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Short Communication

Effect of exogenous vitamin E on proliferation and cytokine production in peripheral blood mononuclear cells from patients with tuberculosis

Jesús Hernández[1]*, Adriana Garibay-Escobar[2], Andrés Mendoza-Mendoza[1,3], Araceli Pinelli-Saavedra[1] and Olivia Valenzuela[2]

[1]*Laboratorio de Inmunología, Centro de Investigación en Alimentación y Desarrollo, A.C. Carretera a la Victoria Km 0.6. Hermosillo, 83000, Sonora, México*
[2]*Departamento de Ciencias Químico Biológicas, Universidad de Sonora, Hermosillo, 83000, Sonora, México*
[3]*Centro Médico, Dr. Ignacio Chávez ISSSTESON, Hermosillo, Sonora, Mexico*

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Micronutrient deficiencies are frequently associated with tuberculosis (TB) worldwide. We tested the effect of exogenous vitamin E on proliferation and cytokine production of peripheral blood mononuclear cells (PBMC) from TB patients and healthy purified protein derivative (PPD) + volunteers. Proliferation was stimulated with mycobacterial antigen (PPD) and evaluated by the incorporation of tritiated thymidine in PBMC cultured with or without 50 μM-vitamin E for 6 d. Cytokine production (IL-2 and interferon (IFN)-γ) was determined by intracellular cytokine staining and by ELISA in the supernatant of PBMC stimulated for 24 h with phytohaemagglutinin or PPD. Our results show that culture with vitamin E increased ($P \leq 0{\cdot}05$) the antigen-induced proliferation of PBMC in TB patients but not in healthy PPD + volunteers. No significant changes in the number of cytokine-producing cells or in the production of IFN-γ were observed with vitamin E treatment. These results indicate that vitamin E may enhance the antigen-specific *in vitro* response of PBMC from TB patients.

Tuberculosis: Vitamin E: Purified protein derivative

Mycobacterium tuberculosis is one of the most important diseases around the world. About two billion people are infected, 10 % develop the active disease and more than two million people die annually[1]. Macrophages eliminate mycobacteria when activated by interferon (IFN)-γ produced by T lymphocytes[2]. However, some bacilli resist killing and survive within macrophages, leading to the active form of the disease[3]. Mycobacteria avoid phagosome maturation and inhibit bacterial antigen presentation, suppress macrophage apoptosis and modulate the production of down-regulating cytokines, such as IL-10, transforming growth factor β, which, in turn, inhibit IFN-γ production by T cells and macrophage activation[4]. The severity of tuberculosis (TB) is increased by several immunodeficiency diseases; the most important is HIV/AIDS[5]. Malnutrition is another important cause of immunodeficiency that increases the risk for TB[6], which is particularly found in developing countries. Individuals with TB present deficiencies of micronutrients, including antioxidants[7]. Patients with TB from Ethiopia were found to have significantly lower concentrations of vitamins C, E and A in their serum than healthy volunteers[7]. In another study of patients with TB from Indonesia, the authors reported that the nutritional status of patients suffering from TB was poor in comparison with healthy controls. Patients were anaemic and exhibited low plasma concentrations of retinol and Zn[8]. Protein malnutrition also alters the abilities of cells to produce cytokines, including IFN-γ, TNF-α and transforming growth factor β in response to purified protein derivative (PPD)[9,10]. Nutrition has been recognized as an important way to modulate the immune response. Some nutrients have shown the ability to alter the production of cytokines[11–13]. Vitamin E increased the proliferation of lymphocytes and production of IL-2 in elderly people, as well as in experimental models of aged animals[14,15]. Vitamin E supplementation has been reported to enhance the production of IFN-γ[16,17]. The aim of this work was to test whether the addition of vitamin E would alter the proliferation and cytokine production of peripheral blood mononuclear cells (PBMC) from TB patients when stimulated with PPD or phytohaemagglutinin (PHA).

Abbreviations: IFN, interferon; PBMC, peripheral blood mononuclear cell; PPD, purified protein derivative; PHA, phytohaemagglutinin; TB, tuberculosis.

* **Corresponding author:** Dr Jesús Hernández, fax +52 662 280 0094, email jhdez@ciad.mx

Methods

Seven patients suffering from TB were diagnosed based on clinical, radiological and bacteriological data by the medical staff of the Department of Health of the state of Sonora, following international criteria. Five men (60·2 (SD 15·6) years old) and two women (50·2 (SD 19·2) years old), with pulmonary TB and under treatment were included in the study. Healthy volunteers (*n* 7) were matched to the patients by age and gender. Patients and volunteers received 0·1 ml (5 units) PPD (Tubersol®; Aventis Pasteur Limited, Ontario, Canada) intradermally. The induration was measured at 72 h and recorded as the average of two perpendicular diameters on mm[18]. Patients and volunteers presented a positive skin reaction to intradermal PPD injection (5·7 (SD 3) mm and 26 (SD 4) mm of reaction in patients and volunteers, respectively). All patients and volunteers were vaccinated with BCG (TB vaccine) when born. All subjects signed a letter of consent to participate in the protocol, which was approved by the ethics committee of the institution and the corresponding state health authorities, following international regulations. Criteria of inclusion were HIV-negative, non-pregnant women, cancer free, not alcohol or drug consumers and not taking a vitamin E supplement. For *in vitro* supplementation, a stock solution of vitamin E was prepared by dissolving α-tocopherol in absolute ethanol. To optimize cellular uptake, vitamin E was then mixed with inactivated fetal bovine serum (16000-044; GIBCO, Grand Island, NY, USA) at a final concentration of 2·31 mM and incubated at 37°C for 1 h in the dark with intermittent vortexing. For supplementation of PBMC, vitamin E was prepared in RPMI-1640 (R4130; Sigma, St Louis, MO, USA) with 10 % fetal bovine serum at a final concentration of 50 μM for 4 h. We chose this concentration based upon previous reports[19]. Before stimulation, cells were washed and resuspended in fresh medium without vitamin E.

Blood (15 ml) was collected into heparin-coated blood collection tubes (Becton-Dickinson, San Jose, CA, USA), overlaid with an equal volume of Ficoll-Hypaque (17-144-02; Amersham Biosciences, Uppsala, Sweden) and centrifuged at 500 ***g*** for 20 min. PBMC were collected from the interface, washed three times in RPMI-1640 and cell viability was determined by the trypan blue dye exclusion method. Cell proliferation was evaluated by [^{3}H]thymidine uptake. A total of 2500 PBMC were placed into 96-well plates (3596; Corning, NY, USA) in a final volume of 200 μl RPMI-1640 medium supplemented with 10 % heat-inactivated fetal bovine serum, 50 mM-2-mercaptoethanol (M7522; Sigma), 100 UI penicillin/ml and 100 μg streptomycin/ml (P4458; Sigma). The cells were stimulated with PHA (12 μg/ml) or PPD (10 μg/ml) for 6 d at 37°C in a 5 % CO_2 humidified atmosphere. Before collection (18 h), cells were pulsed with 1 μCi [^{3}H]thymidine and then collected with a multiple cell harvester (Titratek) onto glass microfibre filter paper (Whatman, Maidstone, Kent, UK). The paper was dried, immersed in vials containing scintillation fluid and the DNA-incorporated radioactivity was measured in a Beckman LS 5000 counter (Beckman Coulter, Fullerton, CA, USA). Results are expressed as counts per min. Serum samples were collected from blood (8 ml), frozen at −70°C and protected from the light until α-tocopherol quantification. The α-tocopherol was quantified from serum and 4×10^6 supplemented or not supplemented PBMC, as described previously[20] with some modifications. The HPLC quantification consisted of a Varian Solvent Delivery module Pro-Star 220, a variable wavelength UV-Vis detector (Model 9050; Varian, Walnut Creek, CA, USA) and a Microsorv C-18 column (R-0089200E3; Varian). The mobile phase was methanol–water (98:2) and detection limit was 0·02 ug/ml. Flow cytometry analysis of intracellular cytokine production by PHA- or PPD-stimulated PBMC was performed as previously described[21] and analysed using CellQuest® software or WinMDI (http://facs.scripps.edu/software.html). The concentration of IFN-γ in the supernatant of PHA- or PPD-stimulated PBMC was determined according to manufacturer's recommendations. Data are expressed as means and standard deviations. Statistical analysis of the data was performed by two-way ANOVA followed by Tukey's test to evaluate differences and interactive effects between vitamin E supplementation and TB patients on several responses (PPD stimulus and unstimulated on proliferation, cell producing cytokines and IFN-γ). Student *t* test was used to analyse the serum concentration of vitamin E on TB patients and healthy volunteers. All analysis was done using the NCSS 2000 package (v.2007, Kaysville, UT, USA).

Results

Serum vitamin E status in TB patients and healthy volunteers was 2·0 (SD 0·8) μg/ml (range 0·82–3·66 μg/ml) and 2·9 (SD 1·1) μg/ml (range 0·87–4·83 μg/ml), respectively. A significant difference ($P \leq 0{\cdot}05$) was observed between TB patients and healthy volunteers. Proliferation of PBMC from TB patients (Table 1) was not significantly different ($P \geq 0{\cdot}05$) with regard to non-stimulated cells; however, proliferation increased significantly (3·3 fold difference, $P \leq 0{\cdot}05$) in the presence of vitamin E. Proliferation of PBMC from healthy PPD+ volunteers was higher as compared with non-stimulated cells ($P \leq 0{\cdot}05$), but, in this case, vitamin E did not increase proliferation ($P \geq 0{\cdot}05$). When comparing the PPD-induced proliferation between healthy PPD+ volunteers and TB patients, it was 3·1 fold higher in healthy volunteers ($P \leq 0{\cdot}05$). No proliferation was observed in unstimulated cells cultured with vitamin E (Table 1). The percentage of cells producing IL-2 (Fig. 1) was higher in PHA-stimulated PBMC than in cells stimulated with PPD or non-stimulated cells ($P \leq 0{\cdot}05$); similarly, the percentage of cells producing IL-2 was higher in those stimulated with PPD than in non-stimulated cells ($P \leq 0{\cdot}05$). These findings were similar in patients and healthy PPD+ volunteers. PBMC from healthy PPD+ volunteers showed an increase in the percentage of cells producing IL-2 in the presence of vitamin E, but this difference was not statistically significant ($P \geq 0{\cdot}05$). The percentage of cells producing IFN-γ was similar to that of IL-2-producing cells. However, patients with TB showed a lower number of IFN-γ-producing cells when stimulated with PHA or PPD as compared with healthy PPD+ volunteers, although the difference was not statistically significant. As described for IL-2, vitamin E did not increase the percentage of IFN-γ-producing cells in healthy PPD+ volunteers stimulated with PPD ($P \geq 0{\cdot}05$). No changes were observed when IFN-γ was quantified by ELISA in the supernatant of either patients or healthy PPD+ volunteers (Fig. 2; $P \geq 0{\cdot}05$). Before supplementation, the α-tocopherol

Table 1. Comparison of the effects of vitamin E in purified protein derivative (PPD)-induced proliferation of peripheral blood mononuclear cells from patients with tuberculosis (TB) and healthy PPD+ volunteers†

(Values are means and standard deviations for seven TB patients or seven healthy volunteers)

	Unstimulated				PPD			
	Non-vitamin E		Vitamin E		Non-vitamin E		Vitamin E	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TB patients	3488[a]	1816	3014[a]	1202	7048[a]	3816	11 398[b]	4826
Healthy PPD+	1789[a]	4826	1905 [a]	3256	22 085[b]*	4826	18 734[b]	7632

[a,b] Mean values within a column with unlike superscript letters were significantly different ($P \leq 0{\cdot}05$). Statistical analyses were performed using two-way ANOVA followed by Tukey's multiple comparison test.

* Mean values were significantly different between TB patients and healthy PPD+ volunteers. *P* values were 0·001004 for vitamin E supplementation, 0·0182 for presence of TB and 0·173517 for vitamin E × presence of TB.

† For details of subjects and procedures, see Methods.

concentration was similar in PBMC from patients and from healthy PPD+ volunteers. After supplementation, α-tocopherol content increased significantly ($P \leq 0{\cdot}05$) in the cells of both patients and healthy PPD+ volunteers. No statistically significant differences were observed between patients and healthy PPD+ volunteers with regard to the α-tocopherol status of their cells (Fig. 3).

Discussion

The ability of vitamin E to modulate the immune response has been demonstrated previously[14–17,19,22]. These effects include an increase in proliferation of PBMC and production of IL-2 *in vitro*[19] and *in vivo* in human subjects[17], a decrease of IL-4 *in vitro* in human subjects[22] and, in some cases, an increase

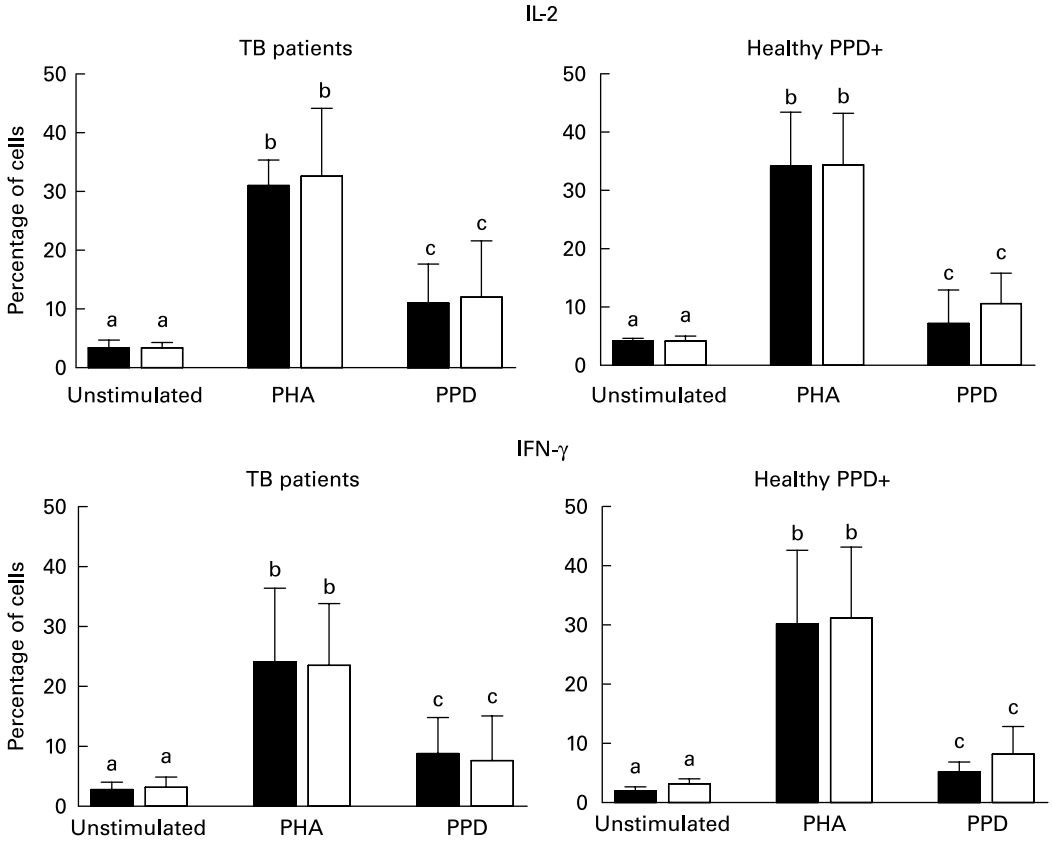

Fig. 1. Cytokine-producing cells. Mean values and standard deviations for seven tuberculosis (TB) patients or seven healthy purified protein derivative (PPD)+ volunteers are represented by vertical bars. Black bars represent cells without vitamin E, and white bars cells with vitamin E. Statistical analyses were performed using two-way ANOVA followed by Tukey's multiple comparison test. [a,b,c] Mean values with unlike superscript letters were significantly different ($P \leq 0{\cdot}05$). *P* values for production of IL-2 on TB patients were 0·617895 for vitamin E, 0·000001 for stimulus, 0·947225 for vitamin E × stimulus; *P* values for production of IL-2 on healthy volunteers were 0·507861 for vitamin E, 0·00001 for stimulus and 0·0679987 for vitamin E × stimulus; *P* values for interferon (IFN)-γ on TB patients were 0·826710 for vitamin E, 0·00001 for stimulus and 0·0·96375 for vitamin E × stimulus. *P* values for IFN-γ on healthy volunteers were 0·434009 for vitamin E, 0·00 001 for stimulus and 0·919051 for vitamin E × stimulus. PHA, phytohaemagglutinin. For details of subjects and procedures, see Methods.

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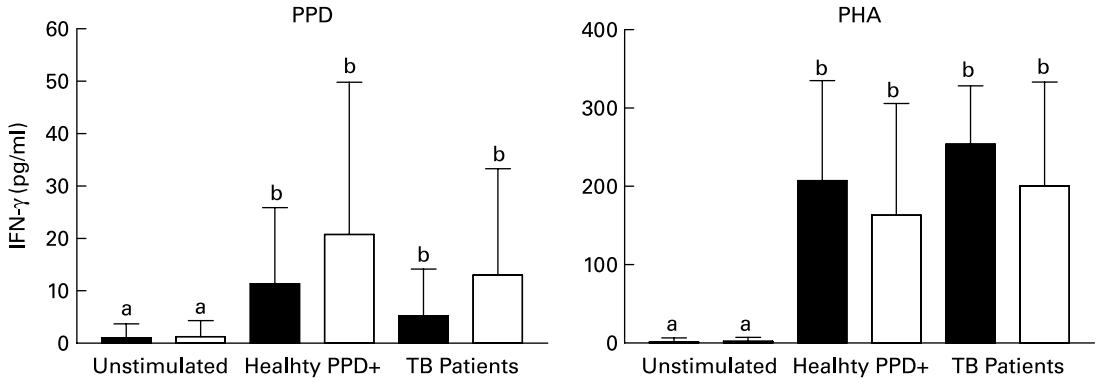

Fig. 2. Production of interferon (IFN)-γ. The production of IFN-γ was determined by ELISA in the supernatant of peripheral blood mononuclear cells stimulated with phytohaemagglutinin (PHA) or purified protein derivative (PPD) for 24 h. Mean values and standard deviations for seven tuberculosis (TB) patients or seven healthy PPD+ volunteers are represented by vertical bars. Black bars represent cells without vitamin E, and white bars cells with vitamin E. Statistical analyses were performed using two-way ANOVA followed by Tukey's multiple comparison test. [a,b] Mean values with unlike superscript letters were significantly different ($P \leq 0{\cdot}05$). *P* values for cells stimulated with PPD were 0·207961 for vitamin E, 0·05 for presence of TB and 0·6847 for vitamin E × presence of TB. *P* values for cells stimulated with PHA were 0·2585 for vitamin E, 0·0001 for presence of TB and 0·71173 for vitamin E × presence of TB. For details of subjects and procedures, see Methods.

in IFN-γ *in vivo* in human subjects and mice[16,17,23] or *in vitro* in young but not in aged mice[23]. Considering these findings, we hypothesized that vitamin E could exert the same effects on PBMC from patients with TB. To test this hypothesis, we evaluated whether vitamin E supplementation would increase proliferation or/and cytokine production (IL2 and IFN-γ) in PBMC from TB patients. As previously described[7], serum α-tocopherol was lower ($P \leq 0{\cdot}05$) on TB patients than on healthy volunteers; in contrast, content in PBMC did not show differences between groups. These results suggest that the lower serum vitamin E did not affect the concentration at cellular level. However, *in vitro* supplementation increased the content of vitamin E on PBMC from TB patients and healthy volunteers. It was interesting to find that vitamin E resulted in higher PPD-induced PBMC proliferation in TB patients, but no changes were observed in PPD+ healthy volunteers. To determine if this increase was associated with the production of IL-2 or IFN-γ, we evaluated the number of cytokine-producing cells. The cells were cultured for 24 h and the results revealed no significant changes in the number of cells producing IL-2 or IFN-γ from patients stimulated with PPD in the presence of vitamin E ($P \geq 0{\cdot}05$). In addition, no significant changes were observed on the IFN-γ detected in the supernatant of cultured cells.

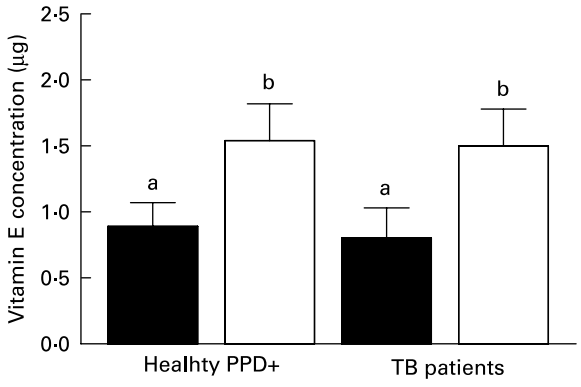

Fig. 3. Vitamin E content in the membrane of supplemented PBMC. Five million peripheral mononuclear cells were supplemented with 50 μM-vitamin E for 4 h and vitamin E concentration was evaluated by HPLC. Mean values and standard deviations of seven tuberculosis (TB) patients or seven healthy purified protein derivative (PPD) + volunteers are represented by vertical bars. Black bars represent cells without vitamin E, and white bars cells with vitamin E. Statistical analyses were performed using two-way ANOVA followed by Tukey's multiple comparison test. [a,b] Mean values with unlike superscript letters were significantly different ($P \leq 0{\cdot}05$). *P* values were 0·000454 for vitamin E, 0·732041 for presence of TB and 0·60654 for vitamin E × presence of TB. For details of subjects and procedures, see Methods.

The ability of α-tocopherol to neutralize oxygen intermediaries[24] and to increase the normal function of the immune system are its most important properties[25]. Vitamin E increases the humoral and cellular immune response. In the cellular immune response, the effects include enhanced proliferation of T cells and production of IL-2[19]. This characteristic has been observed in human subjects and in other species[14,15,19]. In human subjects, vitamin E is especially important in restoring the immune function of aged people. In this population, the increased production of PGE_2 by macrophages is reduced through vitamin E supplementation[26], thereby removing the inhibitory PGE_2 effects. Vitamin E also increased the expression of cell-related genes such as cyclin B, Cdc2 and Cdc6, which are important in the regulation of cell cycle of the cells[23], and improves age-related early T cell signalling in naïve CD4 T cells[27]. We observed that TB patients had diminished reaction to PPD skin test and low PPD-induced PBMC proliferation. Similar results have been observed in other reports, in which they suggest an erroneous immune response provoked by an imbalance in the production of T-helper 1 and T-helper 2 cytokines[28,29]. In agreement with the former study, the present results showed that vitamin E increases PBMC proliferation in TB patients stimulated with PPD, but not in PBMC from healthy PPD+. An important challenge will be to test if vitamin E is able to reverse this phenomenon *in vivo*. Considering that proliferation is associated with production of IL-2, we expected increases of this cytokine, but we did not observe differences. A possibility for this disagreement is that we only evaluated the number of cells producing IL-2 and not production of IL-2 protein. Previous assessments of the effects of vitamin E on

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mice have been focused on the increase in IL-2 production (protein and intracellular expression), as well as on the increment in the number of IL-2-producing cells[19].

Other effects of vitamin E include the reduction of IL-4 at the transcriptional level by blocking NF-κB and activating protein-1[22], as well as increasing IFN-γ[16,17]. The IFN-γ increase has been described in a mouse model[16,23] and in some patients with colorectal cancer[17] who were supplemented with 750 mg vitamin E for 2 weeks. This latter study showed that vitamin E effects include increments in the proportion of CD4:CD8 T cells and IL-2 production. However, it is not yet completely understood how vitamin E controls the production of IFN-γ, but evidence suggests that vitamin E is able to modulate the balance T-helper-1/T-helper-2[22,23]. We expected an increase in the production of IFN-γ in the presence of vitamin E but vitamin E effects were not statistically significant. Future studies must evaluate other concentrations of vitamin E to identify whether these results could represent a significant difference.

In summary, the present work shows that vitamin E enhances the PPD-induced proliferation of PBMC from TB patients, but no changes in cytokine production were observed. We are currently performing experiments in our laboratory to determine how vitamin E could modulate the immune response in patients with TB.

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