only the present generation but also the generations to come.

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β-Carotene Stimulation of the Reaction of Cell Immunity in Mice

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β-carotene, or provitamin A, is an effective drug possessing anti-infectious, radioprotective, and, in some cases, antitumor activities [9,15,17,18]. A variety of defense functions of β-carotene have been shown to be related to a great extent to its stimulatory effects on cell, humoral, and antitumor immunity [2,8,19].

The aim of the present study was to investigate the immunomodulatory influence of synthetic β-carotene and its novel nutritional microgranulated form (a Solnechnyi dry milk product, manufactured by the Research Institute of Milk Production, Stavropol') on the reactions of cell immunity in mice, T-cell mitogen-induced lymphocyte prolifera-

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tion, cytotoxic and suppressor activity of T cells and macrophages, and changes in adhesive properties and the production of reactive oxygen forms in monocyte-macrophages.

MATERIALS AND METHODS

Mice of the BALB/C (H-2^d), C57Bl/6 (H-2^b), and C3H (H-2^k) lines aged 1.5-2 months were used for the experiment.

The preparations tested were either an oil solution containing 0.17-0.34 mg β-carotene or microspheres containing 0.1-0.5 mg of active substance in a capsule made of natural casein (particle size 10-20 μ). For investigation of the effect of β-carotene on the reaction of blast transformation of lymphocytes (RBTL), cell proliferation in a mixed lymphocyte culture (MLC), and adhesiveness of macrophagal cells, C57Bl/6 mice were fed β-carotene in the form of microspheres continuously for 15, 30, and 45 days. In study of the effect of β-carotene on the cytotoxic and supressor activity of lymphocytes in BALB/C mice an oil solution of β-carotene was added to mouse chow during 4-5 days after and 5 days prior to immunization with allogenic cells.

Potter homogenizers were used for preparation of the spleen lymphocyte suspension. T cells were separated by passing 2.5×10^7 spleen cells through a nylon wool column with a 30-min incubation at 37°C and eluting nonadherent cells with an experimental medium [13]. In the adhesion test $2\text{-}3\times10^7$ spleen cells were incubated at 37°C for 45 min in Petri dishes pretreated with fetal calf serum (FCS) [14]. Attached cells, the macrophage-enriched lymphocyte fraction, were then harvested with 0.25% trypsin solution, while nonadherent cells were used in some experiments as a T-cell-enriched lymphocyte suspension. In all experiments spleen lymphocytes and isolated T cells and macrophages were cultured in RPMI-1640 medium supplemented with 10% FCS or bovine serum, 1% 200 M L-glutamate, and 40 IU/ml gentamicin.

Allogen-induced generation of reactive oxygen forms by macrophages was assessed by measuring their chemiluminescence in the presence of luminol with an S1-3603 device, and the data were recorded by a computer [5].

In the RBTL 3×10^5 cells were incubated for 3 days with 1-5 μg concanavalin A (ConA) in 96-well plates (37°C, 5% CO_2). For allogenic MLC 5×10^6 reacting cells were added to 1×10^6 γ-irradiated (15 Gy) stimulatory cells, and the mixture was incubated as described above. At the end of incubation ^{3}H-thymidine was added to the wells

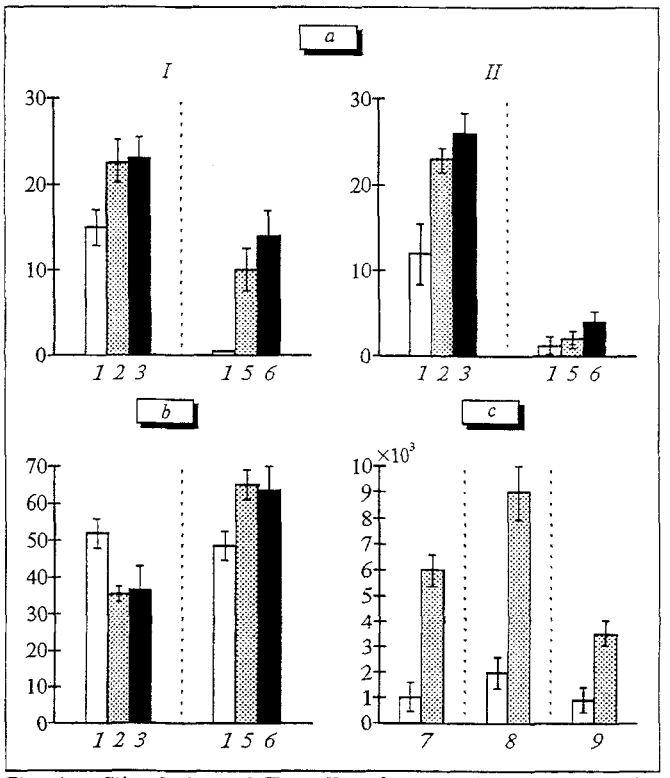

Fig. 1. Stimulation of T-cell and macrophage cytotoxicity (*a*), adhesiveness of macrophagal cells (*b*), and generation of reactive oxygen forms (*c*) induced by β-carotene administration in BALB/c mice before and after intraperitoneal immunization with splenocytes from C3H mice. Ordinate: *a*) cytotoxicity index (%) of T cells non-adherent to nylon wool (*1,2,3*) and of macrophages adherent to plastic (*4,5,6*) in the cytotoxicity test against L-929 (*I*) and VAC-1 (*II*) target cells, 50:1 effector/target ratio; *b*) T cells nonadherent to nylon wool (*1,2,3*) and macrophages adherent to plastic (*4,5,6*); here and in *a*: *1,4*) nontreated mice; *2,5*) and *3,6*) mice treated with β-carotene in a dose of 0.17 mg/mouse in the form of oil solution before and after immunization, respectively; *c*) intensity of spontaneous and *Candida alba*-induced chemiluminescence; *7*) nontreated mice; *8,9*) mice treated with β-carotene in a dose of 0.17 mg/per mouse in the form of oil solution before and after immunization, respectively.

and the cells were transferred onto membrane filters with a harvester, followed by radioactivity determination. The stimulation index was calculated as the ratio between isotope incorporation into the experimental and control samples.

For activation of cell-mediated immunity the BALB/c mice were immunized i.p. with 2.5×10^7 splenocytes from C3H mice irradiated with 15 Gy. Killer activity was assessed in a 16-hour test with ^{51}Cr-labeled target tumor cells. In the cytotoxic test 1×10^4 ^{51}Cr-labeled L-929 and VAC-1 target cells were mixed with 5×10^5 effector lymphocytes in triplicate. The cytotoxic effect was calculated according to the formula: 100(Ex-S)/(M-S), where Ex, M, and S are the experimental, maximal (in the presence of 1% SDS), and spontaneous (without effectors) ^{51}Cr release, respectively.

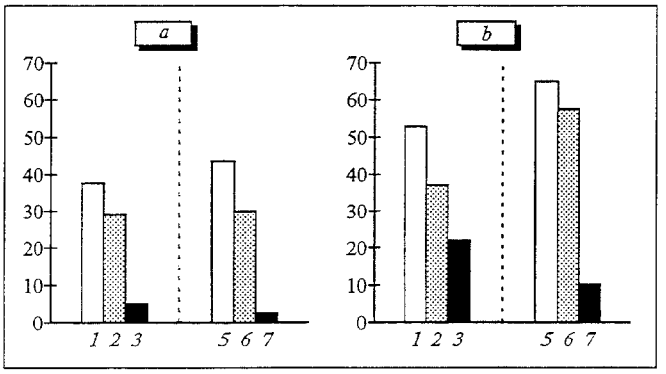

Fig. 2. Abolishment by β-carotene of suppressive activity of T cells inhibiting lymphocyte proliferation in RBTL (*a*) and MLC (*b*). Ordinate: suppressive activity of T cells in BALB/c mice immunized intravenously with splenocytes from C57Bl/6 mice; *1,4*) nontreated mice, *2,5*) and *3,6*) mice treated with β-carotene oil solution in a dose of 0.17 and 0.34 mg active substance/mouse, respectively.

For inducing T-cell suppression in the spleen of the BALB/c mice, these were immunized i.v. with 5×10^7 splenocytes from C57Bl/6 mice irradiated with 15 Gy [1]. Suppressor activity was determined by inhibition of lymphocyte proliferation in RBTL and MLC. To this end either 1.5×10^5 or 2.5×10^7 suppressor cells pretreated with mitomycin C were added to the same number of effector lymphocytes. The percentage suppression was calculated by the formula: $100(a-b)/a$, where a and b are the thymidine incorporation into the control and experimental samples (without and with suppressor cells, respectively).

RESULTS

Long-term supplementation of mouse chow with microspheres containing 0.1-0.5 β-carotene per mouse resulted in an enhanced proliferative response to ConA of both the total spleen lymphocyte suspension and isolated T cells. The maximal ^{3}H-thymidine incorporation into proliferating cells was observed on day 30 of the treatment, followed by a gradual decrease of the β-carotene-stimulated proliferative activity. The dynamics of the proliferative response to ConA correlated with an increase in adhesiveness of macrophagal cells. Addition of β-carotene-free microspheres (placebo) to mouse chow according to the same scheme boosted the proliferative response in both the total spleen lymphocyte fraction and purified T cells, although the stimulation index in RBTL was lower than that in the β-carotene-fed group. The only exceptions are the results obtained for nonfractionated lymphocytes on day 15, and for T cells on days 30 and 45 of preparation administration.

The oil solution of β-carotene administered *per os* to BALB/c mice (0.17 mg per mouse) immunized with splenocytes from C3H mice increased the proliferation of effector cells in the allogenic MLC by 1.7 times and the cytotoxicity of T cells and macrophages isolated from the spleen of immunized mice (Fig. 1, *a*). An enhanced cytotoxicity of T cells against L-929 and VAC-1 cells was elicited by β-carotene administered either 5 days prior to or during 5 days daily after immunization, while the cytotoxicity of macrophages against L-929, but not VAC-1, was noted to rise in both experiments. The β-carotene-induced stimulation of cytotoxic activity of macrophagal cells correlated with their adhesiveness to nylon wool and their ability of producing reactive oxygen forms, detected by chemiluminescence. For example, administration of β-carotene 5 days prior to and during 5 days after immunization reduced the number of nonadherent T cell and increased the share of macrophagal cells attached to the nylon wool (Fig. 1, *b*). When administered 5 days before immunization β-carotene stimulated the generation of reactive oxygen forms induced by phagocytosis of *Candida alba* (Fig. 1, *c*).

Following administration to BALB/c mice of an oil solution containing 0.17-0.34 mg β-carotene per mouse, a lowered level of T-cell suppressor activity induced by intravenous immunization with allogenic splenocytes from C57Bl/6 mice was observed 4 days postimmunization (Fig. 2). The dose-dependent abolishment of T suppression was assessed according to the inhibition of the proliferation of nonfractionated and T cells from BALB/c mice in response to ConA and in allogenic MLC.

The results are in accordance with published data on the stimulation by polyclonal T-cell mitogens of the proliferative response of rat and bovine T cells as well as human $OKT4^+$ cells [7,8,10].

Our previous data on the possible induction by β-carotene of cytotoxic T lymphocytes in MLC are extended with new data on the stimulatory effect of this immunomodulator on the cytotoxic activity of both T cells in the natural killer population and macrophages in allogen-induced killers [4].

The β-carotene-induced increase in adhesiveness of macrophagal cells from normal and immunized mice and their enhanced antitumor cytotoxicity shown here are in conformity with previous reports on the enhanced phagocytotic capacity of human monocytes and augmented secretion of the antitumor cytotoxic factor by murine macrophages caused by β-carotene [6,16]. The enhanced cytotoxic activity of macrophages against L-929 probably resulted from a dramatically increased genera-

tion of reactive oxygen forms by cells of macrophagal lineage for β-carotene administration 5 days prior to allogenic immunization, these being the actual cause of tumor cell death.

Of particular interest are the data on the decreased suppressive activity of T cells inhibiting lymphocyte proliferation in the RBTL and MLC against the background of stimulated cell immunity caused, like immunosuppression, by transplantation antigens. The administration of β-carotene appears to promote a shift in the immunological balance in the murine spleen toward the formation of cytotoxic T cells and macrophages. It may be hypothesized that the stimulatory effect on antitumor immunity is related to the ability of the product of β-carotene enzymatic transformation, vitamin A, to alter the antigen density on the surface of antigen-presenting cells through glycosylation of the cell membranes, as well as stimulation of interleukin-2 secretion by helper cells, thus promoting recognition of transplantation antigens by specific T killers and their accelerated proliferation and differentiation [3,11,12,20,21]. On the other hand, β-carotene is capable of enhancing the secretion of antitumor cytokines in macrophages and of increasing the cytotoxic activity of T cells in the natural killer population by inducing the expression of additional receptors on activated lymphocytes, as mentioned above.

This study demonstrated the activation of cell immunity in mice by synthetic β-carotene administered either in the form of oil solution or in microspheres, providing a basis for developing therapeutic and preventive remedies derived from β-carotene and for their clinical application.

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