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Vitamins C and E prevent AZT-induced leukopenia and loss of cellularity in bone marrow. Studies in mice

J. GARCÍA-DE-LA-ASUNCIÓN[1,2], L. G. GÓMEZ-CAMBRONERO[2], M. L. DEL OLMO[2], F. V. PALLARDÓ[2], J. SASTRE[2] & J. VIÑA[2]

[1]*Department of Anaesthesiology and Surgical Critical Care, Hospital Clinic University, Valencia, Spain, and* [2]*Department of Physiology, School of Medicine, 46010 Valencia, Spain*

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Abstract
A major limitation in the use of AZT for AIDS treatment is the occurrence of side effects, such as leukopenia. The effects of antioxidant vitamins C and E on AZT-induced leukopenia were investigated in mice. Mice were divided into four groups: (1) controls; (2) AZT-treated; (3) treated with AZT plus vitamins C and E; and (4) pre-treated with vitamins and then treated with AZT plus vitamins. Our results demonstrate that AZT causes leukopenia in mice, which was abrogated by administration of vitamins C and E in the pre-treated group. These vitamins prevented the decrease in cellular content induced by AZT in bone marrow and diminished peroxide levels in myeloid precursors in bone marrow. AZT also caused an increase in plasma malondialdehyde and blood oxidized glutathione levels, which was prevented by the administration of antioxidant vitamins. In conclusion, oxidative stress is involved in AZT-induced leukopenia which may be prevented by antioxidant treatment.

Keywords: *Antioxidant treatment, AZT-induced leukopenia, AIDS, bone marrow, peroxide levels, cellularity*

Introduction

AZT (3′-azido-2′,3′-dideoxythimidine) is the most widely used drug, either alone or in combination with other nucleoside or protease inhibitors, in the treatment of acquired immunodeficiency syndrome (AIDS). AZT decreases human immunodeficiency virus replication and increases the number of CD_4+ cells. The incorporation of AZT monophosphate (AZT-MP) into viral DNA results in premature termination of DNA synthesis [1]. A major limitation in its use is the occurrence of severe side effects [2]. Its haematopoietic toxicity leads to macrocytic anemia and leukopenia [3]. This has been attributed to direct inhibition of the erythroid colony forming unit (CFU-E) and the granulocyte macrophage colony forming unit (CFU-GM) activities by AZT or its reduction product AMT (3′-amino-3′deoxythymidine), which may be 5–7 fold more toxic to human bone marrow progenitor cells than AZT [4]. Early studies have shown that *ex vivo* AZT treatment of $CD_{34}+$ bone marrow progenitors causes decreased steady state levels of mitochondrial DNA (mtDNA) [5]. Moreover, exposure of murine bone marrow cells to AZT (50 μM) had a suppressive effect on the growth of granulocyte–monocyte colony forming unit (CFU-GM) derived colonies by suppressive effect on granulocyte–monocyte colony stimulating factor receptor type alpha (GM-CSFR alpha) gene expression [6].

AZT treatment is associated with oxidative damage, causing mitochondrial impairment and affecting cellular functions in several tissues [7,8]. Previously,

Correspondence: J. Viña, Departamento de Fisiología, Facultad de Medicina, Avda. Blasco Ibáñez 17, 46010 Valencia, Spain.
Tel: 34 963864650. Fax: 34 963864642. E-mail: jose.vina@uv.es

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we found that AZT increases mitochondrial production of reactive oxygen species and causes oxidative damage to mtDNA and lipid peroxidation in skeletal muscle, liver and heart [8,9,10]. These toxic effects were prevented by dietary administration of antioxidant vitamins C and E [8,9,10]. Hence, reactive oxygen species are involved in some of the side effects of AZT.

On the other hand, low serum vitamin E levels have been associated with an increase in oxidative stress in HIV-infected individuals, and the vitamin E supplementation suggests that vitamin E may have important immunostimulatory properties [11]. Vitamin E has been shown to protect against bone marrow toxicity, a well established side-effect of AZT. In murine bone marrow cell cultures, and in mice treated with AZT, *d*-alpha-tocopheryl succinate protected against the bone marrow toxicity otherwise seen with AZT [12]. A study of the effects of *d*-alpha-tocopherol on bone marrow cultures from stage IV of AIDS patients revealed similar findings [13]. Research in murine AIDS using a high dose of dietary vitamin E, demonstrated normalization of immune parameters that are altered in HIV/AIDS [14].

The aim of this work was to test if administration of antioxidant vitamins (C and E) protects against AZT-induced leukopenia.

Materials and methods

Young (5 month-old) male OF1 mice from IFFA Credo® (Barcelona, Spain) were fed a standard laboratory diet from Letica® (Barcelona, Spain) and were divided into four groups: (1) controls; (2) mice treated with AZT; (3) mice treated with AZT plus antioxidant vitamins; and (4) mice pre-treated with antioxidant vitamins and then treated with AZT plus antioxidant vitamins. In the last group, treatment with these vitamins was started one month before treatment with AZT to obtain maximum stable plasma and tissue levels of these vitamins. AZT was administered in the drinking water for 40 days (10 mg/kg body weight/day). The mice were dosed with vitamins C and E based on the daily dietary intake. The diet was supplemented with vitamin C (10 g/kg diet) and alpha-DL-tocopherol (0.6 g/kg diet) in order to achieve the following doses daily: 1.25 g of vitamin C/kg of body weight, and 75 mg of vitamin E/kg of body weight. When mice received AZT or AZT plus vitamin-supplemented diet, their food intake and their body weight were similar to those of controls. Peripheral blood was used for the haemogram as well as for measurement of reduced and oxidized glutathione and lipid peroxides. For the study of bone marrow, the mouse femur was removed at 4°C discarding all surrounding tissues, and subsequently 1 ml of potassium phosphate buffer (0.1 M, pH 7.0) flowed through it three times to remove all bone marrow cells. Cell count was performed in these samples by flow cytometry with a flow cytometer Epic Elite II® (Coulter Electronics, Hialeah, FL, USA) and the corresponding cell content was calculated. Peroxide levels were determined by flow cytometry using dihydrorhodamine 123 as fluorochrome [7].

Reduced glutathione (GSH) was measured spectrophotometrically using the glutathione-*S*-transferase assay [15]. Oxidized glutathione (GSSG) was determined by the HPLC method with UV detection at 365 nm that was developed to accurately measure GSSG in presence of an excess of GSH [16]. The GSSG/GSH ratio was calculated as an index of oxidative stress.

Measurement of malondialdehyde (MDA) content in plasma was performed by an HPLC method that determines MDA formed from lipoperoxides [17].

Statistics: Results are expressed as mean ± SD. ANOVA was performed first, and then the sets of data in which F was significant were examined by the Student's t-test.

Results

Our results show that AZT causes leukopenia in mice with a significant decrease (over 35%) in the leukocyte count in peripheral blood (Figure 1). However, leukopenia was completely prevented when mice were pre-treated with antioxidant vitamins C and E and then treated with both AZT and these vitamins. Other haemogram parameters measured were not significantly affected by AZT treatment (Table I). The lack of AZT effect on red blood cells count may be due to the short length of AZT treatment [3].

In the pre-treated group, antioxidant vitamins prevented the decrease in cell content induced by

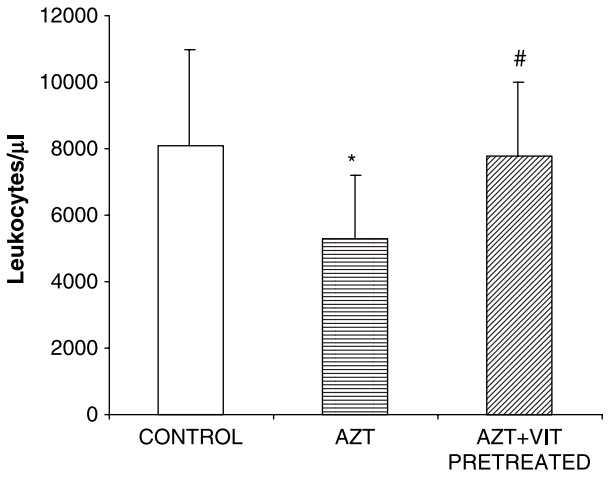

Figure 1. Antioxidant vitamins prevent AZT-induced leukopenia. Results are mean ± SD for 8–9 experiments. Statistical difference is indicated as follows: $^{\star}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. AZT.

Table I. Changes in haemogram from AZT-treated mice and AZT plus antioxidant vitamins-treated mice.

	Control	AZT	AZT + antioxidant vitamins
Erythrocytes ($\times 10^3/\mu l$)	6000 ± 500	7000 ± 1300	6700 ± 4400
Platelets ($\times 10^3/\mu l$)	650 ± 90	620 ± 180	625 ± 64
Haemoglobin (g/dl)	11.4 ± 2.8	12.5 ± 2.2	12.8 ± 1.0
Haematocryt (%)	39 ± 8	39 ± 6	44 ± 4

Results are mean ± SD for 8 experiments.

AZT in the bone marrow (Figure 2A). When vitamins were administered simultaneously with AZT without pre-treatment, only a slight non-significant improvement in cell content was found. Consequently, pre-treatment is required to obtain a complete prevention of AZT-induced leukopenia.

AZT did not change significantly peroxide levels in myeloid precursors from the bone marrow (Figure 2B), but increased blood GSSG levels and plasma MDA levels (Table II). Antioxidant treatment prevented the increase in GSSG and MDA levels in AZT-treated mice. The effect of antioxidant treatment on glutathione redox status was also assessed in control mice and we did not find any significant difference (results not shown).

Administration of antioxidant vitamins diminished significantly peroxide levels in myeloid precursors in the bone marrow (Figure 2B).

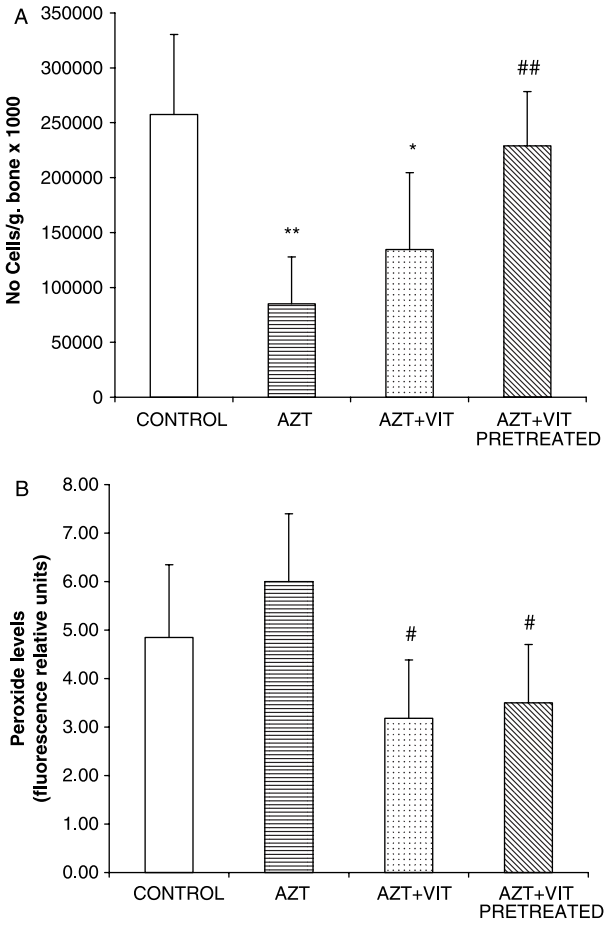

Figure 2. (A) AZT induces a decrease in cell content in bone marrow. Prevention by antioxidant vitamins. (B) Peroxide levels in bone marrow cells. Results are mean ± SD for 5–8 experiments. Statistical difference is indicated as follows: $^{\star}p < 0.05$; $^{\star\star}p < 0.01$ vs. control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ vs. AZT.

Discussion

Previous experimental evidence suggests that AZT therapy is associated with oxidative damage, affecting cellular functions in several tissues and causing oxidative damage particularly to mitochondria [8,18]. A recent report showed that AZT treatment causes a suppression of Mn-superoxide dismutase (Mn-SOD) activity *in vivo* [18]. Furthermore, the content of protein carbonyls in liver and thymus was also increased. It has been previously reported that vitamin E decreased the bone marrow cell toxicity induced by AZT *in vitro* [19].

Our results show a protective effect of antioxidant vitamins against AZT toxicity in peripheral blood and also in bone marrow *in vivo*. This treatment also lowered peroxide levels in myeloid precursors in the bone marrow. Hence, the beneficial effect of vitamins C and E appears to be due, at least in part, to their antioxidant capacity, which may also be involved in the stimulated proliferation of myeloid precursors in the bone marrow reported above. Vitamin C can regenerate other small molecular weight antioxidants, such as vitamin E and glutathione, and this could explain the beneficial effects of our co-treatment [20].

The animals were pre-treated for 1 month with vitamins C and E because previous work has shown that a minimum treatment period of 2–4 weeks is required to obtain maximum stable plasma and tissue levels of these vitamins [21,22]. It is noteworthy that the pre-treatment with antioxidant vitamins is required to improve the cellularity in the bone marrow upon AZT treatment in order to maintain the normal leukocyte count in blood. Accordingly, it seems especially important to increase vitamin E levels before administration of AZT to improve at an optimum level the mitogenic response of immune cells.

Figure 2B shows that both types of anti-oxidant treatment, i.e. pre-treatment and co-treatment

Table II. Effect of AZT on blood glutathione (GSH, GSSG) and MDA levels.

	Control	AZT	AZT + antioxidant vitamins
GSH (nmol/ml)	918 ± 76	948 ± 110	990 ± 175
GSSG (nmol/ml)	34 ± 14	60 ± 29⋆	38 ± 15
GSSG/GSH (× 100)	3.5 ± 1.4	5.5 ± 1.7	3.8 ± 1.3
MDA (pmol/ml)	220 ± 91	370 ± 99⋆	132 ± 58†

Results are mean ± SD for 5–8 experiments. GSH and GSSG were measured in peripheral blood. MDA was measured in plasma from peripheral blood. Statistical difference is indicated as follows. ⋆ $p < 0.05$ vs. control. † $p < 0.01$ vs. AZT.

significantly diminished peroxide levels in the bone marrow to the same extent. Other studies have reported that the mitogenic stimulation of immune cells by tocopherol does not depend on its antioxidant properties, since tocopherol quinone (an oxidized form of tocopherol) and menadione (a quinone without a side chain) also stimulated the mitogenic response [23]. More recently, Simin Meydani and co-workers reported that the difference in the effect of different tocopherol homologues on immune function does not correlate with their antioxidant activity [24]. Hence, it seems that at least part of the beneficial effect of pre-treatment with vitamins may rely on the ability of vitamin E to stimulate the mitogenic response of immune cells independently of its antioxidant properties.

In summary, treatment of mice with AZT causes a remarkable decrease in cell content in bone marrow and leukopenia, and both can be prevented by administration of supra-nutritional doses of vitamins E and C. This finding provides a rationale for treatment of AIDS patients with these vitamins prior to and during AZT administration.

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