

## Vitamin C and Immunity: Influence of Ascorbate on Prostaglandin E<sub>2</sub> Synthesis and Implications for Natural Killer Cell Activity

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*Summary: A regimen of vitamin C in the drinking water of several strains of inbred mice has been observed previously to be without substantial effect on natural killer (NK) cell activity. In the present study, macrophage cultures derived from bone marrow of a number of these strains showed, on the average, a 90-100 % increase in prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production when ascorbate was added. This latter finding might suggest an explanation for the apparent failure of ascorbate to augment NK activity while enhancing interferon production.*

### Introduction

Earlier studies [10] have demonstrated an increased response to interferon induction in mice fed a diet containing vitamin C, and similar findings have been reported [11] in mouse cell cultures. In other studies [4] it has been noted that interferon increased the tumoricidal activity of natural killer cells, effector cells mediating cell-mediated cytotoxicity naturally, that is, in the absence of deliberate prior immunization or sensitization. Subsequent investigation to determine whether ascorbic acid in its role as immunomodulator [12, 13] would augment NK activity revealed that this was not the case [14]. In the present report we have ventured to advance a possible explanation for this paradoxical observation.

### Materials and Methods

C57B1/6, BALB/c and DBA/2 mouse strains originally purchased from the Jackson Laboratory, Bar Harbor, Maine, were bred and raised in the Oregon School of Medicine animal care facility. The method employed for preparing pure cultures of bone marrow-derived macrophages from these strains was essentially that of HAVELL and SPITALNY [6]. Cells were obtained by perfusing femurs and tibiae with 1-2 ml of

Hanks' balanced salt solution. After centrifugation cell pellets were suspended at a concentration of  $2 \times 10^6$  cells/ml and  $35 \times 10$  mm dishes seeded with 1 ml of the cell suspension in culture medium. The growth medium consisted of RPMI-1640 (GIBCO) medium containing 10% horse serum supplemented with 10% of 0.22  $\mu$ m filtered L-cell conditioned medium (*macrophage growth factor*) which was originally used to grow murine L929B cells. In the presence of this growth factor, murine bone marrow cells proliferated and differentiated into macrophages. The purity of the macrophage populations was established by positive esterase activity of the cultured cells with *q*-naphthyl butyrate as substrate and microscopic morphological criteria [5]. After 9 days in culture macrophages usually reached confluency, at which time cultures were replenished with fresh medium containing  $10^{-4}$  M ascorbic acid; control cultures received fresh medium only. After 24 h the medium was removed and cells extracted with 0.2% Triton X-100 in Tris-HCL buffer pH 7.8. Determinations for protein and prostaglandin E<sub>2</sub> were carried out on pools of 4-5 cultures, treated and controls, per mouse. Following protein assessment [8], cell-associated PGE<sub>2</sub> synthesis was evaluated using a modification of the method of SUN [16]. An aliquot of the cell extract was incubated for 10 min with 0.14  $\beta$ Ci of ( $1^{-14}$ C) arachidonic acid, 55  $\beta$ Ci/mmol (New England Nuclear), with shaking. The reaction mixture was adjusted to pH 3.5 with formic acid and extracted twice with ethyl acetate. Unlabeled PGE<sub>2</sub> was then added as a marker. After evaporation and resuspension in ethyl acetate, an aliquot of the extract was applied to a silica gel thin layer plate previously divided into channels, and developed in the solvent system dioxane: toluene: acetic acid (10:20:1). The location of the unlabeled PGE<sub>2</sub> was identified by exposing the plate to iodine vapor, and this sector as well as segments of the remainder of the entire channel were scraped into scintillation vials and counted.

### Results and Discussion

In previous studies [14] mice on a regimen of 250 mg % ascorbate in the drinking water for 4 to 5 weeks failed to demonstrate any increase in natural killer cell activity compared to water controls. This was not anticipated since ascorbic acid had been found previously to enhance the production of interferon, a known NK cell activator [20]. In the present study, an *in vitro* model was developed in an attempt to provide an explanation for this seemingly paradoxical observation.

Tab. I: Prostaglandin E<sub>2</sub> production in bone-marrow-derived macrophage cultures<sup>a</sup>.

Mouse strain	Treatment		% Increase
	Control	Vitamin C ( $10^{-4}$ M)	
BALB/c	7.94	18.2	129
	14.4	23.5	63 (101)
	7.67	16.1	110
DBA/2	26.1	53.6	105 (87)
	38.3	64.5	68
C57Bl/6	9.20	15.3	66 (93)
	3.75	8.20	119

<sup>a</sup> Values shown are expressed as picomols/mg protein/10 min incubation produced by control and ascorbate-treated macrophage cultures derived from bone marrow of individual animals of the various strains. Values are of assays carried out on pools of 4-5 cultures, treated and controls, per mouse. Average % increase of treated over controls for each strain is given in parentheses.

The macrophage is an integral element in both humoral and cell-mediated immunity [15]. Studies on macrophage functions have been carried out most frequently with freshly seeded adherent cell populations obtained from the peritoneal cavity. While attached cells have been considered to be macrophages, these cultures are known to consist of a heterogeneous array of other cell types [9]. To avoid this factor of heterogeneity, we have chosen in our model to examine the effects of ascorbic acid on pure murine macrophage cultures derived from bone marrow and grown to confluency *in vitro*.

In the present experiments, bone marrow-derived macrophage cultures, treated with vitamin C ( $10^{-4}$  M) for 24 h, demonstrated (Table I) substantial increases in PGE<sub>2</sub> production compared to untreated controls. These increases for the various inbred mouse strains were, on the average, of the order of 90 to 100%, level elevations [1] probably sufficient to compromise the ability of interferon to enhance NK activity. The influence of macrophage products such as interferon and prostaglandin on the regulation of natural killer cells is well recognized [7]. It has become evident that while interferon is a major mediator of NK enhancement, prostaglandins are the major inhibitors released from macrophages [18]. The addition of indomethacin to macrophage cultures has been noted to inhibit prostaglandin synthesis and to potentiate NK cytotoxic activity [19]. Also, CHIRIGOS [2] have found that the addition of prostaglandins of the E series (PEG<sub>1</sub> or PEG<sub>2</sub>) to macrophage cultures simultaneously with interferon resulted in marked inhibition of interferon-induced macrophage function. Interferons, in this regard, have been implicated in tumor cell destruction through activation of macrophages and enhancement of NK activity [3, 17].

Previous observations in our laboratory of ascorbate-induced enhancement of interferon synthesis in both mouse cultures and intact animals [10, 11] suggested that vitamin C, conceivably, could participate in defense strategies against malignant disease by involvement of elevated NK activity. It would appear, in this connection, that in the activation of natural killer cells, the balance between the activating (interferon) and inhibiting (prostaglandin) macrophage products may play a determining role in the ultimate level of cytolytic NK cells. Conceivably, the increased PGE<sub>2</sub> levels noted with ascorbate treatment may present a mechanism to explain the failure to demonstrate heightened natural killer cell activity in the intact animal.

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Published in this Journal, Issue No. 4/84, pp. 339-342: The paragraph «Materials and Methods» contained 4 mistakes as to units and nomenclature. The paragraph should read as follows:

#### Materials and Methods

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