EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C3 - Management of scientific committees II; scientific co-operation and networks

Scientific Committee on Food

SCF/CS/NUT/UPPLEV/37 Final

28 November 2000

Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Beta Carotene

(expressed on 19 October 2000)

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FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

ß-Carotene and carotenoids in general are isoprenoid compounds which are not synthesised in animals but biosynthesised by plants and micro-organisms. About 700 naturally occurring carotenoids have been identified so far. About 10% of them can be found in the human diet, and about 20 of these compounds have been found in plasma and tissues of the mammal. The predominant carotenoids observed in the plasma are ß-carotene, lycopene, lutein, ß-cryptoxanthin and α-carotene, accounting for more than 90% of the circulating carotenoids in humans (see Rock, 1997, for specific references).

Some dietary carotenoids, such as ß-carotene, serve as an important source of vitamin A, which is the major known function of carotenoids in humans. ß-Carotene is a hydrocarbon $C_{40}H_{56}$ that has a ß-ionone structure as the terminal ring system at each side of the poliene chain. Carotenoids containing at least one unsubstituted ß-ionone ring and a poliene chain are potential precursors of vitamin A. The preformed vitamin A is only present in animal products (e.g. liver, eggs, milk products), thus, in countries where the intake of animal products is low, carotenoids have to meet (i.e. by 80% or more in Asia and Africa) the vitamin A requirements. Even in developed countries carotenoids usually contribute to vitamin A supply by more than 40% (Woutersen *et al*., 1999).

The best-characterised natural functions of carotenoids are to serve as light-absorbing pigments during photosynthesis and protection of cells against photosensitization. Carotenoids provide considerable coloration and identification for many species, from vegetables to animals. In addition, carotenoids serve several other functions, such as radical quenching, antioxidant and anticarcinogenic activities in different animal sites and are regulators of cell function.

A number of reviews, monographs and comments on the safety of ß-carotene have been published during the last decade (e.g. Bauernfeind *et al*., 1981; Heywood *et al*., 1985; Rock, 1997; IARC, 1998; Omenn, 1998; Palozza, 1998; SCF, 1998; Woutersen *et al*., 1999). Very recently the SCF has set out the scientific data relevant to the safety of use of β-carotene from all dietary sources but limited their conclusions only to food additive uses (SCF, 2000).

2. NUTRITIONAL BACKGROUND AND METABOLISM

In the majority of industrialised countries, fruits and vegetables provide an estimated 2-3 mg/day of pro-vitamin A carotenoids, of which β-carotene is the principal component (Granado *et al.*, 1996). An approximate β-carotene intake of 1.8 mg/day in a randomly selected population of women in the USA has been reported (Chug-Ahuja *et al.*, 1993), the main dietary sources being carrots, orange juice, oranges, tomatoes and dark green leafy vegetables. The average intake in the German National Food Consumption Survey was 1.81 mg/day (Pelz *et al.*, 1998), mainly from carrots. An average β-carotene intake of 1.7-2.1 mg/day has been reported in Finland (Heinonen, 1991), and of 3.0 mg/day in The Netherlands (Goldbohm *et al.*, 1998). Levels of fruit and vegetable consumption, however, vary greatly between individuals and β-carotene intake may be much higher than average in people who regularly consume substantial amounts of foods such as carrots (Gregory *et al.*, 1990; Scott *et al.*, 1996). Some authors have reported that β-carotene intake varies according to seasonal factors, perhaps due to the differing availability of specific fruits and vegetables, or because of factors such as light and heat that may affect the carotenoid content of foods (Olmedilla *et al.*, 1994; Rautalahti *et al.*, 1993; Takagi *et al.*, 1990). The SCF has not recommended the consumption of β-carotene and carotenoids in general, beyond what is needed to supply vitamin A (SCF, 1993).

The Committee has not received detailed information on how much the use of ß-carotene as an additive contributes to the overall intake. However, unpublished data from a Danish and Austrian survey showed that present use levels (Elmadfa *et al.*, 1996; SCF, 1997), combined with the knowledge of the eating habits of the population, suggest an average exposure from ß-carotene, used as food additive, of about 1-2 mg/person/day.

The general mechanism of intestinal ß-carotene absorption in mammals is by passive diffusion of mixed micelles, which are formed during fat digestion in the presence of bile acids. In general, the types and amounts of carotenoids in the plasma reflect those in the diet. Depending on specific conditions, the extent of absorption for ß-carotene reported in the literature varies between 10% and 90% (see Woutersen *et al.*, 1999). Absorption appears to be linear up to intakes of 20-30 mg, but becomes limited at higher intakes. Limiting factors are dependent on the formulation or food matrix, the amount and type of fat co-ingested with the carotenoid and the presence of bile acids. Release from the food matrix into the lipid phase and solubilisation within mixed micelles appears to be the most critical steps in ß-carotene absorption. Dietary fibre and other meal components, together with a number of metabolic factors and subject characteristics (Rock, 1997) may also affect ß-carotene absorption. Important differences in the rates of absorption and intestinal cleavages have been demonstrated between man and laboratory animals (see section 2.1).

The main site of carotenoid metabolism is the intestinal mucosa, at least in rodents, but peripheral tissues such as lung, kidney, liver and fat of several mammals, including humans and rodents, can also convert ß-carotene to retinoic acid (RA) (Wang *et al.*, 1992; Redlich *et al.*, 1996).

ß-carotene can be cleaved in mammalian tissues mainly at the central double bond (C-15,15') yielding two molecules of retinal which may either be reduced to retinol (vitamin A) or further oxidised to RA; an alternative pathway (which can also yields RA, with or without the involvement of intermediate retinal) is the non-central (eccentric) cleavage at eccentric

double bonds (e.g. C-13',14', C-11',12', C-9',10' and C-7',8') (Krinsky *et al.*, 1990; Wang *et al.*, 1992; Wang *et al.*, 1999) to form retinoids and apo-ß-carotenoids, which have structures that are similar to retinoids, the function of these being largely unknown.

Carotenoids are transported in association with the lipoproteins, with a distribution highly correlated to that of cholesterol. Liver and adipose tissue are the main sites of carotenoid deposition. After absorption retinyl esters formed in the enterocyte are incorporated into chylomicrons, before they are secreted into the intestinal lymph and move into the blood stream. In the fasted state about 75% of the ß-carotene is bound to LDL and about 25% to HDL and VLDL. Circulating carotenoid concentrations are found to be lower in smokers than in non-smokers, due in part to the depletion of these compounds by components of cigarette smoke (Handelman *et al.*, 1996).

Carotenoids can act as antioxidants and free radical/reactive species scavengers (Tsuchiya *et al.*, 1993; Everett *et al.*, 1996; IARC, 1998; Omenn, 1998). *In vitro*, carotenoids efficiently quench excited molecules such as singlet oxygen and can scavenge peroxyl radicals; interactions with several other radicals have also been reported and a synergistic antioxidant protection by carotenoids with vitamins E and C has been shown (Edge and Truscott, 1997). The role *in vivo* and in humans is less clear (IARC, 1998; Palozza, 1998; Lambert, 1999). The switch from antioxidant to pro-oxidant behaviour can be, for example, a function of oxygen concentration (Edge and Truscott, 1997; Palozza, 1998). The pro-oxidant activity of ß-carotene has been demonstrated at a high partial pressure of oxygen; because this is highest in the outermost cells of the lung, these cells might be particularly subject to the pro-oxidant effect of ß-carotene (cited in Paolini *et al.*, 1999).

Other effects of carotenoids, which can be related to cancer prevention, are the enhancement of the immune response observed in some experimental models, which may be due to production of tumour specific antigens (IARC, 1998). In addition, carotenoids have been reported to modulate cytochrome P450 metabolism, inhibit arachidonic acid metabolism, inhibit chromosome instability and chromosome damage, influence apoptosis, and affect several other biological processes (see IARC, 1998, and text later on)

Part of the effects of ß-carotene can be mediated by the formation of retinoic acid (RA) that has a key function as a regulator of gene expression, morphogenesis, and growth in vertebrate embryos. Cellular responses to retinoids are generally mediated by two families of nuclear receptors (RARs and RXRs) (Chambon, 1996). Different retinoic acid receptor isotypes display a characteristic pattern of tissue distribution, RARα being the most ubiquitously distributed (Chambon, 1996). RARß plays an important role in lung development and has been proposed to have a tumour suppresser function in lung (Houle *et al.*, 1993). Primary lung tumours and lung cancer cell lines lack RARß expression, and such loss of expression may be an early event in lung carcinogenesis. RARß2 is the most abundant isoform in normal human lung tissue and restoration of RARß2 in a RARß-negative lung cancer cell line has been reported to inhibit tumorigenicity in nude mice (see references in Wang *et al.*, 1999).

2.1. Species differences in ß-carotene metabolism

Most laboratory animals break down ß-carotene in their intestine and thus absorb almost none intact. Hence, rodents have low serum carotenoid levels (about 1/1000 of human levels) that

are not related to dietary intake due to very active dioxygenase cleavage to retinal. In man, roughly 20-75% of the ß-carotene is absorbed intact (Wang *et al*., 1992; Rock, 1997).

Studies have thus indicated that the rat and mouse are not suitable models for studying the uptake of β-carotene into the plasma, with the possible exception of experiments using very high doses or a non-oral way of administration. Similarly, studies in hamsters showed that β-carotene concentrations remained low in animals given dietary β-carotene supplementation, although retinol levels increased, indicating that hamsters are also efficient converters of β-carotene to retinol (cited in IARC, 1998). Rabbits do not appear to absorb β-carotene well and these animals when fed a carotenoid-rich diet showed no carotenoids in the blood and only small increases in liver vitamin A concentrations (cited in IARC, 1998). Strict carnivores obtain a diet rich in pre-formed vitamin A and thus do not depend on provision via carotenoids in the diet. Indeed, cats reportedly lack the enzyme β-carotene-15,15'-dioxygenase and, thus, have a requirement for pre-formed vitamin A in the diet (Bauernfeind *et al*., 1981).

Ferrets (Gugger *et al*., 1992; Wang *et al*., 1992; White *et al*., 1993a; Rock, 1997; Wang *et al*., 1999), the pre-ruminant calf (Poor *et al*., 1992) and the Mongolian gerbil (Krinsky *et al*., 1990; Mathews-Roth, 1993) have been proposed as useful models for human ß-carotene absorption and cleavage as these animals also absorb and release intact ß-carotene from the enterocyte. The ferret studies are particularly relevant to the present report. This animal model partially mimics the absorption and tissue metabolism of ß-carotene in humans. It has been used for studies of tobacco smoking and inhalation toxicology (Sindhu *et al*., 1996), and also it has been used to test the hazard associated with a high dose of ß-carotene and tobacco smoking on lung (Wang *et al*., 1992; Wang *et al*., 1999). Although serum β-carotene levels are normally very low in these animals, dietary supplementation has been shown to increase concentrations to levels similar to those detected in human serum, and also to increase levels in the liver, adipose and other tissues (Ribaya-Mercado *et al*., 1989; Gugger *et al*., 1992; Ribaya-Mercado *et al*., 1992; Ribaya-Mercado *et al*., 1993; White *et al*., 1993a; White *et al*., 1993b; Wang *et al*., 1999). It has to be recognised that no single species provides a good model for studying all aspects of β-carotene in humans (van Vliet, 1996; IARC, 1998), but ferrets are particularly interesting as an example which allows reproducing (to some extent) the problem in the particular tissue (the lungs) pointed out by human trials.

3. HAZARD IDENTIFICATION

A number of epidemiological studies in humans and several animal studies developed during the last third of the past century support the idea that ß-carotene can prevent cancer, cardiovascular diseases and other diseases in humans. However human chemoprevention trials developed the last decade (see Section 3.2) have shown that supplemental ß-carotene actually increases both lung-cancer incidence and mortality in human smokers and, more recently, mechanisms which offer likely explanations of these adverse effects have been derived from experimental studies in appropriate animal models (see Section 3.3).

3.1. Animal studies

3.1.1. Standard toxicological studies

In summary, no adverse effects of high-dose oral β-carotene supplementation have been observed in several standard toxicological studies in various experimental animals (rat, mice, rabbits) (IARC, 1998; Woutersen *et al*., 1999). These studies included acute toxicity, up to 5000 mg/kg bw/day in Sprague Dawley rats (Woutersen *et al*., 1999) and up to 2000 mg/kg bw/day in Wistar rats (Buser, 1992; Strobel, 1994), chronic toxicity/carcinogenicity up to 1000 mg/kg bw/day for life in rats (Hummler and Buser, 1983; Heywood *et al*., 1985) or mice (Buser and Hummler, 1983a; Heywood *et al*., 1985), teratogenicity and reproductive toxicity (up to 1000 mg/kg bw/day for 3 generations, or during days 7 to 16 of gestation, in rats; up to 400 mg/kg bw/day during days 7 to 19 of gestation in rabbits) (Komatsu, 1971, cited in Kistler, 1981; Buser and Hummler, 1982; Heywood *et al*., 1985, and Woutersen *et al*., 1999).

In beagle dogs (Buser and Hummler, 1983b; Heywood *et al*., 1985) no toxic effects (up to 250 mg/kg bw/day for 2 years) were observed. However, this study, in addition to the problem of using a hydrosoluble formula, had a non-explained episode, at week 88 of the study (Buser and Hummler, 1983b), when a dramatic weight loss in dogs after withdrawing ß-carotene was observed.

However, the above studies were not aimed at investigating specific effects in the lung, which now we know appears to be the more sensitive tissue. In addition, species used are particularly unsuitable for oral studies, due to the high efficiency of conversion to vitamin A, such that no significant levels of unaltered β-carotene are absorbed and incorporated into the systemic circulation.

Genotoxicity and modulation of genotoxic effects of β-carotene has been previously reviewed (SCF, 2000), most studies giving negative findings. However there are no good experimental studies especially addressing the genotoxicity of ß-carotene *in vivo* and negative findings in studies designed to asses the anticlastogenic activity of ß-carotene do not provide conclusive evidence on the lack genotoxicity *in vivo*. Positive results obtained in a limited study with synthetic ß-carotene should be evaluated with caution but not dismissed, in view of the pro-oxidant activity of ß-carotene and the evidence of micronucleus induction *in vitro* by synthetic ß-carotene (Xue *et al*., 1998). The latter study also suggests that the genotoxicity *in vitro* of ß-carotene formulations can be modulated by their relative stereoisomer composition. These findings should be taken into account also in the evaluation of studies *in vivo*, which used samples of ß-carotene of different and/or unspecified composition. In summary, the data available are insufficient for an adequate evaluation of the genotoxicity of ß-carotene *in vivo.*

The majority of studies relating to carcinogenicity effects by β-carotene have indeed shown either preventive, or no effect, as it has been previously reviewed (SCF, 2000). However, in contrast to studies relating to tumours at other sites, only one report (Furukawa *et al*., 1999) has described an inhibitory effect of β-carotene supplementation on carcinogen-induced respiratory tract tumourigenesis. Supplementation of the diet with β-carotene at levels up to 0.25% (approximately 250 mg/kg bw/day), for 12 weeks, resulted in a significant reduction of the incidence of benign respiratory tract changes (hyperplasia and papillomas) in hamsters exposed to cigarette smoke (Furukawa *et al*., 1999). The majority of studies have shown no

effect of β-carotene supplementation on experimentally induced respiratory tract tumourigenesis in mice (Murakoshi *et al*., 1992; Nishino, 1995; Yun *et al*., 1995), or hamsters (Beems, 1987; Moon, 1994; Wolterbeek *et al*., 1995).

Two reports in hamsters (Beems, 1987; Wolterbeek *et al*., 1995) and one in ferrets (Wang *et al*., 1999), which have been recently reviewed by the SCF (2000), describe potential enhancement of chemically-induced respiratory tract tumourigenesis, although statistically significant increases in the incidences of malignant tumours have not been reported. The study in ferrets, which was specifically designed to mimic the human trials, is described below.

Study in ferrets

Wang *et al*. (1999) used a ferret model to assess the single or combined effects of cigarette smoke and/or β-carotene supplementation on lung histopathology/biochemistry.

To mimic human trials, by correcting for species differences in ß-carotene absorption, Wang *et al*. (1999) fed ferrets with 2.4 mg ß-carotene/kg per day (15 times higher than the 0.16 mg of ß-carotene/kg per day for the control group fed a low ß-carotene diet). This dose mimics an intake equivalent to 30 mg of ß-carotene per day in a 70-kg human. It was shown that: 1) the plasma level of ß-carotene in the ferrets had a similar increase (17-22 fold) to that observed in human trials (see Section 3.2.2); 2) tissue levels of ß-carotene, retinol and RA in control ferrets were within the range found in the normal human, although this was not the case for the higher plasma levels of retinyl esters in the ferret; 3) the lung architecture and formation of oxidative metabolites from ß-carotene were considered similar in both species (references listed in Wang *et al*., 1999), and 4) the concentration of urinary cotinine equivalents in the smoke-exposed ferrets was similar to that found in humans smoking 1.5 packs of cigarettes per day.

Four groups of 6 males were treated with either 2.4 mg/kg bw/day β-carotene supplementation (in corn oil, fed orally), cigarette smoke exposure (smoke from 10 cigarettes, in a chamber, for 30 minutes, twice daily), both, or neither, for a period of 6 months, at which point they were killed (Wang *et al*., 1999). Histopathological analysis revealed that all β-carotene treated animals showed an increase in cell proliferation and squamous metaplasia in lung tissue, and this was further enhanced in the animals that were also exposed to cigarette smoke. Animals exposed to cigarette smoke alone did not show these changes. The assessed histopathological endpoint, squamous metaplasia, may not be directly related to carcinogenesis, but this study did reveal interestingly related molecular/biochemical changes in the lungs of the animals tested which are discussed later in this report (see Section 3.3).

3.2. Human studies

In humans, doses of 20-180 mg/day ß-carotene have been used to treat patients with erythropoietic photoporphyria, with no evidence of toxicity and without the development of abnormally elevated blood vitamin A.

A substantial amount of epidemiological information linking higher carotenoid intake with lower cancer incidence was accumulated in the 1970s and 1980s. Also noted was the apparent lack of toxicity of ß-carotene in high-dose clinical use against erythropoietic photoporphyria (doses of 20-300 mg/day given for many years) (Mathews-Roth, 1993;

Meyers *et al*., 1996). Thus, these facts, together with the known biological properties of ß-carotene (see above), combined to justify large-scale, cancer prevention trials in humans. However, these trials did not confirm the positive expectations.

3.2.1. Epidemiological studies

3.2.1.1. ß-carotene and incidence of cardiovascular disease

A number of descriptive, cohort and case-control studies have been reviewed (IARC, 1998; Woutersen *et al*., 1999), suggesting that carotenoid and/or ß-carotene rich diets may prevent cardiovascular disease. Recently, the Rotterdam 1999-Study in the elderly (Klipstein-Grobusch *et al*., 1999) confirmed a protective association. However, the finding in numerous observational studies that increased intake of carotenoid-containing diets and higher blood concentrations of carotenoids are associated with reduced risks for cardiovascular disease cannot be interpreted as a specific protective effect of ß-carotene or other carotenoids *per se*.

In the ATBC study (ATBC Study Group, 1994), 11% more total cardiovascular death was seen in men taking ß-carotene. When the analysis was restricted to the 1862 participants who had previously had an MI, men who received ß-carotene alone had relative risks of 1.75 for fatal coronary heart disease and 3.44 for fatal MI. Similarly, an increased number of deaths from cardiovascular disease was seen in the CARET study (Omenn *et al*., 1996a; Omenn, 1998) among men taking supplemental ß-carotene plus retinol (relative risk of 1.26).

ß-carotene and cancer incidence

A number of reviews published in the 1990s have summarised the research on diet and lung cancer during the preceding 25 year period (see Steinmetz *et al*., 1996, and Ziegler *et al*., 1996). The consensus was that observational studies of diet and lung cancer, whether prospective or retrospective, consistently demonstrated reduced risk with increased intake of carotenoids from vegetables and fruits. Further, high levels of ß-carotene in the blood were consistently associated with reduced incidence of lung cancer in prospective studies. The simplest explanation of the epidemiology was that ß-carotene was protective, although other carotenoids or other compounds from vegetables and fruits, and associated dietary patterns had not been adequately explored.

The observational data suggesting cancer preventive effects are most consistent for some types of cancer -lung, oral, pharyngeal and stomach- (IARC, 1998; Woutersen *et al*., 1999), the incidence of which tends to be inversely related to ß-carotene intake or blood concentrations. A review that summarised results from 206 human epidemiological studies confirmed the evidence for a protective effect of greater vegetable and fruit consumption against cancers of the stomach, oesophagus, lung, oral cavity and pharynx, endometrium, pancreas and colon (Steinmetz *et al*., 1996). The types of vegetables or fruit that most often appeared were raw vegetables, allium vegetables, carrots, green vegetables, cruciferous vegetables and tomatoes. A number of interesting substances present in these foods include dithiolthiones, isothiocyanates, indol-3-carbinol, allium compounds, isoflavones, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, vitamin C, D-limonene, lutein, folic acid, ß-carotene, lycopene, selenium, vitamin E, flavonoids and dietary fibre (Steinmetz *et al*., 1996).

A recent case-control study in Greece (Bohlke *et al*., 1999) involved 820 women with histologically confirmed breast cancer who were compared with 1548 control women.

Among postmenopausal women there were no associations between any of the micronutrients evaluated and the risk of breast cancer. Among premenopausal women, ß-carotene, vitamin C and vitamin E were each inversely associated with breast cancer, but after mutual adjustment among the three nutrients only ß-carotene remained significant.

In conclusion, the general assumption is confirmed that individuals who eat more fruits and vegetables, rich in carotenoids, and/or have high levels of serum ß-carotene, have a lower risk for cancer and cardiovascular diseases. However, a possibility could be that ß-carotene may be only a marker of the intake of other beneficial substances in fruits and vegetables, or perhaps other life-style habits. Actually (see below) no clinical trial of ß-carotene as a single agent, has shown a reduction in the risk of cancer at any specific site. On the contrary there is evidence of an increase in the risk for lung cancer among smokers and asbestos workers receiving ß-carotene supplements at high doses, which resulted in blood concentrations an average of 10-15 times higher than normal.

ß-carotene and other diseases

Erythropoietic protoporphyria (EP) (Mathews-Roth, 1993) is a genetic disease of porphyrin metabolism, characterised by abnormally elevated concentrations of protoporphyrin, which acts as an endogenous photosensitizer. As carotenoids can interact and quench photosensitizer triplet states and single oxygen, their efficacy in this disorder appears to be a consequence of the quenching of excited species. Most patients with EP or other photosensitivity diseases benefit from recommended doses for adults of about 180 mg/day, with no serious side effects and no long-term toxicity reported. These photosensitivity diseases are the only current therapeutic use of carotenoids.

Dietary carotenoids have been suggested to reduce the risk of age-related macular degeneration (Seddon *et al*., 1994; Cooper *et al*., 1999), the most common cause of irreversible blindness in people over age 65 in western countries.

Senile cataract is another ocular condition potentially related to oxidation, and ß-carotene has been studied for a possible role in the prevention of this disorder. However the available results are somewhat inconsistent. Carotenoids have also been suggested to be of benefit for several other health outcomes (such us ageing, impaired cognition, rheumatoid arthritis and cystic fibrosis), however the data are scant (IARC, 1998).

3.2.2. Selection of critical data. Prevention trials in humans with ß-carotene supplementation

Six major prevention trials with ß-carotene supplementation have been completed so far (Greenberg *et al*., 1990; Blot *et al*., 1993; ATBC Study Group, 1994; Greenberg *et al*., 1994; Hennekens *et al*., 1996; Omenn *et al*., 1996a). Short-term trials using sputum as a presumed intermediate endpoint were conducted as well with some preliminary promising results (see Omenn, 1998). However, results from the majority of clinical trials reported are not in support of using ß-carotene supplementation as a mean to reduce cancer and cardiovascular disease rates.

The first study (Greenberg *et al*., 1990) showed that supplementation with 50 mg ß-carotene/day for 5 years had no effect on the occurrence of new basal-cell or squamous-cell carcinoma in well nourished patients who had skin cancer previously. However, a 12-year latency period for these cancers diminished the value of these results.

In a second study (Greenberg *et al*., 1994), ß-carotene (25 mg/day), with or without vitamin C (1g/day) and α-tocopherol (400 mg/day) for 5-8 years, was not found to reduce the occurrence of colorectal adenoma in patients who had a prior history of adenomas.

A lack of effect of long term supplementation with ß-carotene on the incidence of malignant neoplasms and cardiovascular disease was reported in 1996 (Hennekens *et al*., 1996).

The Tyler asbestos cohort studied 755 randomised asbestos workers (McLarty, 1992) at Tyler (Texas), receiving 50 mg of ß-carotene together with 25,000 IU retinol/day or placebos. There was no difference in the two groups by criteria of sputum atypia. The ß-carotene was obtained from BASF and it is thought that the 50 mg dosage is almost equivalent to 30 mg of Roche-ß-carotene.

Two notable trials (Blot *et al*., 1993; Li *et al*., 1993) were conducted in China (The Linxian Trials) but they were very complex in design and difficult to compare with the findings (see below) in western populations: the observed effects cannot be directly attributed to ß-carotene supplementation as a combined supplementation was given and low population nutrient intake was interfering.

3.2.2.1. The Alpha-Tocopherol/Beta-Carotene (ATBC) Trial in Finland

The ATBC trial (ATBC Study Group, 1994) involved 29,133 male smokers (age 50-59) with a smoking history averaging one pack/day for 36 years. The 2x2 factorial design evaluated 20 mg ß-carotene (from Roche) and/or 50 IU alpha-tocopherol (vitamin E) daily for 6.5 years. These doses represent a 10-fold and 5-fold excess over the median intake of ß-carotene and α-tocopherol, respectively, in this population. After 2 years of treatment, median serum ß-carotene levels had increased 17.5-fold in the ß-carotene treatment groups.

Results were unexpected. Vitamin E supplementation did not reduce the incidence of lung cancer (relative risk (RR) was 0.98). Participants receiving ß-carotene alone or in combination, had significantly higher lung cancer incidence (RR 1.18; 95%CI 1.03-1.36) and higher mortality (RR 1.08; CI 1.01-1.16) than subjects receiving placebo.

The excess lung cancer incidence was not apparent in the initial 18 months, but the incidence curves significantly diverged thereafter. Subsequent subgroup analysis (see Albanes *et al*., 1996) revealed a higher risk in heavy smokers (20 or more cigarettes/day) (RR 1.25, CI 1.07-1.46) than in light smokers (5-19 cigarettes/day) (RR 0.97, CI 0.76-1.23). Associations with alcohol intake and with non-small-cell histology were also noted. The risk was confined to the heavier drinkers (more than 11 g ethanol per day).

Interestingly, in agreement with earlier observational studies, both dietary intake and serum ß-carotene levels at baseline (before treatment) were found to be inversely related to risk of lung cancer during the trial (Albanes *et al*., 1996).

3.2.2.2. The ß-carotene and Retinol Efficacy Trial (CARET) in the USA

The CARET study (Omenn *et al*., 1996a; see also Omenn *et al*., 1996b, and Omenn, 1998) successfully randomised 18,314 participants. 30 mg ß-carotene and 25,000 IU vitamin A (retinyl palmitate) were administered daily to 14,254 smokers and former smokers (45%

female) aged 50-59 at enrolment, and to 4,060 asbestos-exposed males (age 45-74). After five years of study the median serum ß-carotene levels in the active treatment group was increased by 12-fold (170 ng/ml *versus* 2100 ng/ml).

A total of 388 new cases of lung cancer were diagnosed during the 73,135 person-years of follow- up (mean 4.0 years). The active treatment group had a RR of lung cancer of 1.28 (CI 1.04-1.57), compared with the placebo group. The differences (significant from 24 months of treatment onwards) were greater as the intervention progressed. There were no statistically significant differences in the risks of other types of cancers.

In the active group the RR of death from any cause was 1.17, of death from lung cancer, 1.46, and of death from cardiovascular disease, 1.26.

As in a further analysis from ATBC published in the same issue (Albanes *et al*., 1996), there was an association (less clear trend than in ATBC study) of the excess lung cancer incidence between treatment groups with the highest quartile of alcohol intake, but no association with baseline serum ß-carotene concentrations.

In the CARET study it is not possible to distinguish the ß-carotene effects from those of the vitamin A, since the two compounds were administered in combination.

3.2.2.3. Physicians Health Study

This trial was to test the effect of aspirin on cardiovascular disease incidence (Steering Committee of the Physicians' Health Study Research Group, 1989). ß-carotene was added in a 2x2 design, using 50 mg BASF ß-carotene on alternate days. 22,071 male physicians were followed for a mean of 12.5 years. Those assigned to receive ß-carotene had significantly higher serum concentrations than those given placebo (2240 nmol/l *vs* 560 nmol/l) (4-fold). It has to be noted that this increase is lower compared with that obtained in the two previously considered trials, a situation that could be related to higher basal levels in the PHYS population and/or to a lower bioavailability of ß-carotene compared with the other trials.

In this healthy population, with 50% never-smokers and only 11% current smokers, 170 lung cancers were accumulated over the follow up period. The relative risks were 1.02 (CI 0.93-1.11) for overall mortality, 0.98 (CI 0.91-1.06) for all malignant neoplasms, and 0.93 for lung cancer.

In summary there was no effect of ß-carotene supplementation on total cancer, on total mortality, or on heart disease. Neither was an effect on lung cancer observed, but due to the lower number of cases, the power of the statistical analysis underlying this conclusion is rather weak.

3.3. Mechanisms

In light of the adverse findings in human intervention trials, in which β-carotene supplementation was associated with a promotional effect on lung tumourigenesis in smokers, studies in animals have been carried out to elucidate potential mechanisms by which these effects may have occurred.

Mechanisms have been proposed, which are related to effects in the same target tissue, the lungs, where the adverse effects have been observed in humans.

3.3.1. Effects on P450-related activities. A mechanism for a hypothetical co-carcinogenic effect of ß-carotene

Perocco *et al.* (Perocco *et al.*, 1999) first reported that β-carotene enhanced the transforming effect of benzo(α)pyrene (B[α]P) and cigarette-smoke condensate (tar) on mouse BALB/c 3T3 cells in an *in vitro* cell transformation assay, although β-carotene alone was not transforming in this system. The authors suggested that β-carotene may exert its effects by inducing P450 activities (in particular CYP 1A1/2), with a consequent increase in the metabolism of cigarette smoke constituents. Interestingly, however, β-carotene showed no capacity to enhance the transforming activity of 3-methylcholanthrene (3-MCA), which also requires metabolic activation by CYP1A1.

The same group (Paolini *et al.*, 1999) found that dietary supplementation of rats with 500 mg/kg bw/day β-carotene for 5 days significantly increased lung enzyme activities associated with CYP1A1 and 1A2 (activating aromatic amines, polychlorinated biphenyls, dioxins and PAHs), CYP2A (activating butadiene, hexamethyl phosphoramide and nitrosamines), CYP2B1 (activating olefins and halogenated hydrocarbons) and CYP3A (activating aflatoxins, 1-nitropyrene and PAHs). The authors postulated that these powerful booster (stimulating) effect on phase I carcinogen-bioactivating enzymes might explain why ß-carotene supplementation increases the risk of lung cancer in smokers, probably due to the co-carcinogenic properties of β-carotene and it's capacity to generate oxidative stress.

Other studies (Astorg *et al.*, 1994; Astorg *et al.*, 1997, Basu *et al.*, 1987, Gradelet *et al.*, 1996) showed no ß-carotene enhanced effects on phase I or phase II xenobiotic-metabolising enzymes, but measurements were made in the liver and not in lung tissue (SCF, 2000).

3.3.2. Altered retinoid signalling: a mechanism to enhance lung tumourigenesis after high dose ß-carotene supplementation in smokers

When ferrets (animals that metabolise ß-carotene in much the same way as humans) were given ß-carotene doses equivalents to those used in the clinical trials, changes in ß-carotene metabolism were induced that may promote rather than inhibit tumourigenesis (Wang *et al.*, 1999). This may explain why high-dose ß-carotene supplements unexpectedly increased lung cancer rates in the two cancer human prevention trials.

Ferrets were given a ß-carotene supplement, exposed to cigarette smoke, or both for 6 months (see also Section 3.1.2). Cell proliferation and squamous metaplasia in lung tissue were assessed by examination of proliferating cell nuclear antigen expression and histopathological examination, respectively. ß-carotene and retinoid concentration in lung tissue and plasma were analysed. Expression of genes for retinoic acid receptors (RARs) and activator protein-1 (encoded by c-jun and c-fos genes) in lung tissue specimens was examined. The results clearly showed that a strong proliferative response in lung tissue was observed in all ß-carotene-supplemented animals, and this response was enhanced by exposure to tobacco smoke. The treatment groups had statistically significant lower levels of retinoic acid in lung tissue, and they exhibited 18-73% reductions in RARß-gene expression, without reduction of RARα and RARγ. Ferrets given a ß-carotene supplement and exposed to tobacco smoke had threefold to fourfold elevated expression of the c-jun and c-fos genes.

Decreased lung concentration of retinoic acid may cause diminished retinoic signalling, enhanced lung cell proliferation, and potential tumour formation. Results showed that localised keratinised squamous metaplasia (a precancerous lesion) was observed in all ferrets in the high-dose of ß-carotene, with or without exposure to smoke. Retinoic acid levels are lowered in lung tissue as a result of ß-carotene supplementation, in spite of having increased levels of ß-carotene (by 300 fold). The possibility that some of the eccentric cleavage products of ß-carotene could act as a ligand and interfere with RA requires further investigation. Thus it is possible that ß-carotene supplementation in itself might modify ß-carotene metabolism. Reduction of retinoic signalling could occur after induction of cytochrome P450 enzymes (see section 3.3.1), perhaps by the ß-apo-8'-carotenal (increased by 2.5-fold by smoke exposure).

It can be deduced from the preceding study that diminished retinoid signalling, resulting from suppression of RARß gene expression and overexpression of activator protein-1 could be a mechanism to enhance lung tumourigenesis after high dose ß-carotene supplementation and exposure to tobacco smoke. However, a relationship between the endpoint studied (squamous metaplasia) and lung cancer has not been demonstrated, and the dose-response relationship was not studied. Nevertheless, lung carcinogenesis is associated with an alteration in retinoid signalling involving the AP-1 complex, which mediates the signal from growth factors, inflammatory peptides, oncogenes, and tumour promoters, usually resulting in cell proliferation. AP-1 (c-fos, c-jun) transcriptional activity can be inhibited by RA treatment, thus contributing to the suppression of human bronchial epithelial squamous metaplasia.

In contrast to what occurs with high doses of ß-carotene (even more when smoking), if low levels of ß-carotene are ingested, eccentric cleavage products are produced by the cells (as would be the case when one consumes ß-carotene from a carotenoid enriched diet). This form of carotenoid intake could be beneficial by giving rise to some RA.

Adverse effects of high dose supplemental ß-carotene (alone) cannot be ruled out. The intervention trial (Hennekens *et al.*, 1996) which did not include many smokers, and that did not reveal any increase in incidence of cancer or death, can not be considered conclusive, because precancerous lesions (analogous to those observed in ferrets under high ß-carotene intake) were not considered. They should be further analysed to deduce more definitive conclusions.

3.3.3. The pro-oxidant activity of ß-carotene

The pro-oxidant activity of β-carotene has also been considered as an hypothetical mechanism in lung toxicity (SCF, 2000), taken into account that the relative high partial oxygen pressure in the lung may shift the antioxidant activity of carotenoids into pro-oxidant activity. However, further studies of the pro-oxidant role of carotenoids *in vivo* and *in vitro* will help in testing hypothesis relating to the influence of these compounds in the development of human chronic diseases. In any case, the pro-oxidant activity of β-carotene can be part of the other mechanisms as they are not mutually exclusive.

4. DOSE-RESPONSE ASSESSMENT

No dose-response relationship for ß-carotene effects is available from the intervention trials in humans, as single doses were used in each study, and the conditions were different in the different studies.

The study in ferrets also used a single daily dose. Further studies in ferrets using a range of different ß-carotene doses and a wider range of selected parameters would be appropriate to assist in future toxicological evaluation.

It can be presumed that the effects of ß-carotene are dependent on the specific source of exposure, and that differences will not be unexpected with different matrices or different formulations containing ß-carotene, depending on the composition of accompanying antioxidants and of other components, and also depending on the relative proportion of isomers of ß-carotene. Natural ß-carotene preparations differ from synthetic all-*trans*-ß-carotene in the relative proportion of *trans/cis* isomers. From preliminary studies (see Section 3.1.3), the isomeric form appears important in the genotoxicity and antigenotoxicity of ß-carotene. However, at present there is insufficient information to establish the role of all these factors.

5. DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

Existing evidence from human trials indicates that supplemental ß-carotene (20 mg/day or more) is contraindicated for use in current, heavy smokers. However, there is insufficient scientific basis to set a precise figure for an UL of isolated ß-carotene as no dose-response relationship for ß-carotene effects is available either from the intervention trials in humans or from appropriate animal models. Moreover, it is not possible to be more specific in distinguishing different isomeric forms of ß-carotene or specific formulations.

6. CHARACTERISATION OF RISK

Three general ß-carotene sources can be considered (SCF, 1997): a) natural food sources that may contribute around 2-5 mg/European person/day, b) food additives (1-2 mg/person/day), and c) supplements. The combination of a) and b) sources represents about 3-7 mg/day (or up to 10 mg/day depending on seasonal and diet variations) of ß-carotene exposure. Thus, there may be a very small difference between the levels that may confer health benefits (up to 10 mg/d, mainly from natural sources) and those that may produce adverse effects in smokers in the general population (20 mg/day in the ATBC study). In this situation it seems that the use of ß-carotene as a supplement should be regarded cautiously.

On one hand, human chemoprevention trials carried out in the last decade have shown that all-*trans*-ß-carotene actually increases both lung-cancer incidence and mortality in human smokers and, more recently, mechanisms which offer likely explanations of these adverse effects have been derived from experimental studies in appropriate animal models.

On the other hand, a number of reviews have summarised the research on diet and lung cancer in humans during the preceding 30 year period. The consensus is that they consistently demonstrated reduced risk of lung cancer, with increased intake of vegetables and fruits rich

in carotenoids. Further, high levels of ß-carotene in the blood were consistently associated with reduced incidence of lung cancer. However, these effects can not be attributed to β-carotene as the role of other carotenoids or other compounds from vegetables and fruits, and associated dietary or life style patterns, has not been adequately explored in the epidemiological studies.

Thus, the general assumption that individuals who eat more fruits and vegetables, rich in carotenoids, and/or have high levels of serum ß-carotene have a lower risk for cancer and cardiovascular diseases cannot be extended to specific formulations of ß-carotene.

7. RECOMENDATIONS FOR FURTHER WORK

The lung appears to be the target tissue for future investigations to address the adverse effects of ß-carotene. This tissue is were the tumourigenic effect of ß-carotene was observed in human trials, it depends on ß-carotene metabolites for the regulation of its growing cells, and is where a highest oxygen partial pressure is present, thus potentially enhancing the pro-oxidant properties of ß-carotene.

The ferret (and perhaps other animals that are able to absorb ß-carotene in its intact form), could be suggested as an appropriate model for studying the role of oral ß-carotene in lung carcinogenesis, provided that differences in the total absorption of ß-carotene are taken into account. This would allow establishing dose-response-related effects and/or likely mechanisms for the effects of ß-carotene.

The beneficial effects of diets rich in vegetables and fruits containing carotenoids and other compounds need to be further studied, to be able to set out specific effects of any of single chemical or combination of compounds.

Further study of the data already collected in the developed and ongoing human trials is recommended. Also studies on the biological effects of supplemental ß-carotene from different sources appears of particular interest.

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