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Ascorbic acid suppresses natural killer cell activity in *Bufo marinus**

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ABSTRACT: The effect of ascorbic acid on natural killer (NK) cells in the marine toad *Bufo marinus* has been analyzed in vitro and in vivo. Peripheral blood leukocytes (PBL) which were pre-incubated with ascorbic acid for 1 h at different concentrations (1×10^3, 1×10^4 and 1×10^5 M), exhibited a significant inhibition of NK cell activity (31.8 to 77.3 %). Similarly, injection of ascorbic acid into the dorsal lymph sac of toads at 10 mg kg^{-1} d^{-1} inhibited NK cell activity; 16.1 % at 1 wk and 25.8 % at 3 wk. Suppression of NK activity by ascorbic acid in vivo and in vitro occurs without changing the binding capacity of effector cells to YAC-1 tumor targets.

INTRODUCTION

The antitumor activity of ascorbic acid (vitamin C) has been examined by several investigators, for example Pauling (1980) found that vitamin C inhibits growth of tumors. In other analyses ascorbic acid inhibited the appearance of dimethylbenzanthracene (DMBA) induced papillomas of the skin of toads *Bufo regularis* and also caused regression of established papillomas (Sadek & Abdelmeguid 1982). Amphibians have a well developed immune system that consists of major cellular and humoral components (Cooper 1976). Recently, our studies showed that the immune system of leopard frogs *Rana pipiens* possesses inducible killer (IK) cells (CTL-like) and spontaneous killer (SK) cells (natural killer-like) which effect lytic responses against allogeneic cells (Ghoneum & Cooper 1987) and the tumor cell line YAC-1 (a T cell lymphoma of mouse origin) (Ghoneum et al. 1990). NK cells are considered to be a possible first line of defense in antitumor immunosurveillance (Herberman 1982, Ghoneum et al. 1987).

Factors enhancing NK cell activity could thus be relevant to enhancement of resistance to tumor growth and metastasis. To determine a possible influence of vitamin C on amphibian NK cells, we analyzed the effects of ascorbic acid on toad NK cell activity both in vitro and in vivo.

MATERIAL AND METHODS

Toads. We used *Bufo marinus* of both sexes, purchased from St. Croix Biological, Stillwater, Minnesota, USA. They were kept in plastic pans with water which was changed twice weekly. They were fed meal worms twice weekly.

Preparation of peripheral blood leukocytes (PBL). Toads were sacrificed after anesthetization with ether. Heparinized peripheral blood was obtained by cardiac puncture and the blood was centrifuged in Wintrobe tubes. The buffy coat was carefully removed and washed twice in Amphibian Ringer Solution (ARS) (Ghoneum & Cooper 1987).

Target cells. The YAC-1 tumor cell line (a Maloney leukemia virus-induced mouse T-cell lymphoma of A/Sn mouse origin) was maintained in our laboratory in complete medium (CM) consisting of RPMI-1640, supplemented with 1 % antibiotics (100 U penicillin and 100 µg ml^{-1} streptomycin).

Ascorbic acid. Ascorbic acid was purchased from Sigma Chemical Company, St. Louis, Michigan, USA. For in vitro studies, ascorbic acid was dissolved in phosphate buffered saline (PBS) and adjusted at con-

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centrations of 10^{-3} to 10^{-5} M. PBL were incubated with ascorbic acid for 1 h at room temperature then washed twice with ARS. For in vivo experiments, toads were injected with vitamin C into the dorsal lymph sac at a dose of 10 mg kg^{-1} d^{-1}, and NK cell activity was examined at 1 and 3 wk post-treatment.

Cytotoxic assay. YAC-1 target cells (5×10^6) were labeled with 100 μCi sodium chromate solution (New England Nuclear, Boston, Massachusetts, USA) for 1 h and washed 4 times in 5 ml Hanks' balanced salt solution (HBSS) as previously described (Ghoneum et al. 1987). Target cells (1×10^4) in 0.1 ml CM were pipetted into 96 well-round bottom Linbro plates. Effector cells were suspended at 10×10^6 ml^{-1} ARS, and pipetted into quadruplicate wells to give effector:target (E:T) cell ratios of 25:1, 50:1 and 100:1. Following incubation for 4 h at room temperature, plates were centrifuged at $400 \times g$ for 5 min and 0.1 ml supernatant from each well collected and counted in triplicate in a Gamma counter. The percentage of isotope released was calculated using the following formula:

$$\% \text{ Lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Spontaneous release from target cells was always 8 to 10 % of total release; maximum release was measured by adding 0.1 ml Triton X100; all values in counts min^{-1}.

Conjugate formation. The method previously described by Kumagai et al. (1982) was followed. In brief, PBL (1×10^5) were incubated with YAC-1 target cells (1×10^6) in 1 ml of ARS in 12 × 75 mm glass tubes, sedimented by centrifugation at $130 \times g$ for 5 min and incubated for 1 h at 4 °C. Pellets were resuspended and cytocentrifuged smears stained with Giemsa. The percent of conjugates was examined by counting 200 lymphocytes (bound and free) in triplicate samples.

Lytic units (LU). LUs were calculated from effector titration curves according to our previously demonstrated procedure (Ghoneum et al. 1987). One LU was defined as the number of effector cells required to achieve 10 % lysis; LU × 10^{-7} is the number of LUs in 10 million effector cells.

Statistical analysis. A 2-tailed Student's t-test was used to determine the significance of difference between different experimental groups at $p < 0.05$.

RESULTS

In vitro effects

PBL which were preincubated with ascorbic acid for 1 h inhibited NK cell activity in a dose response relationship: 77.3 % at 1×10^3; 63.4 % at 1×10^4 and 31.8 % at 1×10^5 M ascorbic acid (Table 1). This inhibitory effect was detected at all effector leukocyte target ratios, 25:1, 50:1 and 100:1. Further experiments were conducted to examine the effect of vitamin C on the binding capacity of NK cells to their target tumor cells. Fig. 1A illustrates that treatment with ascorbic acid caused a non significant change in the percent of conjugate formation, as compared with effector cells cultured in CM alone. Trypan blue dye exclusion indicated that ascorbic acid did not significantly affect the viability of effector cells at the concentrations tested.

Table 1. *Bufo marinus*. Natural killer cell activity in peripheral blood leukocytes (PBL) after incubation with ascorbic acid. Cytotoxicity is a 4 h ^{51}Cr-release assay. Data represent mean ± SD of 5 toads in each group. Lytic units (LU's) were calculated from effector titration curves according to our previously demonstrated procedure (Ghoneum et al. 1987)

Conc. (M)	Cytotoxicity (%) 25:1	50:1	100:1	LU	% Inhibition of control
Control	4.3 ± 1.3	10.3 ± 1.3	18.4 ± 3.4	22	–
1×10^3	1.1 ± 0.1	1.8 ± 0.5	4.1 ± 1.8	5	77.3
1×10^4	2.1 ± 0.4	3.6 ± 1.0	7.6 ± 2.8	8	63.4
1×10^5	3.2 ± 0.8	7.2 ± 2.5	13.6 ± 2.3	15	31.8

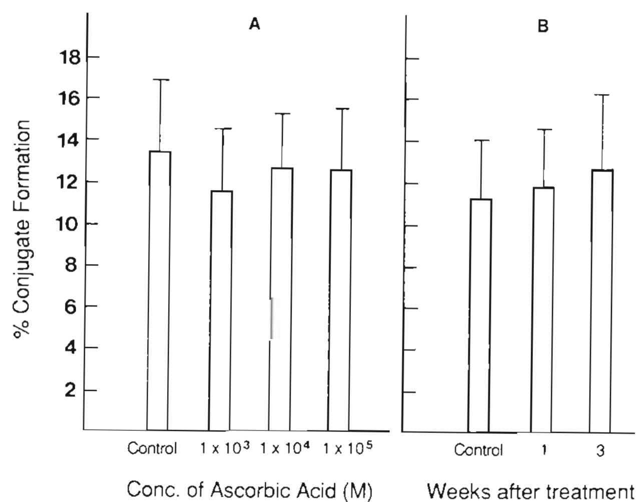

Fig. 1. *Bufo marinus*. Effect of ascorbic acid on percent conjugate formation between effector natural killer (NK) cells and YAC-1 tumor target cells. (A) Peripheral blood leukocytes were treated in vitro with ascorbic acid at varying concentrations for 1 h after which percent conjugates determined. (B) Toads injected with ascorbic acid. Conjugate formation determined 1 and 3 wk post injection

In vivo effects

Data in Table 2 demonstrated that vitamin C inhibits NK cell activity in a time-dependent manner. NK cell activity was inhibited (16.1%) at Week 1 and had decreased further, to 25.8% by Week 3, when compared to controls. Inhibition of NK cell activity was not correlated with changes in the percent of conjugate formation between effector cells and YAC-1 tumor cells. The percent of conjugates was nearly the same in treated and nontreated groups (Fig. 1B).

Table 2. *Bufo marinus*. In vivo effects of ascorbic acid on natural killer cell activity. Cytotoxicity measured as in Table 1. Time represents weeks post ascorbic acid injection. Data represent mean ± SD of 5 toads in each group

Time (wk)	Cytotoxicity (%) 25:1	50:1	100:1	LU	% Inhibition of control
Control	4.8 ±1.9	10.2 ± 2.4	19.1 ± 3.6	21.7	–
1	3.1 ±1.6	8.1 ± 2.5	16.2 ± 3.9	18.2	16.1
3	2.4 ±1.2	7.8 ± 2.9	13.8 ± 2.9	16.1	25.8

DISCUSSION

Ascorbic acid inhibited toad *Bufo marinus* NK cell activity in vivo and in vitro, which agrees with findings using human NK cells (Huwyler et al. 1985). The mechanism by which vitamin C affects NK activity is not fully understood, but could be attributed to the quick reaction of vitamin C with hydroxyl radicals (Anbar & Neta 1967) and oxygen radicals (Nishikimi 1976), where it is oxidized to hydroascorbate. These hydroxyl radical scavengers have an inhibitory function against NK activity (Sythanthiran et al. 1984). Further studies by Huwyler et al. (1985), demonstrated suppression in activity of NK cells after culturing them with vitamin C at 37 °C, whereas NK cells incubated with vitamin C at 4 °C exhibited normal activity. Therefore, ascorbic acid may act as a radical scavenger on NK cells thus suppressing their cytotoxic reactivity without interfering with the binding capacity (of these effectors) to tumor target cells. The effect of vitamin C seems to be cytostatic and not cytotoxic, since trypan-blue exclusion-test demonstrated that vitamin C did not affect the viability of PBL.

The inhibitory effect of vitamin C could also be attributed to its ability to increase the production of Prostaglandin E2 (PGE2) which is known to inhibit NK activity (Siegel & Morton 1984). In contrast, other studies demonstrated an immune modulatory effect of vitamin C as manifested by activation of T cell-mediated immunity and enhancement of interferon (IFN) production, which would not include alterations in NK activity (Siegel & Morton 1983). The reason for such controversy is unknown, but may be attributed to differences in animal models and the way of administering vitamin C – while Siegel & Morton (1984) mixed vitamin C with the drinking water of mice, we injected vitamin C into the dorsal lymph sac of toads. Or, it could be due to the source of NK cells, i.e. murine spleen vs amphibian PBL.

Previous studies demonstrated that ascorbic acid caused regression of skin papillomas in the Egyptian toad *Bufo regularis* (Sadek & Abdelmequid 1982). According to an earlier study by Siegel (1975), vitamin C may protect against some viral infections by enhancing IFN production. It is well-known that IFN exerts anticancer activity in experimental animals (Balkwill 1985). Such conflicting reports make the mechanism of antitumor activity of ascorbic acid more complicated. Since NK cell activity is augmented by IFN, ascorbic acid may exert its effect via several unknown interactions. Much additional study is required to understand the mechanism(s) of action of ascorbic acid on cell mediated immune (CMI) function and its relationship to immune-surveillance.

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