 8 min, and the major portion of the clear supernatant was removed and discarded. The remaining cell-suspension was frozen and stored at -70°C until analysis.

Determination of β -carotene in cells

To a 0.5–1.0 ml cell suspension was added 1.5 ml of an ethanolic solution containing 1% ascorbic acid as antioxidant and echinenon as the internal standard. After brief vortexing and ultrasonication, 4 ml of n-hexane was added. After 10 min incubation, 2 ml of 2% NaCl solution was added, and the tubes were centrifuged at 2500 rpm for 5 min. Of the hexane phase, 3 ml was evaporated under vacuum. The residue was dissolved in 120 μl ethanol and a 25 μl aliquot was injected into the HPLC column (Nova-Pak C18 column 3.9 \times 75 mm, 4 μm , Millipore Co, MA, USA). The apparatus consisted of an autosampler Model SIL-9A (Shimadzu, Japan), an HPLC pump Model 400 (Applied Biosystems 400), and an electrochemical detector Model 5100 A with an analytical cell Model 5011 (Environmental Sciences Assoc, Bedford, MA, USA). The cell was set at detector 1 -0.10 V and detector 2 $+0.55$ V.

The eluent was acetonitrile–dimethylchloride–methanol 70:10:20 with 9 mmol/L Na-perchlorate, and the flow rate was 1 ml/min. Standardization was carried out with a secondary serum standard with a known amount of β -carotene calibrated against a serum sample from the National Institute of Standards and Technology, USA.

The detection limit for BC was 0.09 ng, and the peak height was linear from 0.5 to 170 ng BC. The precision within ($n=6$) and between series ($n=10$) for a 0.2 ml aliquot of a plasma pool was 9.5 CV% and 10.5 CV%, respectively, at the level of 4.9 ng.

Mucosal examination

Oral mucosal alterations were blindly examined and registered according to WHO criteria (Kramer *et al*, 1980; WHO, 1987) by one dentist (K.L.), specialized in clinical dentistry. Lesions clinically diagnosed as leukoplakia were biopsied. The specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections 4 μ m thick were stained with hematoxylin–eosin, van Gieson and PAS. Sections were examined by light microscopy by an oral pathologist (J.H.). Epithelial dysplasias were also diagnosed according to the criteria of WHO (Kramer *et al* 1978).

Statistical methods

The dependence between log-transformed concentration of β -carotene (BC) in cells and explanatory variables were modeled with linear regression. Explanatory variables were serum BC concentration (log-transformed), dietary BC, smoking habits, and AT and BC supplementation groups. All these variables were included in one regression model. Interactions between supplementation groups (AT and BC) were tested by the likelihood ratio test (McCullagh & Nelder, 1989); no interaction was detected.

In the subgroup with oral leukoplakia ($n = 17$) the dependence of concentration of BC in mucosal cells between groups with dysplasia ($n = 7$) and without dysplasia ($n = 10$) was tested with the F -test.

Results

Capsule compliance among participants was high, 98%. On average, the subjects smoked 15 cigarettes/day compared to 21 cigarettes/day at the baseline of the ATBC study (Table 1). The 343 participants were comparable with all participants in the oral study and potential participants in the ATBC study (Table 2).

In the 173 men receiving BC the mean (s.d.) BC concentration in buccal mucosal cells was 7-fold greater compared to the 170 men not receiving BC (7.7 (10.3) mg/kg protein and 1.1 (1.7) mg/kg protein, respectively). The main trial results showed respective serum BC levels in these 343 subjects to be 3.3 mg/l (1.6) ($n = 173$) and 0.27 mg/l (0.3) ($n = 170$), respectively, after 3 years of supplementation. Serum BC levels for all subjects in the main trial were 3.0 mg/l in subjects receiving BC ($n = 11276$), and 0.18 mg/l in subjects without BC ($n = 11314$). The distribution of BC concentration in buccal mucosal cells was wide among the subjects who received BC supplementation (Figure 2, upper panel). The few subjects who had a high cell BC concentration were of

Table 1 Characteristics of participants ($n = 343$)

	BC ^a ($n = 173$)	No BC ($n = 170$)
<i>At the time of the examination (1992–1993)</i>		
Capsule compliance (%)	98.2	98.0
Smoking (cigarettes/day) ^b	15.2 \pm 11.5	14.5 \pm 11.6
<i>At baseline of ATBC study (1985–1988)</i>		
Age (y) ^b	55.8 \pm 4.6	56.2 \pm 4.5
Years of education	10.2	10.4
Smoking (cigarettes/day) ^b	21.0 \pm 9.1	20.0 \pm 9.6
Smoking years ^b	32.5 \pm 8.2	33.0 \pm 8.5

^aBC = β -carotene supplementation 20 mg/day.

^bMean \pm s.d.; differences were not statistically significant.

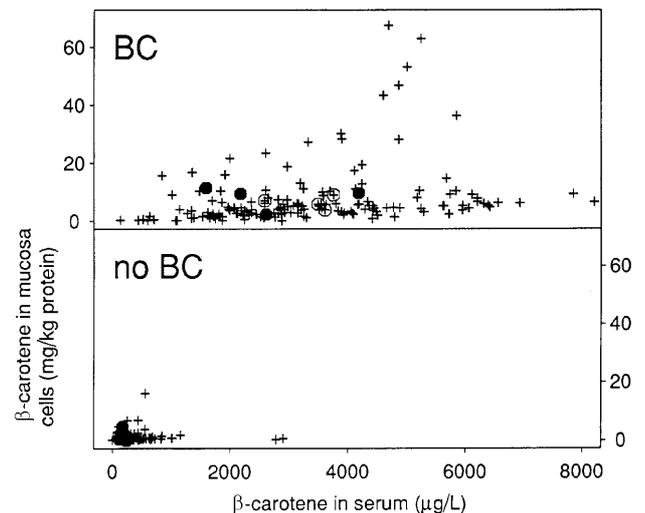
Table 2 Baseline characteristics of participants of the present study, oral study and other potential participants (active in the ATBC study in 1992–93, residents of Helsinki, with ≥ 15 teeth)

	Present study ($n = 343$)	Oral study ($n = 409$)	Rest of sampling frame ($n = 850$)
Age (y) ^a	56.1 \pm 4.4	56.0 \pm 4.5	56.3 \pm 4.6
Smoking (cigarettes/day) ^a	20.6 \pm 9.0	20.5 \pm 9.3	21.5 \pm 9.9
BC ^b			
Recipients	173	195	432
Nonrecipients	170	214	418
AT ^c			
Recipients	161	191	440
Nonrecipients	182	218	410
Alcohol intake (g/day) ^a	19.7 \pm 20.5	20.9 \pm 21.2	22.3 \pm 23.2
Body mass index ^a	26.0 \pm 3.9	26.2 \pm 3.9	26.2 \pm 3.8
Serum BC (mg/l) ^a	0.27 \pm 0.2	0.26 \pm 0.2	0.23 \pm 0.2
Serum AT (mg/l) ^a	12.7 \pm 2.9	12.6 \pm 2.9	12.9 \pm 3.9

^aMean \pm s.d.; differences were not statistically significant.

^bBC = β -carotene supplementation 20 mg/day.

^cAT = α -tocopherol supplementation 50 mg/day.

**Figure 2** β -carotene (BC) concentration (mg/kg protein) in buccal mucosal cells in subjects ($n = 173$) receiving BC supplementation 20 mg/day (BC) and in subjects ($n = 170$) without it (no BC), according to serum BC level (μ g/L). Open circles, subjects with leukoplakia without dysplasia ($n = 10$); solid circles, subjects with leukoplakia with dysplasia ($n = 7$).

the same age and had similar baseline serum BC concentrations as the other subjects. The BC concentration (log-transformed) in buccal cells was explained with a linear model where AT and BC treatment, BC serum concentration (log-transformed), dietary BC at baseline of the ATBC study, and the number of cigarettes smoked per day were used as explanatory variables. The regression equation explained 55% of variation.

Of 343 subjects, 17 (5%) had in their oral mucosae histopathologically diagnosed keratotic lesions clinically diagnosed as leukoplakia, 7 being keratosis with dysplasia and 10 without. The BC concentration in buccal mucosal cells of the 7 subjects with dysplasia in oral leukoplakia did not differ statistically significantly from the concentration in the 10 subjects showing leukoplakia without dysplasia, either among nonsupplemented or supplemented individuals (F -test; $P = 0.74$). In the supplemented group, mean (s.d.) BC concentration in the cells was 7.3 (4.1) mg/kg

protein in the men showing dysplasia and 6.4 (2.2) in those not showing dysplasia, and in the nonsupplemented group 2.6 (2.9) mg/kg protein and 0.9 (0.7), respectively. In the men not showing leukoplakia, cellular BC concentration was 7.7 (10.5) mg/kg protein in the supplemented group and 1.0 (1.7) mg/kg protein in the nonsupplemented group.

AT supplementation did not affect BC concentrations in buccal mucosal cells. Current smokers of more than 10 cigarettes/day had somewhat lower levels of BC in their buccal mucosal cells than did those who smoked less, irrespective of BC supplementation, a difference not statistically significant. Mean (s.d.) BC concentration in the cells of the men who had supplementation and smoked ≥ 10 cigarettes per day was 6.9 (0.8) mg/kg protein, and in the men who had quit smoking 9.3 (2.2) mg/kg protein. Smoking data were collected at time of buccal cell collection for analysis, and individuals who had stopped smoking for at least 6 months before oral examination were labelled quitters. Neither the amount of cigarettes per day nor alcohol intake (g/day) seemed to differ statistically significantly in subjects having dysplasia compared to the rest of subjects (F -test, $P=0.06$ and $P=0.67$, respectively), although the P -value of 0.06 for cigarettes/day was close to conventional significance level in this small sample.

Discussion

The β -carotene concentration in buccal mucosal cells was measured in 343 men, half of whom had taken a daily β -carotene supplement for 5–7 years. A small proportion of those receiving BC supplementation had substantially higher β -carotene concentrations in their mucosal cells than others. The BC concentration of buccal mucosal cells correlated with serum BC level in accordance with the findings of Peng *et al* (1995).

The reproducibility of the measurements was rather poor (mean CV 10.5%). However, the finding that there was a high BC concentration in the cells of some of our subjects is in accordance with the finding of Peng *et al* (1994), who obtained a similar result when measuring BC in the buccal mucosal cells of 154 subjects. These authors attributed their finding to personal use of antioxidant preparations by some subjects. Nevertheless, it is conceivable that some individuals may in fact accumulate much more BC in their mucosal cells than others.

Data on serum BC levels were obtained from the main ATBC study, in which serum BC was measured at baseline ($n=28\,920$), 3 years later ($n=22\,590$), and continuously in a random sample ($n=700-800$ per year). The BC contents of buccal cells were thus measured on a different occasion and later than was the serum level. It should be noted, however, that the ATBC study serum BC levels, after increasing about 17-fold within 4 months after the start of supplementation, remained constant throughout the supplementation period (ATBC Study Group, 1994b). In the study of Albanes *et al* (1992) serum BC increased 10-fold in individuals during a shorter period (2 months) of BC supplementation (20 mg/day).

The ATBC study was factorial in design, with half of both recipients and nonrecipients of BC also receiving AT, but the AT supplement (50 mg/day) did not affect the BC concentration of buccal cells. In the study by Willett *et al* (1983), AT supplementation (800 IU/day) caused a small decrease in plasma total carotenoids.

In the present study, the BC content of mucosal cells was not statistically significantly related to the current daily amount of smoking, although the tendency was in accordance with the finding of Davis *et al* (1983) of an inverse relationship between amount of smoking and serum BC concentration. BC concentration in buccal mucosal cells has not previously been compared with daily amount of smoking. Peng *et al* (1995) compared BC levels of buccal mucosal cells between smokers and nonsmokers, finding lower levels in smokers. It is possible, however, that BC concentration in buccal mucosal cells in the present study showed no significant association with daily amount of smoking because serum levels had been high for up to 6 years before the oral mucosa was sampled.

The BC concentration in cells was measured only once in the present study. Peng *et al* (1994, 1995) have demonstrated little intra-individual variation in repeated measurements. Furthermore, a few days' interval in taking the capsules probably does not affect the intracellular concentration, since BC has a serum half-life of about 2 weeks (Albanes *et al*, 1992). Our cell samples were immediately frozen and subsequently stored at -70°C . Studied by Nierenberg & Nann (1992) and Peng *et al* (1994) indicate that BC in cells remain stable during prolonged storage. Our BC dose (20 mg/day) corresponds to that used in most short-term studies. Gilbert *et al* (1990), Cooney *et al* (1991) and Peng *et al* (1994) used 30 mg/day, while the dose in the study of Stich *et al* (1986) was three times higher.

Potential confounding factors were effectively eliminated by design and analyses in the present study. Since the subjects were of uniform age (55–74 years) and all were males who lived in an urban environment, both randomization and multivariate analyses were applied. Furthermore, the dose of the antioxidant supplement is known to have been the same in all subjects, and the participants used no other antioxidant preparations (ATBC Study Group, 1994b).

Keratotic mucosal changes diagnosed clinically as leukoplakia were encountered in 5% of our 343 subjects. This prevalence of leukoplakia was in accordance with the finding (6%) of Axéll (1976) in a study among nearly 10 000 Swedish men from which 57% were tobacco users. BC has been presumed to be an effective protective agent against oral epithelial dysplasia (Garewal & Schantz, 1995). However, studies by Lippman *et al* (1993) and Liede *et al* (1998) suggest that BC supplementation may not, in spite of its possible leukoplakia-preventive effect, prevent oral epithelial dysplasia.

The other intervention agent in our trial, AT, has also been studied as a potential preventive regimen against oral leukoplakia and cancer. In animals it has prevented experimentally induced oral cancer (Shklar *et al*, 1987, 1990). In humans AT has reduced prevalence of oral leukoplakia when used either alone (Benner *et al*, 1993) or in combination with retinol plus β -carotene (plus riboflavin) (Zaridze *et al*, 1993). However, in a study by Kaugars *et al* (1994) many patients with leukoplakia unexpectedly showed oral carcinoma after treatment with AT plus BC plus ascorbic acid. Long-term low-dose (50 mg/day) AT supplementation seemed not to have any significant effect on the prevalence of oral leukoplakia in smokers in our previous study (Liede *et al*, 1998).

Based on the present study, the final answer to the role of mucosal cell BC concentration in smokers' oral dysplasia cannot be provided; the number of subjects should have

been in the thousands. However, we found no evidence to support an effect of cellular BC concentration on precancerous lesions among the few subjects having them in their oral mucosae.

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