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SHORT COMMUNICATION

Ascorbic acid and β-carotene as modulators of oxidative damage

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Naturally occurring antioxidants are extensively studied for their capacity to protect organisms and cells from damage induced by oxygen reactive species. In fact, oxidative stress is considered a cause of aging, degenerative disease and cancer. We have focused our attention on two agents, ascorbic acid and β-carotene, commonly considered to be antioxidants, but whose protective activity against cancer is insufficiently known. This paper reports on the ability of these agents to act against damage induced by H_2O_2 and bleomycin, in Chinese hamster ovary cells cultivated *in vitro*. Cytogenetic and cytofluorimetric analyses were performed. Both vitamins proved effective in reducing H_2O_2-induced sister chromatid exchanges, but increased H_2O_2- and bleomycin-induced chromosomal aberrations. Cytofluorimetric data, in contrast, showed that ascorbic acid and β-carotene act as scavengers of endogeneous and H_2O_2-induced oxygen species.

Numerous exogenous agents and endogenous processes are capable of generating free radicals *in vivo*. Among these molecules the reactive oxygen species are produced not only as a consequence of normal metabolism, but also during irradiation or metabolic activation of specific chemicals (1). Many defence mechanisms within the organism have evolved to limit the levels of damage induced by oxidative stress. Among these mechanisms are enzymes (superoxide dismutase, catalase, glutathione peroxidase) and also dietary components, such as vitamins, which exhibit scavenging or chelating activity against oxidant molecules. As oxidation phenomena in biological systems are considered a cause of aging, degenerative diseases and cancer, particular attention has been focused on the possibility of modulating the detrimental effects of oxidative stress through the use of free radical scavengers able to minimize cellular injury, particularly DNA damage.

Epidemiological studies and experimental analyses have been addressed to the identification of natural dietary constituents capable of favourably modulating carcinogenic and mutagenic processes. We have recently postulated (2) that ellagic acid, a naturally occurring phenolic lactone, is able to counteract active oxygen species produced by known mutagens, consequently reducing their mutagenic activity in Chinese hamster ovary (CHO*) cells *in vitro*.

Among vitamins, whose antioxidant activity is thought to offer protection to DNA from oxidative free radical damage, carotenoids and vitamin C have been extensively studied (3,4). While much literature is available concerning the reducing activity of ascorbic acid against mutagens such as ethylmethansulfonate and *N*-methyl-*N*-nitrosoguanidine (5,6) and UV and 4-nitroxyquinoline (7), in 1984 Shamberger (8) summarized the genotoxic effects of vitamin C in different test systems, according to its capacity, at higher doses, to function as a pro-oxidant, itself generating oxygen radicals.

Experimental and epidemiological studies are also present in literature about the anticarcinogenic and chemopreventive activities of ascorbic acid, although the data are often conflicting and inconclusive. Recently Drake *et al.* (9) have proposed that high dietary ascorbic acid intake protects against gastric cancer.

β-Carotene, an important pro-vitamin A, is considered an efficient antioxidant (3) acting as a quencher of singlet oxygen and free radicals. Experiments in animals have also suggested that β-carotene is able to reduce DNA and chromosomal damage induced by alkylating agents such as ethylnitrosourea (10) and methylmethanesulfonate (11), and also by pro-carcinogens as benzo[*a*]pyrene (12) and cyclophosphamide (13). β-Carotene has been extensively used in cancer-chemopreventive studies, in which it displayed suppressing activity against oral and colon tumors (14,15).

The aim of this paper is to investigate β-carotene and ascorbic acid scavenging activity, and their effectiveness as modulators of DNA damage induced by two oxidants (H_2O_2 and bleomycin), in order to find a rational basis for a potential use of such vitamins in cancer therapeutic approaches.

Cytogenetic and cytofluorimetric experiments were performed in CHO cells cultivated *in vitro*. CHO cells are routinely cultured in our laboratory (2). For sister chromatid exchanges (SCE) and chromosomal aberration (ChAb) analysis, cultures were treated with the appropriate concentration of ascorbic acid or β-carotene; after 15 min in combined treatment, H_2O_2 was added. This treatment, performed in NaCl (0.9%), lasted 30 min. The cultures were then incubated in fresh complete medium containing ascorbic acid or β-carotene and bromodeoxyuridine (BrdUrd) (for sister chromatid differentiation) at a final concentration of 5×10^{-6} M. Bleomycin treatment was performed in complete medium and lasted 1 h. All agents were prepared immediately before each experiment. Ascorbic acid (Sigma) and β-carotene (Roche) were dissolved in distilled water. H_2O_2 was dissolved in NaCl (0.9%) from a 30% stock solution. Bleomycin (Rhone-Poulenc) was dissolved in phosphate-buffered saline (PBS) lacking Ca^{2+} and Mg^{2+}. 2′,7'-Dichlorofluorescein diacetate (DCFH-DA) was dissolved in ethanol. (For concentration of reagents see figures and tables.) The cells were fixed 26 and 30 h after BrdUrd treatment for SCE analysis and 16 h after for ChAb analysis. Colchicine

***Abbreviations:** BrdUrd, bromodeoxyuridine; DCFH, dichlorofluorescein; PBS, phosphate-buffered saline; CHO cells; Chinese hamster ovary cells; ChAb, chromosomal aberrations; SCE, sister chromatid exchanges; MI, mitotic index; DCFH-DA, 2′,7'-dichlorofluorescein diacetate; a.u., arbitrary units.

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Table Ia. Effect of ascorbic acid on H_2O_2-induced SCE (fixing time 26 h)

Treatment	MI/1000	M_1	M_2	M_3	SCE ± SE per cell	R	*P*
Control	52	3	97	0	6.2 ± 0.3		
H_2O_2 (10^{-4}M)	72	2	98	0	17.8 ± 1.1		
Ascorbic acid 1 (10^{-6} M)	44	3	97	0	7.0 ± 0.4		
Ascorbic acid 2 (10^{-5} M)	118	0	100	0	8.2 ± 0.6		
H_2O_2 + ascorbic acid 1	70	1	99	0	12.6 ± 1.5	28%	<<0.01
H_2O_2 + ascorbic acid 2	56	1	99	0	11.2 ± 1.3	35%	<0.001

Table Ib. Effect of ascorbic acid on H_2O_2-induced SCE (fixing time 30 h)

Treatment	MI/1000	M_1	M_2	M_3	SCE ± SE per cell	R	*P*
Control	119	0	100	0	5.7 ± 0.4		
H_2O_2 (10^{-4} M)	80	2	98	0	17.8 ± 1.9		
Ascorbic acid 1 (10^{-6} M)	102	0	100	0	5.5 ± 0.5		
Ascorbic acid 2 (10^{-5} M)	106	0	100	0	6.3 ± 0.4		
H_2O_2+ascorbic acid 1	118	4	96	0	8.5 ± 1.2	50%	<<0.001
H_2O_2+ascorbic acid 2	40	5	95	0	8.6 ± 0.4	50%	<<0.001

R, percentage of reduction; *P*, two-tailed *P* value. Alternate *t*-test.

(5×10^{-7} M) was always added 2 h before fixing time. Giemsa-Hoechst technique, as previously described (2), was used for differential staining of sister chromatids.

For SCE analysis 40 second mitoses and for ChAb analysis 100–200 first mitoses were scored from coded slides for each point in each experiment. All experiments were repeated three times. For each experimental point, 1000 cells were scored for the mitotic index (MI) and 100 metaphases for first (M_1), second (M_2) and third and successive (M_3) mitosis determination (proliferation index).

For cytofluorimetric analysis, cells were seeded 15 min in PBS lacking Ca^{2+} and Mg^{2+} containing DCFH-DA (5 μM). The medium was then removed and the cells were seeded in NaCl (0.9%) with ascorbic acid or β-carotene and H_2O_2, in combined treatment, for 30 min. The cells were then trypsinized and analyzed in a FACSTAR cytometer (Becton Dickinson) equipped with a 5-watt argon laser (Coherent) (488 nm emission).

For SCE analysis, means and standard errors were determined. For SCE and ChAb analysis, mutagen-treated cultures and mutagen plus antioxidant-treated cultures were compared by Student's *t*-test. For cytofluorimetric analysis, the mean of fluorescence intensity was expressed in arbitrary units (a.u.) and the standard deviation was determined. Mutagen-treated cultures and mutagen plus antioxidants-treated cultures were compared by Student's *t*-test.

Ascorbic acid and β-carotene failed to induce SCE and ChAb in our experimental conditions at the tested doses (Tables I–IV). Furthermore, neither agent affected mitotic and proliferation (M_1–M_3) indices.

H_2O_2 induced a significant increase in ChAb (25% abnormal cells) and a slight increase in SCE (16–21 SCE per cell) (Tables I–IV), in accordance with data in the literature. It is well known that in biological systems H_2O_2 is able to induce DNA damage by itself and/or through the highly reactive oxygen and radical species (16,17). Such damage includes single- and double-strand breaks, base destruction and cross linking. Some of these lesions may result in the generation of ChAb and SCE.

Conversely, bleomycin was unable to induce SCE (data not shown), but was a good inducer of ChAb (28% abnormal cells). Bleomycin, a glycopeptide antibiotic used in the treatment of several different neoplasms, causes DNA strand breaks and micronuclei, but not gene mutations and SCE (18). It has been postulated that bleomycin interacts with molecular oxygen and iron to produce superoxide anion and other oxygen metabolites (19).

Both ascorbic acid and β-carotene used in combined treatment with H_2O_2, significantly reduced (25–50% reduction) SCE rate, particularly in the second fixing time (Tables I and II). Both vitamins were used in combined treatments to analyze their effects on induced ChAb. Tables III and IV show that ascorbic acid and β-carotene significantly increase H_2O_2-induced ChAb, particularly isochromatid breaks, while MI are unaffected.

In combined treatment with bleomycin (Table III), ascorbic acid does not increase the percentage of abnormal cells, but total aberrations are significantly increased, particularly isochromatid breaks. In contrast, β-carotene (Table IV) does not significantly affect total aberrations induced by bleomycin, though isochromatid breaks are increased at the highest dose.

Furthermore we performed a quantitative assay by cytofluorimetric analysis to measure oxidative products, i.e. oxygen and radical species. Figures 1 and 2 show the fluorescence distribution of cells previously treated with DCFH-DA. The histograms represent the number of cells (ordinate) as a function of fluorescence intensity (abscissa). Figure 1A shows that ascorbic acid, at the doses used, does not induce oxidative species, but, at the highest doses, reduces the endogenous oxidants produced by oxidative burst. In Figure 1B, cells treated with H_2O_2 show a significant increase of fluorescence intensity; in combined treatment (H_2O_2 and ascorbic acid) the cells show a significant reduction of fluorescence intensity according to the dose of ascorbic acid, compared with H_2O_2 treatment.

Figure 2A shows the activity of β-carotene versus endogenous oxidants. It seems to be less efficient than ascorbic acid, though the reduction is significant (Figure 3). In combined treatment with H_2O_2 and β-carotene at the doses used, the cells show an evident reduction of fluorescence intensity

Table IIa. Effect of β-carotene on H_2O_2-induced SCE (fixing time 26 h)

Treatment	MI/1000	M_1	M_2	M_3	SCE ± SE per cell	R	*P*
Control	62	16	83	1	8.0 ± 0.6		
H_2O_2 (10^{-4} M)	40	26	74	0	16.0 ± 1.9		
β-Carotene 1 (10^{-6} M)	84	3	97	0	6.2 ± 1.6		
β-Carotene 2 (10^{-5} M)	164	0	100	0	4.4 ± 0.3		
H_2O_2+β-carotene 1	52	6	94	0	11.8 ± 1.1	25 %	<0.02
H_2O_2+β-carotene 2	64	23	77	0	10.5 ± 1.3	35%	<0.02

Table IIb. Effect of β-carotene on H_2O_2-induced SCE (fixing time 30 h)

Treatment	MI/1000	M_1	M_2	M_3	SCE ± SE per cell	R	*P*
Control	45	6	94	0	5.4 ± 0.8		
H_2O_2 (10^{-4} M)	29	8	92	0	21.4 ± 1.4		
β-Carotene 1 (10^{-6} M)	42	13	87	0	5.1 ± 0.5		
β-Carotene 2 (10^{-5} M)	50	10	90	0	4.4 ± 0.5		
H_2O_2+β-carotene 1	138	22	78	0	13.5 ± 2.2	35%	<<0.01
H_2O_2+β-carotene 2	70	22	78	0	12.6 ± 1.9	40%	<<0.001

R, percentage of reduction; *P*, two-tailed *P* value. Alternate *t*-test.

Table III. Effect of ascorbic acid on H_2O_2 and bleomycin-induced ChAb

Treatment	Ascorbic acid (M)	Cells scored	% Abnormal cells	Aberrations per 100 cells						MI/1000
				Gaps	Chromosomal breaks	Isochromosomal breaks	Chromosomal exchange	Dicentric rings	Total (-gaps)	
–	–	200	6	5	2	–	–	–	2	80
–	10^{-6}	200	7	4	1	1	2	–	4	83
–	10^{-5}	200	11	7	2	3	1	–	6	89
H_2O_2 (10^{-4}M)	–	200	25	4	4	10	14	4	32	61
H_2O_2 (10^{-4}M)	10^{-6}	200	39	4	9	19	17	15	60*	76
H_2O_2 (10^{-4}M)	10^{-5}	200	39	5	9	29	18	9	65*	78
Bleomycin (1 μg/ml)	–	200	28	9	7	13	5	2	27	62
Bleomycin (1 μg/ml)	10^{-6}	200	29	8	11	16	4	14	42*	70
Bleomycin (1 μg/ml)	10^{-5}	200	23	4	4	25	11	4	44*	52

*Significant according to Student's *t*-test *P*

Table lV. Effect of β-carotene on H_2O_2 and bleomycin-induced ChAb

Treatment	β-Carotene (M)	Cells scored	% Abnormal cells	Aberrations per 100 cells						MI/10000
				Gaps	Chromosomal breaks	Isochromosomal breaks	Chromosomal exchange	Dicentric rings	Total (-gaps)	
–	–	200	5	3	2	–	–	–	2	108
–	10^{-7}	200	4	3	1	–	–	–	1	99
–	10^{-6}	200	8	6	2	–	–	–	2	102
H_2O_2 (10^{-4} M)	–	200	27	6	1	7	4	13	25	98
H_2O_2 (10^{-4} M)	10^{-7}	200	34	8	5	25	5	13	48	129
H_2O_2 (10^{-4} M)	10^{-6}	200	42	6	7	25	17	26	75*	114
Bleomycin (1 μg/ml)	-	200	20	2	8	21	6	10	45	98
Bleomycin (1 μg/ml)	10^{-7}	200	22	4	16	19	15	6	56	74
Bleomycin (1 μg/ml)	10^{-6}	200	24	4	3	36	2	14	55	64

*Significant according to Student's *t*-test, $P < 0.05$.

(Figure 2B). In Figure 3 we report the values of mean cell fluorescence ± SD, expressed in a.u. The fluorescence intensity varies from 353.7 in the case of H_2O_2 treatment to 181.5 in the case of combined treatment with the highest dose of ascorbic acid; in the case of β-carotene, the value varies from 589.5 to 281.5 with the highest dose of β-carotene. These data show the capacity of both agents to scavenge oxygen species produced by H_2O_2 treatment, with a consequent reduction in mean fluorescence intensity.

In our experimental conditions we showed the ability of both vitamins to strongly reduce SCE induced by H_2O_2. These data are in agreement with the alleged scavenger activity of

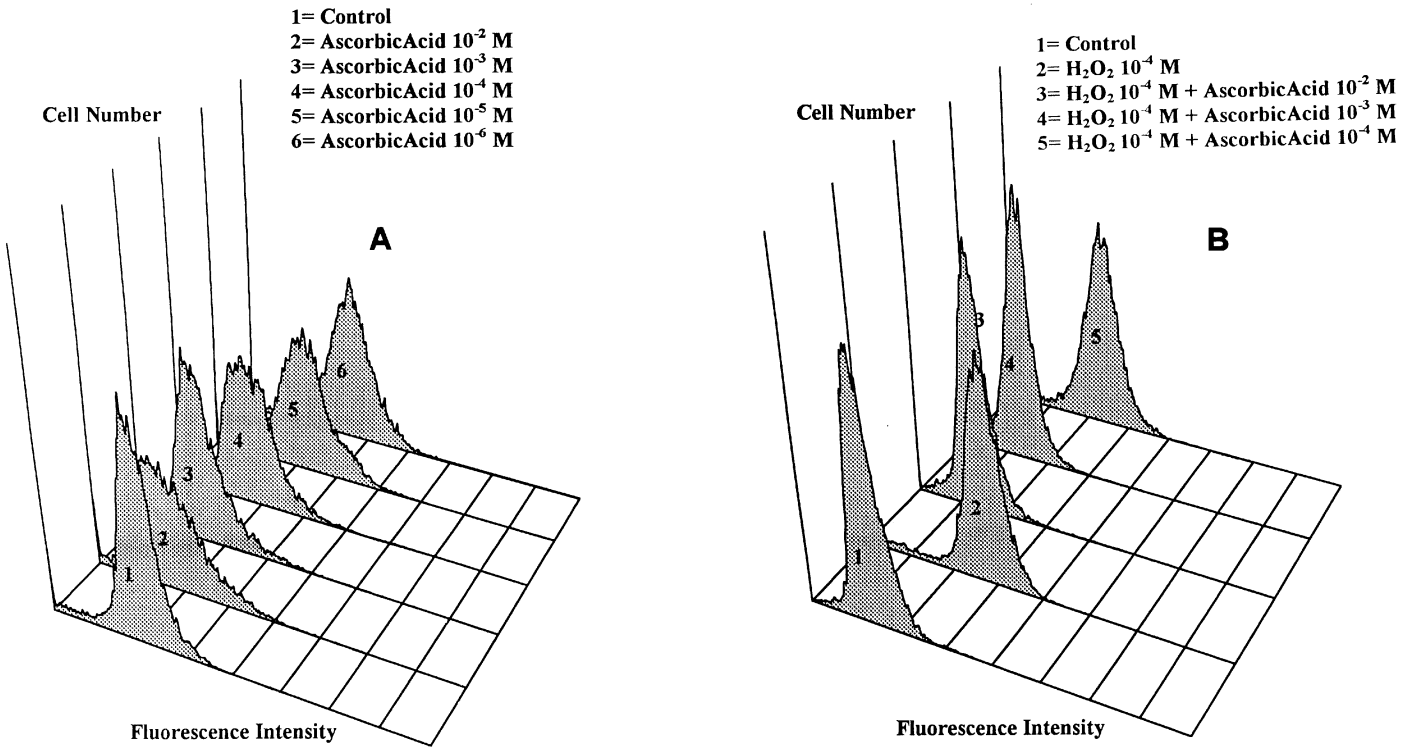

Fig. 1. Flow cytometric histograms of DCFH oxidation in cells treated with: (**A**) ascorbic acid; (**B**) ascorbic acid and H_2O_2.

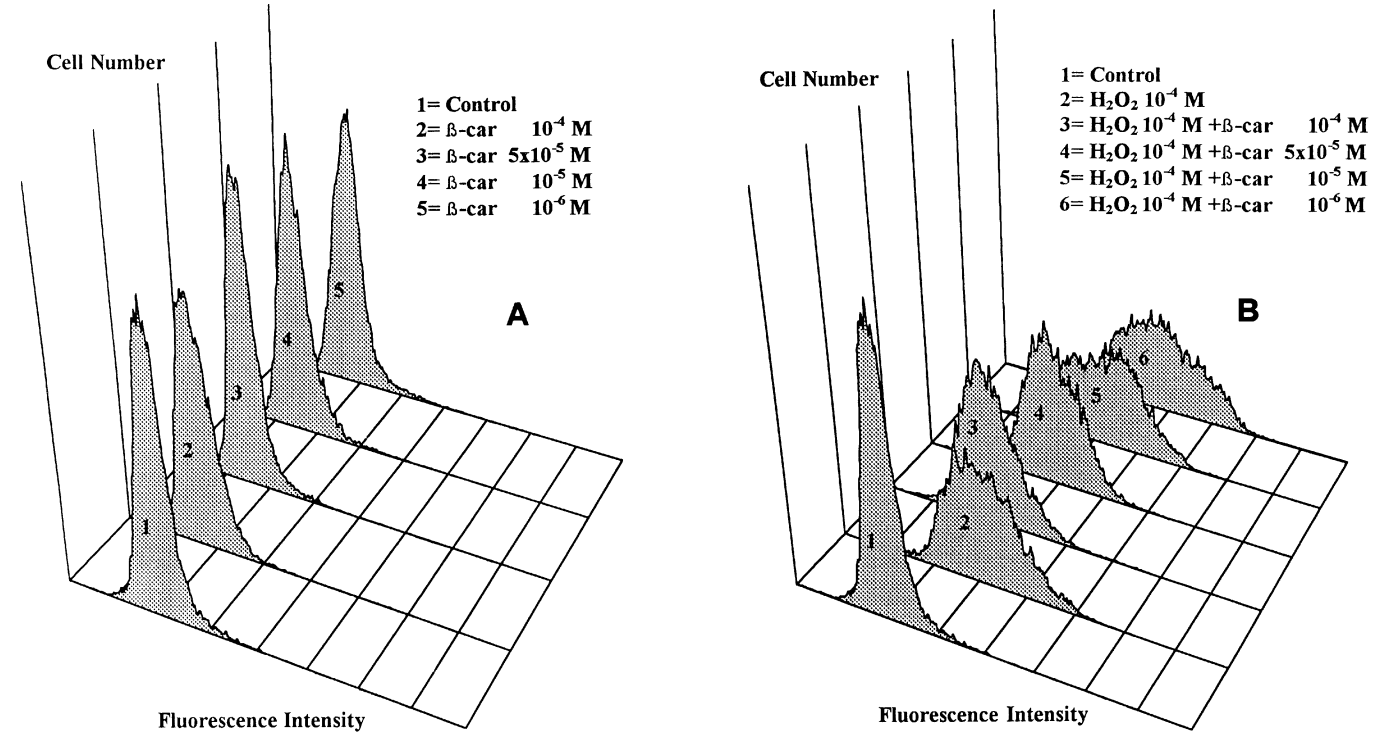

Fig. 2. Flow cytometric histograms of DCFH oxidation in cells treated with: (**A**) β-carotene; (**B**) β-carotene and H_2O_2.

ascorbic acid and β-carotene, which can lead to a reduction of active species responsible for chromosome damage. The ability of both ascorbic acid and β-carotene to 'trap' oxygen species generated by H_2O_2 within the cells is demonstated in the cytofluorometric test (DCFH-test) where we found a drastic reduction of fluorescent cells in combined treatments compared with H_2O_2 alone. This test was first described as a fluorometric assay of H_2O_2 by Keston and Brandt (20). It was recently used in several studies on the effect of reactive oxygen species in cell cultures (21–23). The assay is based on the premise that DCFH-DA crosses cell membranes and is intracellularly hydrolyzed to nonfluorescent DCFH. In the presence of reactive oxygen species DCFH is rapidly oxidized to highly fluorescent dichlorofluorescein quantified as fluorescent cells. With this

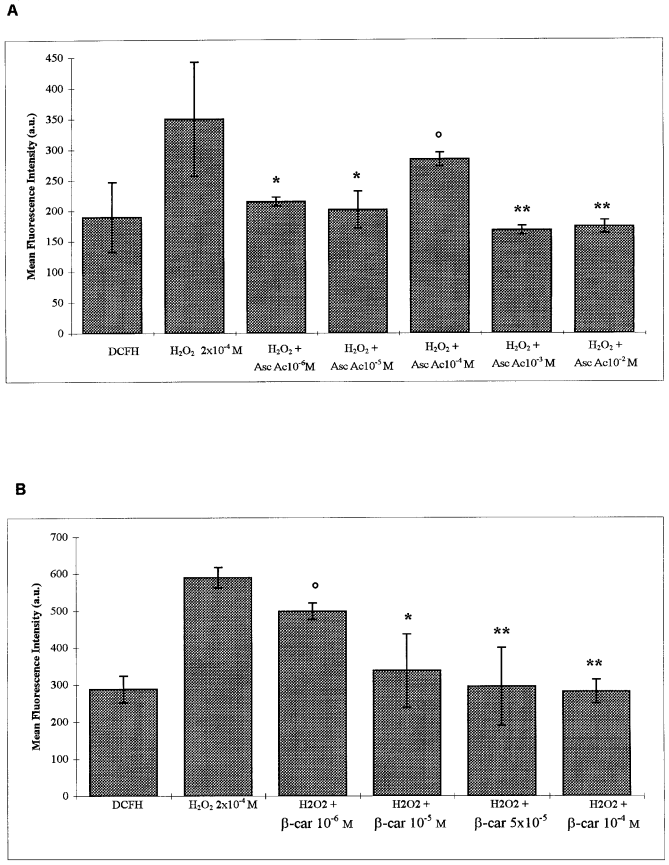

Fig. 3. Mean cell fluorescence ± SD in cells treated with: (**A**) H_2O_2 and ascorbic acid; (**B**) H_2O_2 and β-carotene. ° not significant to Student's *t*-test; * significant to Student's *t*-test, $P < 0.05$; ** significant to Student's *t*-test, $P < 0.01$.

test, we are able to exclude that ascorbic acid, even at the highest concentrations, produces H_2O_2 and free radicals through auto-oxidation but, on the contrary, causes a dose-related reduction of endogenous H_2O_2 due to oxidative metabolism.

In our DCFH-test, bleomycin failed to increase fluorescent cells. In fact, as reported by Lopez-Larraza *et al.* (24), bleomycin acts during the initial 15 min of treatment by free radical production; nevertheless it is also inactivated by the same reactive oxygen species produced (25,26); probably this mechanism of action renders the identification of the oxygen species by DCF production impossible. More intriguing is the situation concerning induced ChAb. Ascorbic acid and β-carotene, particularly at the highest dose, significantly increase all the types of H_2O_2-induced aberrations. These data agree with those obtained by Anderson *et al.* (27) in humam lymphocytes, suggesting that H_2O_2 induces chromosome aberrations through hydroxyl radicals ($^{\bullet}OH$) which are not scavenged by ascorbic acid and β-carotene. On the other hand, using the DCFH-test it is still unclear if hydroxyl radicals stimulate the formation of fluorescent DCF (28).

In combined treatment, ascorbic acid increased bleomycin-induced aberrations, in agreement with data shown by Anderson *et al.* (27) with the Comet assay; conversely, Anderson *et al.* (29) showed that, at the highest doses, ascorbic acid abolished ChAb and decreased micronuclei induced by bleomycin in human lymphocytes.When we used β-carotene, no effect was observed on chromosome aberrations induced by bleomycin. Salvadori *et al.* (30) reported a potentiation of the clastogenicity of bleomycin by β-carotene, on CHO cells.

Probably these contradictory data can be accounted for by the hypothesis of Buettner and Mosely (31). They proposed that ascorbate protects the cells from bleomycin toxicity rendering it inactive prior to DNA binding. But when bleomycin reaches DNA prior to interacting with ascorbate, it initiates DNA strand scission. On the other hand Salvadori *et al.* (30) proposed that bleomycin is destroyed by the reactive oxygen species produced by itself, and the presence of a scavenger, such as ascorbic acid and β-carotene, could cancel or reduce this detoxification.

On the basis of these data, we have to conclude that the exact mechanism of action of ascorbic acid and β-carotene is still unclear, although the DCFH test has shown the ability of both agents to scavenge, at least, oxygen species produced by the mutagen used. Furthermore we underline the importance of coupling to this type of analysis, several tests of chromosomal damages in order to verify the extent to which the activity of entrapment of oxidative molecular species leads to an effective reduction of damage at the chromosomal level. In our work, this proved true for SCE, but not for ChAb. Contradictory results in this direction are liable to render less certain studies aimed at establishing the true effectiveness of this type of compound in the chemoprevention of cancer.

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