The Role of Macrophages in Increased Mitogen Response of Rat Splenic Lymphocytes Following *in Vitro* Incubation with Vitamin E

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(Received March 2, 1995)

Summary The role of macrophages (M ϕ) in the enhancement of lymphocyte proliferation by α -tocopherol (VE) was investigated using rat splenocytes. The proliferation of whole splenocytes was significantly higher than that of M ϕ -depleted splenocytes at all concentrations of concanavalin A (Con A; 0.5–10 μ g/ml). When whole and M ϕ -depleted splenocytes were preincubated with VE (2 μ g/ml) for 24 h, the proliferation of whole splenocytes was significantly enhanced compared to that of whole splenocytes preincubated with medium alone. In contrast, M ϕ -depleted splenocytes did not show any increase of proliferation following *in vitro* pretreatment with VE. When the splenic M ϕ pretreated with both VE (2 μ g/ml) and Con A (10 μ g/ml) for 24 h were further incubated with splenic lymphocytes, their proliferation was significantly enhanced compared to that of splenic lymphocytes cultured with splenic M ϕ pretreated with Con A. In this experiment, the medium containing 2-mercaptoethanol (2-ME) had the ability to enhance splenic lymphocyte proliferation, which masked the enhanced effect of VE on splenic lymphocyte proliferation. Furthermore, *in vitro* treatment of VE could not decrease the production of prostaglandin E₂, but could enhance the production of interleukin 1 from splenic M ϕ . These results suggest that M ϕ play an important role in the proliferation of splenic lymphocytes following *in vitro* incubation with VE, which is closely associated with the action of VE as an immunomodulator rather than antioxidant.

Key Words vitamin E, lymphocyte proliferation, macrophage, interleukin 1

Previous investigation in our laboratory found that high vitamin E (VE) diets could enhance both splenic lymphocyte and alveolar macrophage (AM) functions in rats (1). Bendich et al. have studied the dietary VE requirement for optimum immune responses (2). Tanaka et al. also found that dietary supplementation of

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VE induced the enhancement of helper T-cell activity in mice (3). It is known that VE deficiency causes increased production of prostaglandin E₂ (PGE₂), which depresses T-cell functions via an increase in the cellular cAMP level (4,5). Macrophages (M ϕ) are known to be major prostaglandin (PG)-producing cells and also play a critical role in the regulation of immune responses by releasing monokines which affect lymphocyte functions (6-8). It has been reported that high VE diet decreases the production of PGE₂ from peritoneal M ϕ and enhances the cellular immune functions (1,9). However, the precise mechanisms by which VE stimulates lymphocyte proliferation remains unclear. Namely, it is not known whether VE firstly enhances M ϕ function, and then stimulates lymphocyte proliferation, or whether VE concomitantly stimulates both lymphocyte and M ϕ functions.

In this experiment, the *in vitro* effect of VE on the proliferations of both whole splenocytes and macrophage-depleted splenocytes was investigated. In addition, ethanol has been often used in *in vitro* experiments as a solvent to dilute fat-soluble vitamins (10). However, since it is known that the lower concentrations of ethanol enhance lymphocyte functions (11), VE was diluted with fetal bovine serum (FBS) to avoid the additional effect of ethanol in this experiment.

MATERIALS AND METHODS

Preparation of splenic lymphocytes. The spleen was removed from male F344 rats, 4 weeks old, obtained from Japan SLC (Shizuoka, Japan) and then minced with scissors. Splenocytes were dissociated by passing through a stainless steel screen. The whole splenocytes were cultured for 1 h at 37°C. Then, macrophage-depleted splenic lymphocytes and M ϕ were separated by adhering to the glass. The rate of M ϕ in M ϕ -depleted splenocytes was less than 5%, which was checked by nonspecific esterase staining. Both cells were washed with Hanks' balanced salt solution (HBSS) and adjusted to 1×10^7 cells/ml with RPMI 1640 medium containing 5% FBS.

Dilution of VE. VE (α -tocopherol; Eisai Co. Ltd., Tokyo) was dissolved in FBS by gentle vortexing and incubating in the dark for 20 min at 37°C as described by Narayanareddy and Murthy (12). Then, VE solution was filtered and diluted with RPMI 1640 medium to obtain a final concentration of VE (2 μ g/ml). The final concentration of FBS in media with or without VE was 5% (v/v).

Proliferation of splenocytes. The response of splenocytes to concanavalin A (Con A) was determined as described previously (13). Whole splenocytes and M ϕ -depleted splenocytes were co-cultured in the media with or without VE (2 μ g/ml) for 72 h at the various concentrations of Con A (0-10 μ g/ml). Then, 1 μ Ci of [³H]thymidine (Specific activity 25 Ci/mmol, New England Nuclear, Boston, MA) was added 20 h prior to the termination of culture. Their cells were harvested with a Mash II Harvester and the incorporated radioactivity was measured by using a scintillation counter (LSC-703, Aloka Corp., Tokyo).

Effect of 2-ME on proliferation of splenic lymphocytes. M ϕ -depleted splenocytes were incubated with Con A (5 μ g/ml) in medium containing 2-mercaptoethanol (2-ME; 50 μ M) or VE (2 μ g/ml) for 72 h. Then, their proliferation was measured by the incorporation of 3 H-thymidine.

Interleukin 1 (IL1) production from splenic M ϕ . IL1 production from splenic M ϕ was assessed by proliferation of normal thymocytes from a C3H/HeJ mouse as described previously (14). Splenic M ϕ were separated from whole splenocytes by adhesion to glass. From nonspecific esterase staining, the rate of M ϕ in isolated cells was $86.4 \pm 6.4\%$. Their M ϕ (1×10^6 cells/ml) were cultured for 24 h with Con A (5 μ g/ml) in the presence or absence of VE (2 μ g/ml). The supernatant of these cultures were harvested and used for the assay of IL1 activity. Briefly, thymocytes (1×10^7 cells/ml) of C3H/HeJ mouse were incubated with 50 μ l of their supernatant and 150 μ l of culture medium for 48 h. Then, their proliferation was measured by the incorporation of 3 H-thymidine.

PGE₂ production from splenic M ϕ . Splenic M ϕ were resuspended in RPMI 1640 medium with 5% FBS and placed in a flat-bottomed Microtiter plate (Corning Costar Corp., New York) at a density of 5×10^5 cells/ml. After 24 h, the supernatant were collected and frozen at -80°C . The amount of PGE₂ in the culture media of splenic M ϕ incubated with Con A (5 μ g/ml) or lipopolysaccharide (LPS; 10 μ g/ml) for 24 h was determined by the method of Shono et al. (15). Anti-PGE₂ antibody suspension was mixed with 0.1 ml of ^{125}I -labeled PGE₂. Then, 0.1 ml of either known amounts of PGE₂ or supernatant of cultured M ϕ was added. After 24 h, immune complexes were precipitated and the amount of PGE₂ in the supernate was estimated from standard curve.

Statistical analysis. Experimental results were analyzed by Student's *t*-test (two-tailed).

RESULTS

Effects of splenic M ϕ on the proliferation of rat splenocytes following in vitro incubation with various concentrations of Con A

Figure 1 shows the effect of M ϕ on splenic lymphocyte proliferation following *in vitro* incubation with various concentrations of Con A (0–10 μ g/ml) for 72 h. Proliferation of M ϕ -depleted splenic lymphocytes was significantly lower at all concentrations of Con A compared to those of whole splenocytes. Maximum proliferation in both whole splenocytes and M ϕ -depleted splenocytes was shown at 5 μ g/ml of Con A.

In vitro effect of VE on the proliferation of whole and M ϕ -depleted splenocytes following in vitro incubation with Con A

The enhancing effect of VE (2 μ g/ml) supplementation on splenic lymphocyte proliferation was shown in whole splenocytes, but not in M ϕ -depleted splenocytes (Fig. 2). When splenic M ϕ pretreated *in vitro* with both VE (2 μ g/ml) and Con A

Fig. 1. Proliferations of whole (○) and Mφ-depleted (●) splenocytes following *in vitro* incubation with various concentrations of concanavalin A (Con A) for 72 h. Significantly different from whole splenocytes; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 2. *In vitro* effect of vitamin E (VE; 2 μg/ml) on the proliferation of whole and Mφ-depleted splenocytes with Con A (5 μg/ml) for 72 h. □, medium; ■, VE. Significantly different from whole splenocytes incubated with medium alone; ** $p < 0.01$.

(5 μg/ml) for 24 h were added to the culture of splenic lymphocytes without Mφ, the proliferation of splenic lymphocytes was also significantly enhanced compared to that of splenic Mφ pretreated with Con A only (Fig. 3A).

Effect of 2-mercaptoethanol (2-ME) on the proliferation of splenic lymphocytes following in vitro incubation with Con A

In the present experiment, 2-ME was used to increase proliferation of rat splenic lymphocytes in the stimulation with Con A *in vitro*. 2-ME may have some effects on the proliferation of splenic lymphocytes cultured with both VE and Con A, as shown in Fig. 2. Then, the effect of 2-ME on the proliferation of splenic lymphocytes was investigated. As shown in Fig. 3B, the proliferation of splenic

Fig. 3. Effects of 2-mercaptoethanol (2-ME, ▨) and vitamin E (VE, ■) on splenic Mφ function (A) and lymphocyte proliferation (B). After splenic Mφ were incubated with medium containing 2-ME (50 μM) or VE (2 μg/ml) for 24 h, they were cultured with splenic lymphocytes with Con A (5 μg/ml) for 72 h. The effects of 2-ME and VE on splenic Mφ were estimated by the increase of splenic lymphocyte proliferation. * $p < 0.05$ (vs. 2-ME). Effects of 2-ME and VE on splenic lymphocyte proliferation were estimated by *in vitro* stimulation with Con A (5 μg/ml).

lymphocytes incubated *in vitro* with medium containing 2-ME (50 μM) was similar to that of splenic lymphocytes incubated with medium containing VE (2 μg/ml).

Production of IL1 and PGE₂ from splenic Mφ following in vitro incubation with both VE and ConA for 24 h

Production of IL1 from splenic Mφ was measured by proliferation of mouse thymocytes as described in MATERIALS AND METHODS. As shown in Fig. 4, the supernatant of splenic Mφ cultured with both VE (2 μg/ml) and Con A (5 μg/ml) for 24 h significantly enhanced the proliferation of mouse thymocytes compared to that of the supernatant of Mφ treated with Con A (5 μg/ml) alone. On the contrary, the production of PGE₂ from splenic Mφ was unaffected by *in vitro* incubation with Con A (5 μg/ml) for 24 h and was comparable to that of splenic Mφ cultured with medium alone for 24 h (Fig. 5). Furthermore, *in vitro* supplementation of VE to the splenic Mφ cultures did not suppress PGE₂ produc-

Fig. 4. Proliferation of mouse thymocytes by the supernatant of MØ cultured with Con A (5 µg/ml) or Con A (5 µg/ml)+VE (2 µg/ml) for 24 h. □, medium (Stimulation with Con A); ■, VE (Stimulation with Con A and VE). Significantly different from medium; ** $p < 0.01$.

Fig. 5. Effect of VE (2 µg/ml) on PGE₂ production from splenic MØ cultured with medium, Con A (5 µg/ml) or LPS (5 µg/ml) for 24 h. □, medium; ■, VE.

tion even in Con A stimulation. In the case of *in vitro* incubation with LPS to induce higher production of PGE₂ from splenic MØ, the treatment with VE could not suppress PGE₂ production by splenic MØ (Fig. 5).

DISCUSSION

In the present study, we found that the proliferation of splenocytes without adherent cells, mainly splenic MØ, is significantly lower than whole splenocytes at all concentrations of Con A, as shown in Fig. 1. This finding agrees with the result of Corwin, et al. (16). Furthermore, they found that the impaired proliferative response of splenic lymphocytes without adherent cells was restored to a considerable degree by the *in vitro* addition of VE (1 µg/ml). We have also found that the supplementation of VE *in vitro* could induce a significant increase in splenic

lymphocyte proliferation with Con A in whole splenocytes, but not in M ϕ -depleted splenocytes. The discrepancy between the above report and ours may be explained by the difference in the optimum concentration of Con A to induce a higher proliferation of splenic lymphocytes following the depletion of adherent cells (M ϕ) from whole splenocytes. For instance, the concentration of Con A to induce the maximum proliferation of splenic lymphocytes in whole splenocytes was 5 μ g/ml. On the contrary, the concentration of Con A in the report of Corwin et al. was 0.125 μ g/ml, which was considerably lower compared to that of this experiment.

In the next experiment, we investigated whether or not M ϕ pretreated with both VE (2 μ g/ml) and Con A (5 μ g/ml) *in vitro* for 24 h had the ability to enhance the proliferation of splenic lymphocytes. The addition of splenic M ϕ pretreated with both VE and Con A to splenic lymphocyte culture could induce even higher proliferation of splenic lymphocytes compared to that of splenic M ϕ treated with Con A alone as shown in Fig. 3A. This result suggests that VE has the ability to stimulate splenic M ϕ directly.

Furthermore, there are many reports showing that VE also stimulates lymphocyte functions such as NK activity and mitogenesis (1, 9). As shown in Fig. 2, the proliferation of splenic lymphocytes without M ϕ had been scarcely increased by *in vitro* treatment with VE and was similar to that of splenic lymphocytes cultured with medium alone. Although this result indicates that VE does not stimulate splenic lymphocyte proliferation, it appears that VE has the ability to stimulate the proliferation of splenic lymphocytes as shown in Fig. 3B. In this experiment, the culture medium contained 2-ME. The addition of 2-ME to culture media has been found to enhance the DNA synthetic response of lymphocytes to mitogens (17, 18). As shown in Fig. 3B, the addition of 2-ME to the culture media induced a higher proliferation of splenic lymphocytes comparable to that of VE. This result suggests that the action of VE to enhance lymphocyte proliferation may be masked by the addition of 2-ME to the culture media. In addition, since splenic M ϕ were highly activated by *in vitro* incubation with VE compared to that of 2-ME as shown in Fig. 3A, VE may activate M ϕ function as an immunomodulator rather than an antioxidant.

It is known that M ϕ are important cells to modulate host immune functions via IL1 and PGE₂ (19, 20). As shown in Fig. 4, splenic M ϕ following the *in vitro* treatment with VE showed significantly higher production of IL1 compared to that of splenic M ϕ treated with medium alone. However, the production of PGE₂ from splenic M ϕ was not increased by *in vitro* incubation with Con A and was comparable to that of splenic M ϕ incubated with medium alone. Meydani et al. have shown that phytohemagglutinin (PHA) induces PGE₂ production from peripheral blood mononuclear cells of elderly subjects (21). This conflicts with our finding reported here. The discrepancy is probably due to the difference in the ages of the subjects or animals examined in the experiment, the different stimulating agents used such as PHA and Con A, and the difference of culture condition (the media used in our experiment contained 2-ME). In addition, although VE is known as a strong

antioxidant and an inhibitor of prostaglandin synthesis, the addition of VE to the splenic M ϕ culture did not decrease the production of PGE₂ from splenic M ϕ as shown in Fig. 5. This result is in agreement with the report by Sakamoto et al. (22) who found that the addition of VE to the M ϕ culture had little effect on A23187-stimulated PGE₂ production. In the case of LPS stimulation, which induces higher production of PGE₂ from splenic M ϕ , *in vitro* supplementation of VE could not also decrease the production of PGE₂ from splenic M ϕ . Not only higher concentrations of VE (>2 μ g/ml) but also the presence of VE within the cell membrane may be needed to induce the inhibitory effect of VE on PGE₂ production from splenic M ϕ (23). Further work is needed to clarify the *in vitro* effect of VE on the production of PGE₂ following stimulation with mitogens.

From the above results, the present study suggests that the action of VE to enhance splenic lymphocyte proliferation with Con A is mainly mediated by the activation of splenic M ϕ , which is associated not with decreased production of PGE₂, but with increased production of IL1 as an immunomodulator.

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