ONCOLOGY

Cytotoxicity of Carotenoid Preparations in Human Epidermoid Carcinoma Cell Culture

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Effects of various β-carotene preparations on the viability and proliferation of A431 cells were studied. Colloid water dispersion of β-carotene stabilized with proxanol and containing the minimal number of components was shown to exhibit the highest level of incorporation in the cells, to be nontoxic, and to weakly inhibit cell growth.

Key Words: *carotenoids; cytotoxicity; cell culture*

The number of studies of the biological function of carotenoids *in vivo* and *in vitro* is increasing year by year [7,8,11]. At present β-carotene is widely used in the food and pharmaceutical industry and in agriculture as a prophylactic component of various agents. However, the data on the effects of carotenoids on humans and experimental animals are contradictory [7], primarily due to differences in the dosage forms of carotenoids used in the studies. The spectrum of these forms is broad and includes suspensions and solutions in vegetable oil, aqueous dispersions, liposomal agents, and carotenoid solutions in different organic solvents and even crystals [2,3,5,9]. The shortcomings of these preparations are the low concentration of carotenoid in aqueous "solution"; the large size of dispersion particles, responsible for poor incorporation of carotenoids in the cells; toxicity and independent effects of the added ingredients in these products.

The purpose of this study was to develop a β-carotene preparation suitable for introducing in a cell culture and to investigate its biological properties.

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MATERIALS AND METHODS

A431 cells were cultured in a mixture of Eagle's medium and medium 199 in a 1:1 ratio with 10% fetal calf serum (FCS) and 50 μg/ml gentamicin. In the viability test the cells were grown to almost the confluent state (90-95%), after which a suspension in Hanks' solution was prepared. Test samples were incubated with the test preparations for 2 h at 37°C with stirring on a shaker. Viability of cells was assessed by the method of vital stain exclusion, using 0.5% trypan blue solution.

For the study of the effects of preparations on cell proliferation, the cells were inoculated in culture dishes (60 mm in diameter) in a dose of 5×10^5 cells per dish in the basic growth medium. After 24 h the FCS content was reduced from 10 to 6.5%, the culture medium was replaced, and preparation was added. The concentration of FCS was reduced to prolong the duration of culture growth phases. At certain times the number of grown cells was counted in the cultures in a hemocytometer and the protein content measured [10].

Incorporation of ^{3}H-thymidine in DNA was assessed by adding labeled thymidine in a concentration of 1 μCi/ml to the incubation medium on

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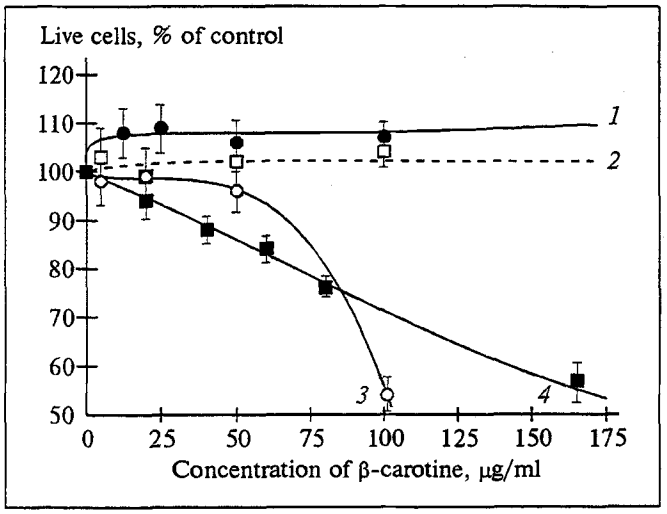

Fig. 1. Effects of various β−carotene preparations on the viability of A431 cells. *1*) proxanol−stabilized preparation; *2*) La Roche preparation; *3*) BASF preparation, *4*) Vetoron.

day 2 of incubation and incubating the culture for 18 h at 37°C. After sedimentation with trichloroacetic acid, the level of radioactivity was measured in a liquid scintillator (LKB).

For incorporation of β-carotene in liposomes we used a method described previously [1]. To prepare a dispersion of β-carotene stabilized with proxanol-286, 50 ml of a 2% β-carotene solution and 10% proxanol-286 in chloroform, heated to 50°C, was added to 100 ml distilled water heated to 70°C. The chloroform was removed under vacuum during incubation at 70°C and intensive stirring. The excess carotene precipitated in the form of crystals was filtered off. The concentration of carotenoids in colloid dispersions was measured by spectrophotometry from the absorption of carotene extract in hexane or petroleum ether, using the extinction coefficient 2550 at wavelength 451 nm for a 1% β-carotene solution. After sterilization by filtration (0.45 μ) the concentration of β-carotene in the resultant "solution" was 0.5-0.6%. The particle size in the resultant dispersion was assessed from light scattering by photon-correlation spectroscopy using an Autusizer 2c Malvern laser nephelometer (He-Ne laser, 633 nm, 10 mW).

To assess the accumulation of β-carotene, the cells were washed three times in phosphate buffer, dried, and extracted with a mixture of hexane and isopropanol in a 3:2 ratio. The concentration of β-carotene in lipid extracts was determined by spectrophotometry.

Synthetic crystalline β-carotene (98%) manufactured by the Moscow Experimental Vitamin Plant, water-soluble β-carotene (La Roche, BASF, Akva-MDT - brand name Vetoron), and proxanol-286 manufactured by the Research Institute of Organic Intermediate Products and Stains, Dolgoprudnyi, were used in the study.

RESULTS

The viability test with A431 cells showed (Fig. 1) that the microcrystalline forms of β-carotene (the proxanol-stabilized and La Roche preparations) were nontoxic in concentrations up to 100 μg/ml. Solubilized forms of β-carotene (Vetoron, BASF) including Tween-80 as emulsifying agent or other surfactants were characterized by pronounced toxicity when used in high concentrations. Carotenoid-containing liposomes obtained by sonication were toxic: at a β-carotene concentration of 2 μg/ml cell viability fell 22%, the degree of toxicity depending linearly on the duration of sonication. We believe this effect was due to the accumulation of lipid peroxides in the course of preparing the liposomes, because ultrasound treatment of carotenoids with a high affinity for oxygen [4] causes various products of interactions between oxygen and pigments promoting phosphatidylcholine peroxidation to accumulate [6].

In further studies of the cytotoxicity of β-carotene preparations we investigated the influence of a dispersion stabilized with proxanol-286 on the proliferative activity of cultured cells (Table 1). The addition of β-carotene in a concentration of 5 μg/ml led to a negligible (5 to 10%) decrease of proliferative activity as regards both cell count and protein content. Only when the concentration was increased fourfold did the inhibition of the prolifer-

TABLE 1. Effect of β−Carotene Dispersions on Proliferation of A431 Cells ($M \pm m$)

Time of incubation with agent, h	Control		β-Carotene			
			5 μg/ml (9.3 μM)		20 μg/ml (37 μM)	
	N	C	N	C	N	C
24	1250±66	0.60±0.03	1190±35	0.57±0.03	1070±27	0.52±0.03
48	2350±73	1.39±0.06	2240±46	1.27±0.05	2130±39	1.12±0.03
72	3170±121	1.51±0.07	2850±108	1.36±0.06	2630±151	1.32±0.02

Note. N: number of cells×10^{-3}/dish; C: content of protein, mg/dish.

TABLE 2. Content of β−Carotene in A431 Cells during Culturing in Media with Different Concentrations of FCS ($M \pm m$)

Serum concentration, %	β-carotene/flask, μg	Protein/flask, mg	β-carotene/protein, μg/mg
0.5	0.35±0.03	0.44±0.05	0.80
2.5	0.37±0.03	0.42±0.04	0.88
5.0	0.18±0.02	0.42±0.05	0.43
10.0	0.19±0.01	0.44±0.03	0.43

Note. Cultures in the logarithmic phase of growth were incubated with 20 μg/ml β−carotene for 24 h in a mixture of Eagle's medium and medium 199 (1:1) with different contents of FCS.

ative activity of cultured cells increase to 19%. Proxanol in a concentration of up to 0.3% did not inhibit the proliferation of cell cultures, and its concentration in the experiments did not surpass 0.02%.

The absence of a toxic effect was once more confirmed by experiments with the incorporation of ^{3}H-thymidine in cells treated with carotene in a 20 μg/ml concentration for 48 h; the incorporation did not reliably differ from the control level (0.45×10^3 cpm) and was 0.48×10^3 cpm per 10^3 cells.

The affinity of β-carotene for cultured cells is an important index of its biological functions. The results of studies of the biological functions of β-carotene are presented in Fig. 2. Accumulation of proxanol-stabilized β-carotene by A431 cells was 0.64 μg/mg protein at a β-carotene concentration of 37 μM in the culture medium. For the La Roche preparation this value was still lower: 0.16 μg/mg. Hence, the use of proxanol for the preparation of the water-soluble form of carotenoids ensures a high level of β-carotene incorporation in cultured cells, the magnitude of this incorporation being comparable to, and even higher than that for other forms [2,5]. This may be due to the properties of the polymeric surfactant and the size of the dispersion particles (about 0.2 μ). Moreover, in contrast to the La Roche preparation, which contains many other chemicals, the number of components in the proxanol-treated agent is minimal.

The incorporation of carotene from a colloid dispersion in cultured cells depended on the concentration of serum in the growth medium (Table 2). It may be assumed that at a 5-10% concentration of serum some particles of the β-carotene dispersion do not participate in binding to cells, forming associates with lipoproteins and proteins. The mechanism of this effect is slated for future study.

In conclusion we would note that there are reports of attempts at using various organic solvents for introducing β-carotene into cell cultures; the most successful of such attempts was the development of a stabilized microemulsion making use of tetrahydrofuran [2], but how this emulsion was

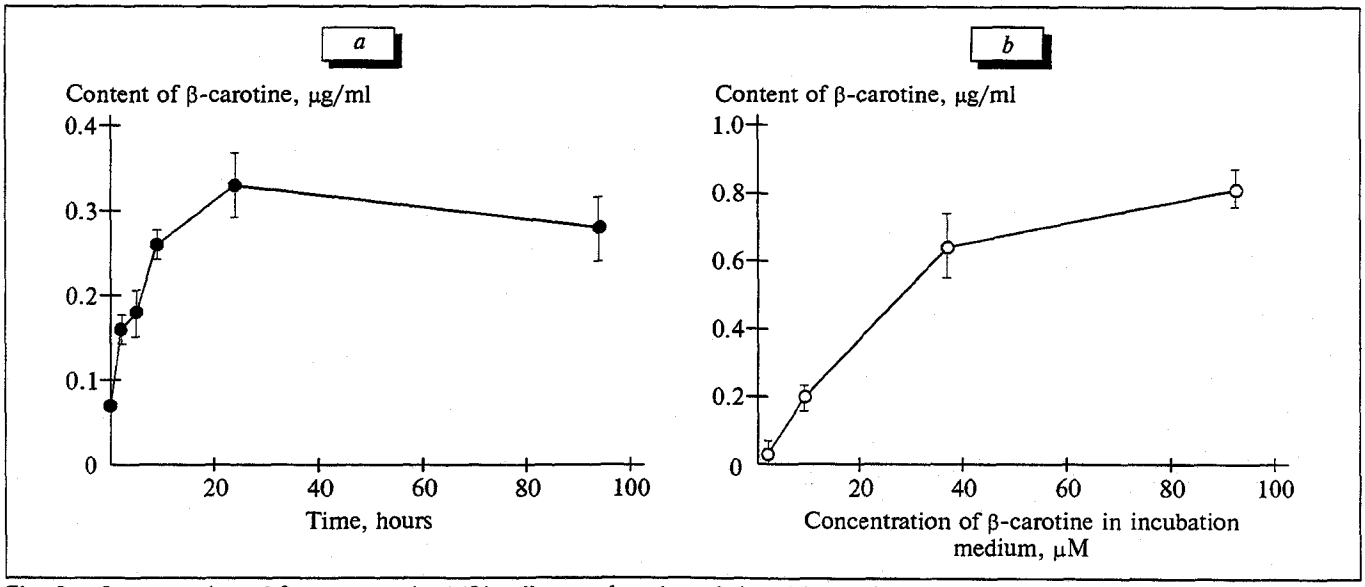

Fig. 2. Incorporation of β−carotene in A431 cells as a function of time (*a*) and the concentration of β−carotene in the culture medium (*b*). Confluent (90−95%) cultures were transplanted to medium with 3% FCS. *a*) after 18 h β−carotene was added in a final concentration of 10 μg/ml; *b*) β−carotene was added at the same time as the medium was replaced and after 18 h the β−carotene content determined in the cells.

formed is not clear. We believe that the use of a carotenoid dispersion with proxanol holds better promise, for this polymer has been long used in the food and medical industry for the stabilization of perfluorocarbons as components of artificial blood, and the method of preparing the agent on the basis of proxanol is simple and easily reproducible.

REFERENCES

1. A. D. Bangham, M. M. Standish, and J. C. Watkins, *J. Mol. Biol.*, **13**, 238 (1965).
2. J. S. Bertram, A. Pung, M. Churley, *et al.*, *Carcinogenesis*, **12**, 671 (1991).
3. Y. V. Bukin, D. Y. Zaridze, V. A. Draudin-Krylenko, *et al.*, *Eur. J. Cancer Prev.*, **2**, 61 (1993).
4. M. P. Di, S. Kaiser, and H. Sies, *Arch. Biochem. Biophys.*, **274**, 532 (1989).
5. P. Grolier, V. Azais-Braesco, L. Zelmire, and H. Fessi, *Biochim. Biophys. Acta*, **1111**, 135 (1992).
6. A. K. Jana, S. Agarwal, and S. N. Chatterjee, *Radiat. Res.*, **124**, 7 (1990).
7. N. I. Krinsky, *Annu. Rev. Nutr.*, **13**, 561 (1993).
8. N. I. Krinsky, *Experientia Suppl.*, **62**, 227 (1992).
9. M. A. Leo, C. Kim, N. Lowe, and C. S. Lieber, *Hepatology*, **15**, 883 (1992).
10. O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11. E. J. Rousseau, A. J. Davison, and B. Dunn, *Free Radic. Biol. Med.*, **13**, 407 (1992).

Intravitam Quantitative Assessment of the Intracellular Distribution of Doxorubicin in Tumor Cells

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Intravitam quantitative assessment of the intracellular distribution of doxorubicin was carried out in anthracycline-sensitive tumor cells of CaOv human ovarian carcinoma grown in a monolayer culture *in vitro* and in P388 leukemia cells transplanted to mice *in vivo*. The intracellular content of antibiotic unbound to DNA was assessed from the total fluorescence of cells at the end of incubation. The DNA-bound active fraction of intracellular doxorubicin makes up the bulk of intracellular doxorubicin in both cell lines, whereas only less than 30% of anthracycline accumulated in a cell is not bound to DNA. The results confirm that the predominant accumulation of doxorubicin in cell nuclei may characterize their sensitivity to anthracyclines.

Key Words: *doxorubicin; tumor cells; intracellular distribution*

The intracellular content of antitumor drugs is an important parameter determining their biological activity. This is indeed true for the anthracycline antibiotics, which for many years have been widely used in the chemotherapy of malignant tumors. Nevertheless, the mechanism of action of anthracyclines, and, specifically, the factors governing the development of resistance, are still not clear. For example, some authorities claim that anthracycline resistance correlates with a decrease of their content in the cell [6,12]. But such a relationship is not always observed [3,5], and the intracellular distribution of the antibiotics appears to be a more important factor, specifically, the amount interacting with DNA and accumulated in the nucleus [4,9], or the ratio of the nuclear to cytoplasmic fractions of the drug [8,11].

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