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Dietary carotenoids inhibit aflatoxin B_1-induced liver preneoplastic foci and DNA damage in the rat: Role of the modulation of aflatoxin B_1 metabolism

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To study the effects of carotenoids on the initiation of liver carcinogenesis by aflatoxin B_1 (AFB_1), male weanling rats were fed β-carotene, β-apo-8'-carotenal, canthaxanthin, astaxanthin or lycopene (300 mg/kg diet), or an excess of vitamin A (21 000 RE/kg diet), or were injected i.p. with 3-methylcholanthrene (3-MC) (6 × 20 mg/kg body wt) before and during i.p. treatment with AFB_1 (2 × 1 mg/kg body wt). The rats were later submitted to 2-acetylamino-fluorene treatment and partial hepatectomy, and placental glutathione *S*-transferase-positive liver foci were detected and quantified. The *in vivo* effects of carotenoids or of 3-MC on AFB_1-induced liver DNA damage were evaluated using different endpoints: liver DNA single-strand breaks (SSB) induced by AFB_1, and *in vivo* binding of [^{3}H]AFB_1 to liver DNA and plasma albumin. Finally, the modulation of AFB_1 metabolism by carotenoids or by 3-MC was investigated *in vitro* by incubating [^{14}C]AFB_1 with liver microsomes from rats that had been fed with carotenoids or treated by 3-MC, and the metabolites formed by HPLC were analyzed. In contrast to lycopene or to an excess of vitamin A, both of which had no effect, β-carotene, β-apo-8'carotenal, astaxanthin and canthaxanthin, as well as 3-MC, were very efficient in reducing the number and the size of liver preneoplastic foci. In a similar way as 3-MC, the P4501A-inducer carotenoids, β-apo-8'-carotenal astaxanthin and canthaxanthin, decreased *in vivo* AFB_1-induced DNA SSB and the binding of AFB_1 to liver DNA and plasma albumin, and increased *in vitro* AFB_1 metabolism to aflatoxin M_1, a less genotoxic metabolite. It is concluded that these carotenoids exert their protective effect through the deviation of AFB_1 metabolism towards detoxication pathways. In contrast, β-carotene did not protect hepatic DNA from AFB_1-induced alterations, and caused only minor changes of AFB_1 metabolism: seemingly, its protective effect against the initiation of liver preneoplastic foci by AFB_1 is mediated by other mechanisms.

Introduction

Since the hypothesis of Peto *et al.* (1) in 1981 that β-carotene might reduce the incidence of cancer, evidence has accumulated from epidemiologic studies, both prospective and retrospective, that people eating more fruits and vegetables rich in carotenoids, or having higher blood concentrations of β-carotene, had a lower risk of developing cancer, especially lung cancer (2,3). At the same time, numerous experimental studies on animal models demonstrated that β-carotene, and also canthaxanthin, a carotenoid without provitamin A activity, could inhibit, attenuate or delay the onset of chemical- or UV-induced cancers in various target tissues (4,5). Moreover, the lack of toxicity of β-carotene (6) allowed its safe use as dietary supplement. β-Carotene was thus considered an outstanding candidate for cancer chemoprevention, especially for lung cancer, and large-scale intervention studies have been conducted in different countries, in which β-carotene was administered alone or in combination with other nutrients. Unfortunately, three of these studies (7–9) failed to detect any protective effect of β-carotene, or of a combination of β-carotene and vitamin A, on the incidence of diverse cancers, and two of these studies (7,8) even showed that these compounds could *increase* the incidence of lung cancer, a result that was opposite to what was expected. However, as these two studies were conducted in 'at risk' populations for lung cancer (smokers, ex-smokers or people exposed to asbestos), and the diet supplements were given at a stage when lung carcinogenesis was likely to have already reached the later phases, their negative results cannot be generalized to the whole population, and should not definitely rule out the possible role of β-carotene in cancer prevention, especially in the early phases of carcinogenesis. Last, many carotenoids other than β-carotene exist in the human diet, and can exert effects different from those of β-carotene.

Although their antioxidant properties and their provitamin A activity have often been considered as clues to their protective effects, the mechanisms by which carotenoids act on carcinogenesis are only partially known. Carotenoids enhance gap-junction cellular communications (10) and stimulate the immune system (11), which could explain their action on the phases of promotion and progression of carcinogenesis. The possible effects of carotenoids on cancer initiation have been supported by the demonstration of their anti-genotoxic properties, both *in vivo* and *in vitro* (4,12), but little is known about how they act. Despite the fact that the modulation of carcinogen metabolism is one of the most important ways by which many protective components prevent the action of indirect carcinogens (13,14), the effects of carotenoids on xenobiotic-metabolizing enzymes (XME*) have been rarely studied. Only recently (15–17), we have shown that two non-provitamin A oxocarotenoids, canthaxanthin and astaxanthin, and a provitamin A apocarotenoid, β-apo-8'-carotenal, are powerful inducers of the 1A1 and 1A2 isozymes of cytochrome P450, as well as 4-nitrophenol-uridinediphosphoglucuronosyl-transferase (4NP-UGT) in rat liver, an inducing profile similar to that of classical inducers such as 3-methylcholanthrene (3-MC) or β-napthoflavone (BNF). Moreover, we have shown

*Abbreviations: 2-AAF, 2-acetylaminofluorene; AFB_1, aflatoxin B_1; AFM_1, aflatoxin M_1; AFQ_1, aflatoxin Q_1; AFP_1, aflatoxin P_1; AFB_{2a}, aflatoxin B_{2a}; BNF, β-napthoflavone; CYP, cytochrome P450; DMSO, dimethylsulfoxide; GST-P, glutathione *S*-transferase, placental form; 3-MC, 3-methylcholanthrene; 4NP-UGT, 4-nitrophenol-uridine diphosphoglucuronosyltransferase; RE, retinol equivalents; ROS, reactive oxygen species; SSB, single-strand breaks; XME, xenobiotic-metabolizing enzymes.

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that cytochrome P450 1A (CYP1A) induction by these carotenoids was mediated by the Ah receptor (18). In contrast with these three carotenoids, feeding rats with β-carotene induced no change of their liver XME (15,17). Aflatoxin B_1 (AFB_1) is a powerful hepatocarcinogen having a complex metabolism that involves several cytochrome P450 isozymes and transferases, such as UGT and glutathione transferases (19,20). Its mutagenic, genotoxic and carcinogenic action can be modulated by effectors of these enzymes (19,21). In particular, CYP1A inducers, such as 3-MC, indole-3-carbinol or BNF, considerably alter AFB_1 metabolism (21–23) and exert an efficient protection against AFB_1 mutagenicity (22), carcinogenicity and genotoxicity in rats or trouts (24–26). The CYP1A inducer carotenoids (canthaxanthin, astaxanthin and β-apo-8'-carotenal) are thus potential chemopreventive components against AFB_1.

The present work was designed to study the preventive effects of several provitamin A (β-carotene, β-apo-8'-carotenal) or non-provitamin A (canthaxanthin, astaxanthin, lycopene) carotenoids (Figure 1) on the appearance of liver preneoplastic foci initiated by AFB_1 in rats, using the Solt and Farber's resistant hepatocyte model of hepatocarcinogenesis (27), in which the carotenoids were added to the diet during the initiation phase, i.e. before and during the administration of AFB_1.We have also looked, as a comparison, at the effects of treating the rats with an excess of dietary vitamin A or with 3-MC. As binding to DNA is the first event leading to initiation, we investigated the effects of these treatments on the *in vivo* binding of AFB_1 to liver DNA was investigated. The effects of these treatments on AFB_1-induced liver DNA single-strand breaks (SSB) was sought because transient DNA SSB arise in a second stage as a consequence of repair, and are also an important marker of genotoxicity. Finally, the way by which AFB_1 is metabolized *in vitro* by liver microsomes from rats fed with carotenoids or treated by 3-MC was investigated.

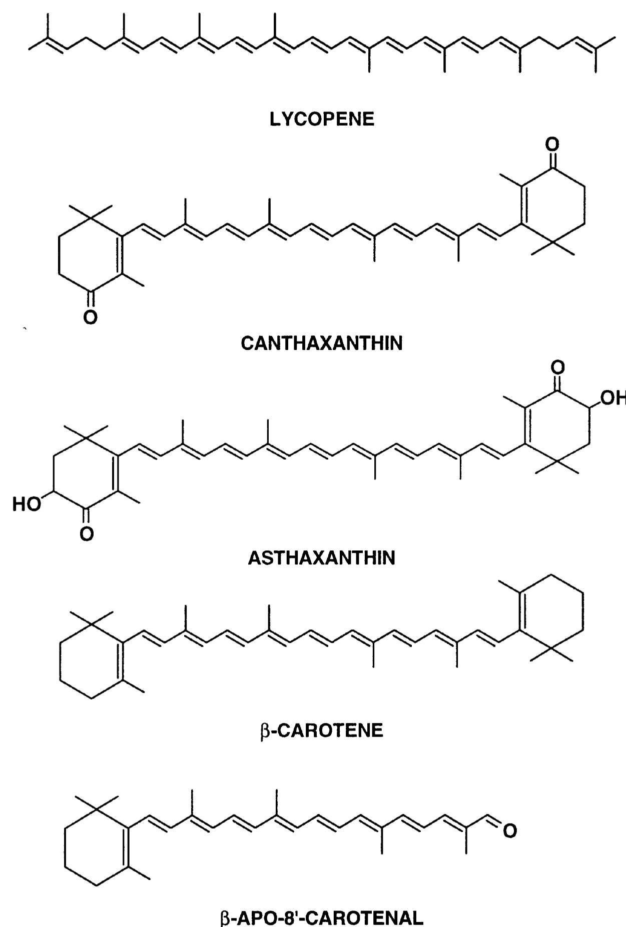

Fig. 1. Structure of the carotenoids studied.

Materials and methods

Chemicals

Commercial synthetic carotenoid preparations from Hoffmann–La Roche (Basel, Switzerland) were used for administration to rats: 10% β-carotene and 10% canthaxanthin cold water-dispersible powders, 8% astaxanthin powder and 20% β-apo-8'-carotenal oil suspension. A placebo powder (containing all ingredients except carotenoid, i.e. sucrose, corn starch, gelatin, ascorbyl palmitate, vegetable oil, DL-α-tocopherol) was also provided by Hoffmann–La Roche. A 5% lycopene oleoresin from tomato, in vegetable oil, was provided by Makhteshim Chemical Works (Beer-Sheva, Israël). According to the manufacturer's analysis, lycopene, mainly all-*trans*, but including small amounts of *cis* isomers, accounted for 94.6% of the carotenoids of the oleoresin; the remainder was β-carotene (2.8%) and a carotenoid tentatively identified as a lycopene epoxide (2.6%). The purity of synthetic canthaxanthin, astaxanthin, β-apo-8'-carotenal and β-carotene in the commercial preparations from Hoffmann–La Roche was 98% or more. Pure carotenoids used as HPLC standards were also provided by Hoffmann–La Roche. Retinyl palmitate [75 000 retinol equivalents (RE)/g] was obtained from Sigma. Aflatoxin B_1, aflatoxin M_1 (AFM_1), aflatoxin Q_1 (AFQ_1), aflatoxin P_1 (AFP_1), aflatoxin B_{2a} (AFB_{2a}), 2-acetylaminofluorene (2-AAF) and 3-MC were also obtained from Sigma, and DMSO from Merck Chemicals. [^{3}H]- and [^{14}C]aflatoxin B_1 were from Moravek Biochemicals (Brea, CA). Anti-rabbit biotinylated immunoglobulin and streptavidin-alkaline phosphatase were purchased from Amersham. Anti-rat GST Yp was from Biotrin International (Dublin, Ireland).

Animals and diets

Male SPF Wistar rats, 24- to 27-day-old, from Iffa-Credo (Lyon, France), were housed in individual stainless steel cages and maintained at 22°C, constant humidity and with a 12-h light–dark cycle. During the experiments described below, they were fed *ad libitum* semi-liquid purified diets, as described previously (15), which provided 1800 RE of vitamin A/kg diet and 50 mg of vitamin E/kg diet.

Effects of carotenoids, vitamin A or of 3-MC on AFB_1-induced liver preneoplasia

The experimental protocol design is illustrated in Figure 2. From the start of the experiment, 90 rats were allotted to nine groups of 10 (designated as C, MC, VA, BC, CX, AC, LY, EQ and AX), which were submitted to the following experimental treatments during the first 3 weeks of experiment: groups BC and CX received 3 g of 10% β-carotene powder or 10% canthaxanthin powder/kg diet, respectively. Groups AC and LY were given 1.5 g/kg of 20% β-apo-8'-carotenal oil suspension or 6 g/kg of 5% lycopene oleoresin, respectively. Group AX received 3.75 g/kg diet of 8% astaxanthin powder. In these five groups, the dietary concentration of carotenoids was 300 mg/kg. Group VA was given an additional dietary vitamin A supplement of 21 000 RE/kg diet as retinyl palmitate (RP) (i.e. a total supply of 22 800 RE/kg). Group C was the control group. As the 8% astaxanthin powder contained 0.2% ethoxyquin, an additional control group, was included in the experiment, the diet of which contained 15 mg ethoxyquin (EQ)/kg, i.e. twice the level of the AX diet. The rats of the group MC were injected i.p. with 20 mg of 3-MC/kg body wt, on days 10, 11, 12, 16, 17 and 18 after the start of the experiment (i.e. the 3 days preceding each i.p. injection of AFB_1, see later). In order to equalize the diet composition of the groups during the first 3 weeks of experiment, 3 g/kg of placebo powder were included in the diet of groups C, AC, LY, VA and MC, and 3.75 g/kg in the diet of group EQ (to match its composition with that of group AX diet). The carotenoid powders and oleoresin were mixed with water and corn oil as described previously (15), and the resulting emulsion-like mixtures were stored frozen and added to the daily prepared diets. Emulsions containing supplemental vitamin A or ethoxyquin were similarly prepared. Drinking water was supplied *ad libitum*. Food intake was recorded daily during the first 3 weeks, and the rats were weighed once a week throughout the experiment.

All rats were submitted to two i.p. injections of 1 mg AFB_1/kg body weight on days 13 and 19 after the start of the experiment. AFB_1 was solubilized in 50% dimethylsulfoxide (DMSO) in water. All rats received the control diet during the 4th, 5th and 6th weeks of experiments. During the 7th and 8th weeks, 50 p.p.m. of 2-AAF was added to the diet of all groups after dissolution in corn oil. In the middle of the 2-AAF treatment period (on day 50), all rats

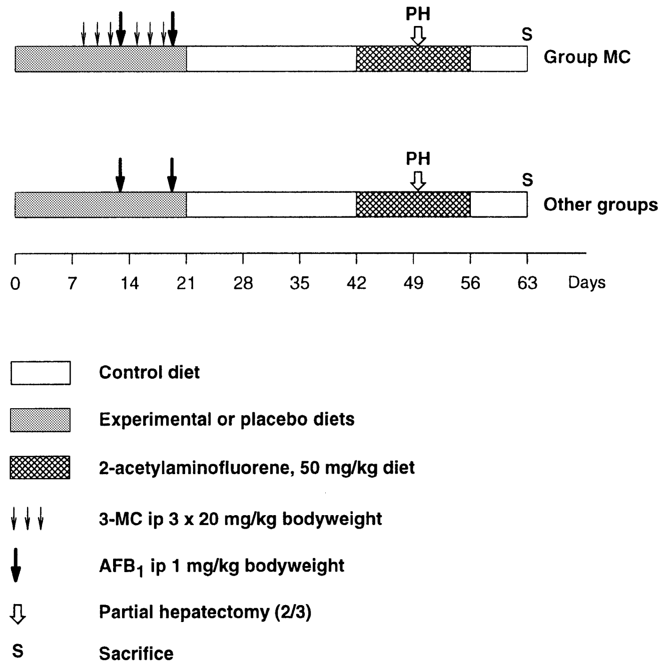

Fig. 2. Schematic representation of the experimental protocol of hepatocarcinogenesis. 3-MC, 3-methylcholanthrene; AFB_1, aflatoxin B_1.

were submitted to 2/3 partial hepatectomy under ether anesthesia. One piece (ca. 0.5 g) of liver was immediately frozen in liquid nitrogen and stored at –75°C for carotenoid analysis. One week after the end of the 2-AAF treatment period (after 64 days of experiment), the rats were killed and the livers rapidly removed. Four slices ~4 mm thick were taken up: two from the right anterior lobe and two from the caudate lobe, the two slices from each lobe being distant by at least 50 mm. The slices were immediately frozen in isopentane at –150°C and stored at –20°C. Serial freeze-cut sections were prepared from each slice, and the placental form of glutathione *S*-transferase (GST-P) was detected by an immunohistochemical method using the streptavidin–biotin–alkaline phosphatase complex (28). Morphometric analysis of one section/slice (i.e. four sections/rat) was performed with the aid of the automated image analysis software SAMBA (TITN-Alcatel, Grenoble, France) coupled to a video-camera equipped with a zoom AF Micro-Nikkor 60-mm lens. Locally developed programs performed the measurement of the total area of each section and of the areas of detected foci. The system allowed the detection of foci sections $>10^{-2}$ mm^2. For each rat (four pooled liver sections), an estimation of the total number of foci/cm^3 liver, as well as of the number of foci of each size class/cm^3 liver, was calculated by the method of Pugh *et al.* (29), and the fraction of liver volume occupied by foci was estimated using the theorem of Delesse (30). The mean volume of foci was deduced from these estimates. Liver carotenoids and retinyl esters were assayed simultaneously by HPLC with a double-wavelength spectrophotometric detector, as described previously (15).

Effect of carotenoids or of 3-MC on AFB_1-induced liver DNA single-strand breaks

From the start of the experiment, 30 rats have been allotted to five groups of six (designated as C, BC, CX, AC and MC). During 2 weeks, groups BC, CX and AC were fed with diets containing 300 mg/kg of β-carotene, of canthaxanthin or of β-apo-8'-carotenal, respectively, as described above. The rats of the group MC were injected i.p. with 3-MC (20 mg/kg body weight, 3 consecutive days) and killed 24 h after the last injection. Group C was the control group. In each group, three rats were injected i.p. with 2 mg AFB_1/kg body weight (AFB_1 was dissolved in 50% DMSO in water) and were killed 4 h later, and three rats did not receive any genotoxic treatment. The liver of all rats were removed immediately after sacrifice, and 2 g liver samples were homogenized in Merchant's buffer (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM Na_2HPO_4, 8.1 mM KCl and 0.53 mM Na_2EDTA, pH 7.4)(31). The homogenate was filtered and the nuclei were separated by centrifugation (450 *g*, 10 min, 4°C). About 10^6 nuclei were loaded on a polycarbonate filter (2 µm pore size, 25 mm diameter, Millipore) and lysed with 6 ml of a lysis buffer (2% SDS, 100 mM glycine, and 20 mM Na_2EDTA, pH 10). DNA was then eluted through the filter, in the dark, by an eluting buffer (20 mM Na_2EDTA, pH 12) at a flow rate of 0.035 ml/min. Ten fractions were collected (1 h 30 min/fraction, 15 h for total elution). The filter was shaken with 5 ml of eluting solution for 40 min at 37°C, then sonicated, in order to solubilize the DNA remaining on it at the end of the elution. After adding 1 ml of sodium phosphate buffer (0.125 M, pH 4.7) to 1 ml of each collected fraction, DNA was assayed fluorimetrically with Hoechst 33258 reagent (32), using an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Elution profiles were obtained by plotting the percentage of total DNA remaining on the filter versus the fraction number. Experimental data suggest that the alkaline elution kinetics consists of two phases. In the initial phase, the elution rate is determined by the size of the DNA induced by the genotoxic treatment. In the second phase of the elution additional strand breaks occur caused by DNA alkaline hydrolysis. An elution rate, constant *K*, representing the first phase elution rate, was calculated using the formula: $K = -\ln$ (DNA retained on the filter after 3 h of elution/DNA deposited on the filter)/eluted volume (ml).

Effect of carotenoids or of 3-MC on the binding of AFB_1 to liver DNA and plasma albumin

The design of this experiment, as well as the dose of AFB_1 used, were identical to those used in the experiment on DNA single-strand breaks. Twenty rats were allotted to five groups of four (designated as C, BC, CX, AC and MC). During 2 weeks, groups BC, CX and AC were fed with diets containing 300 mg/kg of β-carotene, of canthaxanthin or of β-apo-8'-carotenal, respectively, as described above. The rats of the group MC were injected i.p. with 3-MC (20 mg/kg body wt) on the 3 days preceding sacrifice. Group C was the control group. At 14 days after the start of the experiment, the rats were injected i.p. with 2 mg ^3H-AFB_1/kg body weight, (specific activity: 11.7 µCi/µmole; AFB_1 was dissolved in 50% DMSO in water). Exactly 2 h later, blood samples were taken from the abdominal aorta under ether anesthesia, and plasma was obtained by centrifugation and stored at –75°C. The livers were quickly removed, weighed, cut into pieces of ~2 g, frozen in liquid nitrogen and stored at –75 °C. Liver DNA was extracted and purified as described by Fiala *et al.* (33). Absorption at 230, 260 and 280 nm, and radioactivity counting (using a liquid scintillation counter) were performed on aliquots of DNA samples. Each sample was counted twice. The liver DNA samples obtained were of good purity (A260/A280 of ca. 1.9, A260/A230 of ca. 2.3). The binding of AFB_1 to plasma albumin was measured according to Wild *et al.* (34).

In vitro *metabolism of AFB_1 by microsomes from carotenoid- or 3-MC-treated rats*

Thirty rats were allotted to six groups of five and fed for 2 weeks with control diets (groups C and MC) or with diets containing 300 mg/kg of β-carotene (group BC), canthaxanthin (group CX), astaxanthin (group AX) or β-apo-8'-carotenal (group AC), as described above. The rats of group MC were injected i.p. with 3-MC (3×20 mg/kg body wt) on the 3 days preceding being killed. All rats were killed after 2 weeks of experiment, and liver microsomes were prepared as described previously (15) and stored at –75°C. In a total volume of 250 µl, hepatic microsomes (0.375 mg of microsomal protein) were incubated with 10 µM of [^{14}C]aflatoxin B_1 (0.05 µCi) in 80 mM Tris/60 mM HCl buffer, pH 7.4, containing 2 mM NADPH and 6 mM $MgCl_2$, for 30 min at 37°C. At the end of the incubation time, the reaction was stopped with 100 µl of cold methanol. The proteins were sedimented by centrifugation (10 min, 14000 r.p.m.) and allowed to stand overnight in Soluene (Packard), and the associated radioactivity was measured in a Packard scintillation counter. The total radioactivity associated with the supernatant was measured on an aliquot. Another aliquot was used to analyze the radiolabeled AFB_1 metabolites by reverse-phase HPLC, using a C18 Nucleosil N225 column (5 µm, 250×4.6 mm, at 40°C) coupled to a Flo-One (Radiomatic) radioactivity detector. The mobile phase was made of two solvent mixtures, system A [H_2O/acetic acid, 99.75/0.25, (v/v), pH 3.5] and system B [acetonitrile/ethyl acetate/ H_2O, 15/6/79, (v/v/v)], used as follows: 90% A and 10% B for 1 min, then 100% B for 30 min, at the flow rate of 1 ml/min. AFB_1 metabolites were identified by comparing their retention times with those of commercial standards (AFB_1, AFM_1, AFQ_1, AFB_{2a} and AFP_1), and quantified by radioactivity measurement.

Statistical analysis

All data were submitted to analysis of variance, the error term being the between-rat mean square. Log transforms were used in some cases in order to homogenize the group variances (see Results). Differences versus the control group C were assessed by the Dunnett's test ($P \leq 0.05$). The Student's *t*-test was used in some cases for comparing two means (see Results). Calculations were made with the SAS system (Cary, NC).

Results

Effects of carotenoids, of vitamin A and of 3-MC on AFB_1-induced liver preneoplasia

Four rats from four different groups (MC, CX, AC, BC) died during the days following partial hepatectomy. The liver

contents of retinyl esters and of carotenoids at partial hepatectomy are shown in Table I. The vitamin A liver store was increased by 3-week feeding of an excess of vitamin A, and of the provitamin A carotenoids β-apo-8'-carotenal and β-carotene, but also, although slightly, by feeding lycopene. 3-MC treatment decreased it significantly. Although their administration in the diet had ceased 4 weeks before partial hepatectomy, carotenoids, except astaxanthin, were still present in the liver at very significant levels. The results of the morphometric analysis of preneoplastic foci are summarized in Table II and Figure 3. Four of the carotenoids studied (canthaxanthin, astaxanthin, β-apo-8'-carotenal and β-carotene) and 3-MC decreased significantly the initiating effect of AFB_1: these treatments reduced the number of GST-P-positive foci by 64, 55, 70, 59 and 76%, respectively, with respect to the control group, and their size by 88, 82, 69, 77 and 87%, respectively, inducing a shift of their size distribution towards smaller foci. Accordingly, the fraction of liver volume occupied by GST-P-positive foci was reduced by 92, 85, 89, 82 and 95%, respectively. The rats fed lycopene (group LY), an excess of vitamin A (group VA) or ethoxyquin (group EQ) did not differ significantly from the control group C for any of the three morphometric parameters measured. As compared with group EQ, astaxanthin reduced the number of GST-P-positive foci, as well as the mean focal volume and the percentage of liver volume occupied by foci.

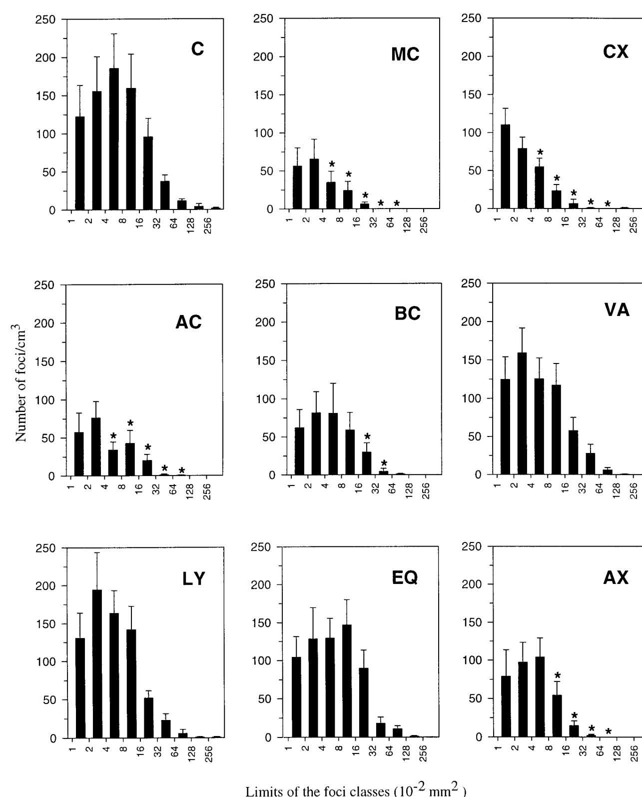

Fig. 3. Size distributions of liver GST-P-positive foci in the different groups (number of foci of each class/cm^3 liver, means of nine or 10 rats ± SEM). The size classes are indicated by limits of diametral section area of the foci, which are supposed to be spherical; these limits (in 10^{-2} mm^2) form a geometric series, the lower limit being excluded from each class, and the higher included. C, control; MC, 3-methylcholanthrene; CX, canthaxanthin; AC, β-apo-8'-carotenal; BC, β-carotene; VA, vitamin A; LY, lycopene; EQ, ethoxyquin; AX, astaxanthin. *Significantly different from control group C (Dunnett's test, $P \leqslant 0.05$).

Table I. Liver contents (μg/g) of carotenoids and retinyl esters at partial hepatectomy[a]

Experimental groups	Carotenoids[c]	Retinyl esters[d]
C	n.d.	62.2 ± 2.0
MC	n.d.	48.7 ± 2.8*
CX	20.0 ± 4.4	57.3 ± 2.8
AC	14.7 ± 1.4	133.2 ± 4.4*
BC	9.2 ± 1.4	263.8 ± 12.8*
VA	n.d.	297.4 ± 14.8*
LY	15.8 ± 3.0	86.0 ± 4.4*
EQ	n.d.	69.1 ± 2.2
AX	0.10 ± 0.03	61.8 ± 3.1

[a]Values are means ± SEM; n.d., not detected.
C, control; MC, 3-methylcholanthrene; CX, canthaxanthin; AC, β-apo-8'-carotenal; BC, β-carotene; VA, vitamin A; LY, lycopene; EQ, ethoxyquin; AX, astaxanthin.
[c]The carotenoids assayed were canthaxanthin, β-apo-8'-carotenal, β-carotene and astaxanthin for groups CX, AC, BC and AX, respectively.
[d]Retinyl esters are the sum of retinyl palmitate, stearate, oleate and linoleate.
*Significantly different from the control group C (Dunnett's test on log transforms, $P \leqslant 0.05$).

Table II. Morphometric analysis of liver GST-P-positive foci initiated by AFB_1 (2×1 mg/kg body wt) in the resistant hepatocyte model[a]

Experimental groups[b]	No. of rats	No. of foci/cm^3 liver	Mean focal volume (10^{-3} mm^3)[c]	Fraction of liver volume occupied by foci (mm^3/cm^3)[c]
C	10	773 ± 169	107.8 ± 46.8	55.4 ± 17.6
MC	9	187 ± 61*	14.5 ± 3.6*	2.8 ± 0.9*
CX	9	275 ± 49*	13.3 ± 2.1*	4.2 ± 1.2*
AC	9	231 ± 66*	33.8 ± 12.7*	6.1 ± 2.0*
BC	9	318 ± 107*	24.3 ± 4.3*	9.5 ± 4.0*
VA	10	617 ± 107	39.6 ± 10.9	25.5 ± 8.0
LY	10	711 ± 116	56.7 ± 25.8	31.5 ± 10.6
EQ	10	627 ± 130	50.0 ± 10.0	31.9 ± 8.4
AX	10	351 ± 99*,**	19.6 ± 2.3*,**	7.3 ± 2.4*,**

[a]Values are means ± SEM.
[b]C, control; MC, 3-methylcholanthrene; CX, canthaxanthin; AC, β-apo-8'-carotenal; BC, β-carotene; VA, vitamin A; LY, lycopene; EQ, ethoxyquin; AX, astaxanthin.
[c]ANOVA was made on log transforms of data.
*Significantly different from control group C (Dunnett's test, $P \leqslant 0.05$).
**Group AX significantly different from group EQ (Student's *t*-test, $P \leqslant 0.05$).

Effect of carotenoids and of 3-MC on AFB_1-induced liver DNA single-strand breaks

The effect of carotenoids and of 3-MC on AFB_1-induced liver DNA single-strand breaks are summarized in Table III and Figure 4. AFB_1 treatment induced liver DNA SSB in the control group C. Feeding the rats with canthaxanthin or β-apo-8'-carotenal, or injecting them with 3-MC significantly protected the hepatic DNA from AFB_1-induced SSB: *K*-values were reduced by 74, 72 and 90%, respectively. In contrast, β-carotene did not significantly decrease AFB_1-induced SSB. Noticeably, 3-MC treatment increased, though not significantly, liver DNA SSB in rats that were not treated by AFB_1. Moreover, DNA SSB in 3-MC-treated rats were significantly reduced by AFB_1 treatment: AFB_1 appears to antagonize the small DNA-damaging effect of 3-MC.

Table III. Liver DNA elution rate constants of *K*[a]

Experimental groups[b]	Rats not treated with AFB_1	Rats treated with AFB_1
C	14.0 ± 5.5	78.7 ± 3.7*
MC	35.0 ± 11.7	8.0 ± 2.1*,**
CX	12.7 ± 5.5	20.3 ± 4.5**
AC	13.7 ± 9.0	22.3 ± 10.3**
BC	15.2 ± 1.5	74.5 ± 17.3*

[a]K= 10^3 ln (fraction of DNA remaining on the filter after 3 h)/eluted volume (ml); values are means of three rats ± SEM.
[b]C, control; MC, 3-methylcholanthrene; CX, canthaxanthin; AC, β-apo-8'-carotenal; BC, β-carotene.
*Significantly different from the *K*-value of rats of the same group, but not treated with AFB_1 (Student's *t*-test, $P \leq 0.05$); **significantly different from the *K*-value of rats of the control group C, treated with AFB_1 (Dunnett's test, $P \leq 0.05$).

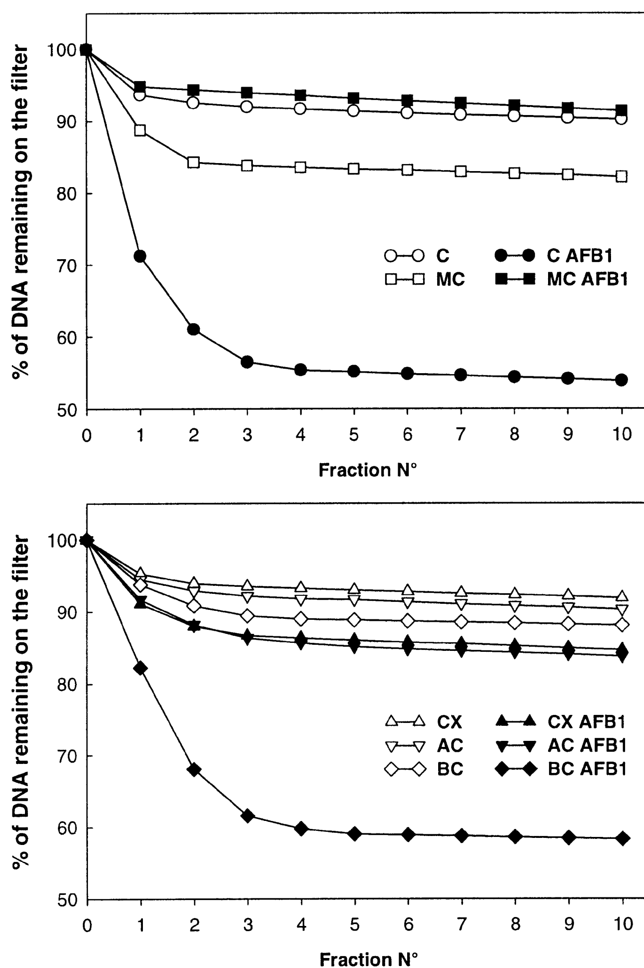

Fig. 4. Liver DNA alkaline elution profiles of rats treated with 3-methylcholanthrene (top) or fed with carotenoids (bottom), and treated or not treated with AFB_1. Each point is the mean of three rats. C, control; MC, 3-methylcholanthrene; CX, canthaxanthin; AC, β-apo-8'-carotenal; BC, β-carotene.

Effect of carotenoids or of 3-MC on the binding of AFB_1 to liver DNA and plasma albumin

The effects of feeding carotenoids to the rats or treating them i.p. with 3-MC on the binding of AFB_1 to liver DNA and plasma albumin, are presented in Figure 5. 3-MC, canthaxanthin and β-apo-8'-carotenal decreased the binding of AFB_1 to liver DNA (by 55, 50 and 60%, respectively) and to plasma albumin (by 61, 60 and 65%, respectively). In contrast, β-carotene feeding had no effect on the binding of AFB_1 to liver DNA or plasma albumin. These results are quite similar to those obtained on the effects of 3-MC or carotenoids on liver DNA SSB (see above).

In vitro *metabolism of AFB_1 by microsomes from carotenoid- or 3-MC-treated-rats*

The microsomal metabolism of aflatoxin B_1 was strongly modified by carotenoids (Table IV, Figure 6). In the presence of microsomes from rats fed canthaxanthin, astaxanthin or β-apo-8'-carotenal, or i.p. injected with 3-MC, the metabolism of AFB_1 was enhanced (although not significantly in the AX group), principally towards the formation of aflatoxin M_1, a less genotoxic hydroxylated metabolite, which was increased by 11-, 6-, 9- and 18-fold, respectively. Other metabolites were also enhanced: the formation of aflatoxin B_{2a} was significantly increased by canthaxanthin (1.6-fold) and 3-MC (1.6-fold), and unknown metabolites bound to proteins were increased by canthaxanthin (2.1-fold), astaxanthin (1.9-fold) and 3-MC (2.2-fold). Feeding β-carotene did not enhance the overall metabolism of aflatoxin B_1, but increased the formation of aflatoxin P_1 (1.8-fold).

Discussion

Few studies have been published on the antimutagenic, anti-genotoxic or anticarcinogenic effects of carotenoids towards

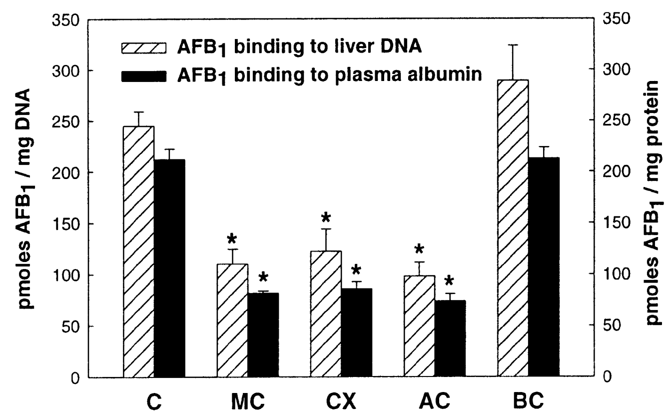

Fig. 5. The binding of AFB_1 to liver DNA and to plasma albumin of rats fed with carotenoids or treated with 3-methylcholanthrene. Each bar is the mean of four rats ± SEM. C, control; MC, 3-methylcholanthrene; CX, canthaxanthin; AC, β-apo-8'-carotenal; BC, β-carotene. *Significantly different from control group C (Dunnett's test, $P \leq 0.05$).

Table IV. *In vitro* AFB1 metabolism catalyzed by liver microsomes from rats fed with carotenoids or with vitamin A, or treated by 3-MC[a]

Experimental groups[b]	Metabolites bound to proteins	Unknown 1	AFB_1 Tris–diol	AFB_{2a}	AFM_1	AFQ_1	Unknown 2	AFP_1	AFB_1
C	8.6 ± 1.0	7.1 ± 0.4	11.6 ± 1.3	3.9 ± 0.3	3.9 ± 0.3	4.3 ± 0.5	1.8 ± 0.4	5.1 ± 0.6	176 ± 2
MC	19.3 ± 0.3*	9.9 ± 0.8	9.5 ± 1.1	6.4 ± 0.4*	69.1 ± 2.0*	3.6 ± 0.4	3.7 ± 0.3	2.3 ± 0.6	98 ± 3*
CX	18.1 ± 2.2*	10.4 ± 1.4	14.7 ± 0.8	6.3 ± 0.9*	41.7 ± 4.8*	5.9 ± 0.9	4.0 ± 0.8	5.9 ± 1.0	115 ± 11*
AX	16.2 ± 1.3*	7.4 ± 1.8	12.4 ± 1.2	4.5 ± 0.6	23.5 ± 3.8*	5.2 ± 0.3	3.6 ± 0.8	5.1 ± 0.7	144 ± 11
AC	12.1 ± 2.3	8.3 ± 1.4	12.7 ± 1.1	4.0 ± 0.7	35.9 ± 6.5*	5.1 ± 0.7	5.1 ± 1.1*	4.8 ± 0.5	134 ± 12*
BC	14.8 ± 2.1	9.7 ± 1.3	10.7 ± 1.0	3.7 ± 0.6	6.0 ± 1.2	5.4 ± 0.3	2.1 ± 0.4	9.0 ± 1.3*	160 ± 8

[a]Values (means of five rats ± SEM) are expressed in pmol/min per mg microsomal protein.
[b]C, control; MC, 3-methylcholanthrene; CX, canthaxanthin; AX, astaxanthin; AC, β-apo-8'-carotenal; BC, β-carotene.
*Significantly different from control group C (Dunnett's test, $P \leq 0.05$).

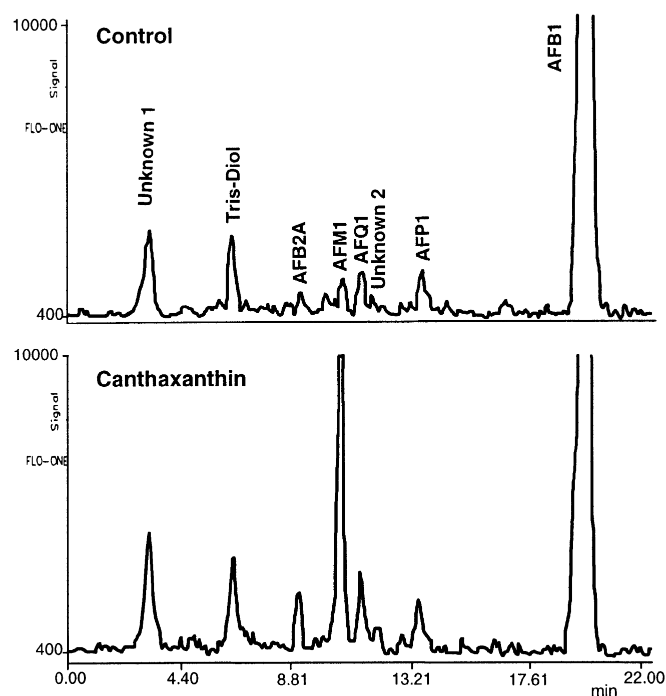

Fig. 6. HPLC chromatogram of aflatoxin B_1 metabolites formed by incubating aflatoxin B_1 with liver microsomes of control rats (top) or of rats fed canthaxanthin (bottom).

AFB_1. Some carotenoids inhibit the mutagenicity of AFB_1 in the Ames' test (35) and others the *in vitro* formation of AFB_1–DNA adducts in the presence of rat liver microsomes (36). Crocetin, an acyclic apocarotenoid from saffron flower and *Gardenia* fruit, reduces AFB_1 toxicity and AFB_1–DNA adducts, and increases glutathione *S*-transferase activity in rat liver or in cultured fibroblast cells (37–40). Very recently, carotenoid-rich extracts of carrots, tomato paste or orange juice have been shown to reduce the number of liver preneoplastic foci induced by AFB_1 in the rat, when administered together with the carcinogen (41). In contrast to these protective effects, β-carotene at lower concentration (1–20 μM) was found to increase AFB_1 binding to DNA in woodchuck hepatocytes (42). Moreover, in a recent cross-sectional study in carriers and non-carriers of hepatitis B virus, plasma α- and β-carotene, which reflect dietary intake, were found to be positively associated with the detection rate of AFB_1–DNA adducts in urine, whereas plasma lycopene was negatively associated (43). Taken together, these results show chemopreventive potencies of some carotenoids against AFB_1 in some experimental models, but the effects and the mechanisms involved are likely to be different between different carotenoids, between *in vivo* and *in vitro* models, and between different species.

The modulation of AFB_1 metabolism and carcinogenicity by various inducers of XME has been extensively studied. Roughly speaking, two types of inducers act through two types of chemopreventive mechanisms: phenobarbital-like inducers, such as phenobarbital (44), phenolic antioxidants such as BHA or BHT (45), ethoxyquin (46) or allyl sulfides (47), reduce the AFB_1 carcinogenicity by increasing its detoxication via the induction of glutathione *S*-transferase, which catalyzes the conjugation of aflatoxin-8,9-epoxide, the main genotoxic AFB_1 metabolite, with glutathione (19). 3-MC-like inducers as 3-MC, dioxin or BNF increase AFB_1 hydroxylation to AFM_1 via CYP1A induction, and also increase AFM_1 glucuroconjugation via 4NP-UGT induction (19). We did not observe any inhibitory effect of ethoxyquin on the carcinogenic action of AFB_1, most probably because the level of ethoxyquin that we administered to the rats (15 p.p.m.) was well below the level previously found as the threshold dose for enzyme induction and chemoprevention, i.e. at 500 p.p.m. (46). As a consequence, it appears clearly that the inhibition of AFB_1 carcinogenicity observed in rats that have been fed with the astaxanthin powder is only due to astaxanthin, not to the ethoxyquin contained in the powder (7.5 p.p.m. of the diet).

One of the main result of this study is that feeding rats with CYP1A induces the carotenoids, canthaxanthin, astaxanthin and β-apo-8'-carotenal, inhibits the initiation of liver preneoplasias by AFB_1 and reduce *in vivo* AFB_1-induced liver DNA single-strand breaks and the *in vivo* binding of AFB_1 to liver DNA. Like the classical CYP1A inducer 3-MC, these carotenoids enhance the *in vitro* metabolism of AFB_1 to AFM_1, which is a less genotoxic metabolite. 3-MC, BNF and other inducers of this type, such as indole-3-carbinol or 3,3′,4,4'-tetrachlorobiphenyl have been shown to inhibit AFB_1 carcinogenic, clastogenic and mutagenic effects (22,24–26,48–50). These inducers increase AFM_1 formation through CYP 1A1/2 induction (19–23). Although CYP1A2 can activate AFB_1 to AFB_1-8,9-epoxide in man, especially at low molecular concentrations (19,20), CYP1A2 catalyses the hydroxylation of AFB_1 to AFM_1 in mice (51). The induction of phase II enzymes by CYP1A inducers can also contribute to AFB_1 detoxication: 3-MC and BNF also induce 4NP-UGT, which catalyzes AFM_1 conjugation to glucuronic acid (19). Indole-3-carbinol, and to a lesser degree BNF, were shown to enhance the conjugation of AFB_1 epoxide through the induction of Yc2 glutathione transferase subunit, an isoenzyme with a high

specificity towards AFB_1-8,9-epoxide (50). Canthaxanthin, astaxanthin and β-apo-8'-carotenal, just as 3-MC, only marginally induced chlorodinitrophenol-GST, but strongly induced 4NP-UGT, in rat liver (15–17). More specific effects of carotenoids on GST isoenzymes have not been investigated to-date.

Even for CYP1A1/2 inducers, mechanisms other than enzyme induction can be involved in the inhibition of the effects of AFB_1: thus, BNF has been shown to inhibit *in vitro* and *in vivo* AFB_1–DNA binding in trout liver, at dietary levels that do not induce CYP1A enzymes (26). At low doses, BNF only acts by inhibiting CYP-catalyzed AFB_1 activation (26). Some carotenoids have been shown to inhibit *in vitro* the activation of AFB_1, but the results are not consistent. Thus, β-carotene and canthaxanthin inhibited AFB_1-induced mutagenesis in *Salmonella typhimurium*, in the presence of rat liver S9, at concentrations of ~100 μM, and β-cryptoxanthin at 10 μM or less, whereas lycopene had no effect (35). β-carotene, β-apo-8'-carotenal, β-cryptoxanthin and lutein inhibited the *in vitro* binding of AFB_1 to DNA in the presence of rat liver microsomes, at concentrations of 100 μM (36), whereas canthaxanthin and astaxanthin had no effect. In our previous works on the effects of carotenoids on XME, some carotenoids, when fed to rats for 2 weeks at the level of 300 mg/kg diet, reached high concentrations in rat liver: ~100 μM for β-carotene and lycopene (15,16), >200 μM for β-apo-8'-carotenal (17), and >500 μM for canthaxanthin (15,16), but only 1–3 μM for astaxanthin (16). Among the three CYP 1A inducer carotenoids that we found to actively inhibit the liver preneoplasia induced by AFB_1 and alter its metabolism, one (astaxanthin) is present in liver only at a very low concentration, and two (canthaxanthin and astaxanthin) show no inhibitory effect on *in vitro* AFB_1–DNA binding, even at higher concentration (36). Thus, it seems unlikely that inhibition of P450-dependent AFB_1 activation plays a significant role in the *in vivo* inhibitory effects of CYP1A inducer carotenoids on the initiation of preneoplastic foci by AFB_1. Our results support the hypothesis that enhancement of AFM_1 formation and conjugation via CYP1A1/2 and 4NP-UGT induction are the main mechanisms by which canthaxanthin, astaxanthin and β-apo-8'-carotenal protect AFB_1 from genotoxicity and initiating actions.

At variance with carotenoids which induce CYP1A, β-carotene did not grossly alter AFB_1 metabolism and did not protect the hepatic DNA from AFB_1-induced alterations. These results are consistent with our previous works, showing no change of liver XME in rats fed with β-carotene (15,17), and in particular, no enhancement of liver CYP 1A or CYP 2B-associated activities and of liver GST. The increase of AFP_1 formation by liver microsomes of rats fed β-carotene indicates, nevertheless, some unknown change in liver XME. However, this change is apparently of too small intensity significantly alter the balance between AFB_1 activation and detoxication, since feeding β-carotene did not decrease AFB_1-induced DNA alterations in liver. Yet, feeding β-carotene reduced AFB_1-induced enzyme-altered foci as efficiently as the inducer carotenoids, but seemingly via a quite different, and still unknown, mechanism. As a first hypothesis, the antioxidant properties of β-carotene are often referred to as the reason for its protective effects against the action of carcinogens, which often involves reactive oxygen species (ROS) and oxidative damage. As a matter of fact, lipid peroxidation (52) and oxidative DNA damage (53) have been observed in rat liver following AFB_1 administration, and ROS have been found to be involved in AFB_1-induced cell injury in cultured rat hepatocytes (54). The question remains, however, whether ROS and oxidative DNA damage play a role in AFB_1 mutagenicity and carcinogenicity, since AFB_1 induces other mutagenic DNA adducts. Moreover, this hypothesis cannot explain why β-carotene is protective against AFB_1 initiating action whereas lycopene, which is also a good antioxidant, is not. On the other hand, β-carotene is mostly converted to vitamin A in the rat, and it could also act through its provitamin A activity. Apparently, this is not the case in our experiment, since an excess of vitamin A in the diet, which induces a greater increase of vitamin A hepatic store than does β-carotene, has no effect on the induction of preneoplastic foci by AFB_1. Some modulation of AFB_1 activation by dietary vitamin A has been observed by other authors, but only with a very large dietary overload (55). Alternatively, the fact that β-carotene has been shown to exert protective effects against a variety of mutagens and carcinogens, including both indirect carcinogens as benzo[*a*]pyrene (56), dimethylbenzanthracene (57) and cyclophosphamide (58), and direct-acting mutagens such as methyl-methanesulfonate (59), methylnitrosourea (57) or ethylnitrosourea (60), suggests that it can act through mechanisms other than XME modulation. Particularly, the role of cell cycle in DNA repair, as well as the role of cell proliferation and apoptosis in cancer initiation, have been recently stressed (61), including the model of AFB_1-initiated liver GST-P-positive foci (62). Moreover, recent works have shown that carotenoids could modulate these phenomena (63,64). However, this effect must depend on the system used, since lycopene, which has been shown to be a potent inhibitor of cell proliferation of several cancer cell types (65), has no effect on the initiation of liver enzyme-altered foci by AFB_1.

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