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Cellular Immunity and Tumor Growth in Vitamin E-Sufficient and -Deficient Mice

Haruki KATO,* Ryuki KIN, Mari TANIGAWA,
Masahiro UEDA, Toshikazu YOSHIKAWA,
Shigeru SUGINO, and Motoharu KONDO

The First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kamikyo-ku, Kyoto 602, Japan

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Summary Effects of vitamin E (VE) on tumor growth and *in vitro* lymphocyte proliferation as markers of cellular immunity in CDF_1 mice were examined in the VE-deficient state as well as in the VE-sufficient state attained by pretreatment with VE. Oral (585 mg of dl-α-tocopheryl nicotinate per 100 g diet) or intraperitoneal (0.5 ml of saline-diluted d-α-tocopherol every other day at a dose of 40 mg/kg) supplementation with VE did not enhance *in vitro* lymphocyte proliferation to concanavalin A, phytohemagglutinin, or lipopolysaccharide, suppress the growth of Meth A fibrosarcoma, or prolong the survival of tumor-bearing animals, even though the serum VE value was maintained 1.5 to 2.4 times higher in the group fed the VE-sufficient diet and about 3 times higher in the group injected with VE than the value for the group fed the control diet. On the other hand, VE-deficiency produced by a basal VE-deficient diet (0.16 mg of d-α-tocopherol per 100 g diet) seemed to be disadvantageous for the tumor-bearing host in view of the decrease in *in vitro* lymphocyte proliferation to phytohemagglutinin or lipopolysacharide, rapid tumor growth, and shortened survival of tumor-bearing mice.

Key Words: cellular immunity, tumor growth, vitamin E, mitogens, vitamin E deficiency

Antioxidants, such as selenium [1, 2], vitamin E (VE, d-α-tocopherol) [3], and ascorbic acid [4], prevent chemical carcinogenesis in experimental animal systems. Breakage of DNA by oxygen radicals could be one of the causes of chemical carcinogenesis, and was reduced by the treatment of antioxidants [5]. On the other hand, administration of massive amounts of antioxidant VE promoted carcinogenesis [6], namely, an antioxidant could be a prooxidant in excessive

*To whom correspondence should be addressed.

dosage. Therefore, VE seems to have an appropriate dosage at which it is useful for the prevention of chemical carcinogenesis.

VE has various kinds of effects on immune responses. Tengerdy *et al.* [7, 8] and Campbell *et al.* [9] reported that humoral immune responses were enhanced by the administration of VE in chickens and in mice. Resistance against *E. coli* infection was augmented by the oral administration of VE [10]. Tanaka *et al.* [11] observed enhancement of antibody (hemagglutinin) production and suggested that VE stimulated the helper activity of T-lymphocytes. Antibody-dependent cellular cytotoxicity [12] and phagocytosis of neutrophils [13, 14] were also enhanced. Accordingly, it would be interesting to determine whether or not VE suppresses established tumor cell growth *in vivo*, because VE could modify various kinds of immune responses, and these enhanced immune responses might play some roles in tumor suppression. Yasunaga *et al.* [15] reported suppression of tumor growth in mice when they were given an appropriate dose of VE prior to or at the same time of tumor inoculation. Herein, we report the effects of VE on tumor growth and *in vitro* lymphocyte proliferation as marker of cellular immunity in VE-deficient mice especially, as well as in those made VE-sufficient by pretreatment with VE.

MATERIALS AND METHODS

Diet. The basal VE-deficient diet (Oriental Kobo Ind., Tokyo) contained on a weight-weight basis 2.0% vitamin mixture (0.16 mg (0.23 IU) of d-α-tocopherol/100 g), 38.0% corn starch, 10.0% alpha-maltstarch, 5.0% granulated sugar, 25.0% vitamin-free casein, 6.0% purified lard, 6.0% mineral mixture, and 8.0% powdered filter paper. To this diet, 20 mg (20 IU) of dl-α-tocopheryl acetate per 100 g diet was added as the control diet and 585 mg (500 IU) of dl-α-tocopheryl nicotinate per 100 g diet as the VE-sufficient diet. D-α-Tocopherol and HCO60 as a solvent of VE were donated by Eisai Co., Tokyo.

Animals and treatment with VE. Male 4-week-old CDF_1 (BALB/c × DBA/2) mice were purchased from Charles River, Atsugi. In one experiment they were orally given the basal VE-deficient diet, the control diet or the VE-sufficient diet from 4-weeks of age throughout their lives. In another experiment, one group was intraperitoneally (i.p.) injected every other day from the same age with 40 mg (44 IU) of d-α-tocopherol dissolved in 0.5 ml saline/kg. The same volume of HCO60 diluted with saline was also injected i.p. in another group for the same period.

Measurement of VE. Mice were killed by decapitation. Whole blood was collected, clotted at room temperature, and processed sera were stored at $-70°C$ until used. VE was measured by high performance liquid chromatography [16].

In vitro lymphocyte proliferation. The spleens were aseptically removed from the mice killed by decapitation, washed with RPMI 1640 medium (Nissui Seiyaku Inc., Tokyo) supplemented with 10% heat-inactivated new-born calf serum (Handai Biken, Suita), 8.2 mM l-glutamine (Wako Pure Chemical Ind., Osaka), and 1% penicillin-streptomycin-fungizone mixture (MAB, Walkersville, MD), and then

minced with scissors into pieces 1–4 mm in diameter. The minced tissue was forced through a 60-mesh stainless steel wire screen and suspended in the medium. After centrifugation, erythrocytes were lysed by resuspension of the cell pellet in ammonium chloride-potassium lysing buffer (8.290 g NH_4Cl, 1.0 g $KHCO_3$, and 0.0372 g EDTA per liter, pH 7.4). The cells were washed twice with the buffer and resuspended in the medium. These spleen cells (5×10^5 cells/well) were stimulated with concanavalin A (Con A, Pharmacia Fine Chemicals, Piscataway, NJ) at a final concentration of 5 μg/ml, phytohemagglutinin (PHA, Pharmacia Fine Chemicals) at 25 μg/ml or lipopolysaccharide (LPS, *S. typhosa* 0901, Difco Laboratory, Detroit, MI) at 100 μg/ml in 96-well U-bottomed plastic tissue culture plates (Nunc, Roskilde, Denmark) for 48 h at 37°C in 5% CO_2, after which 1 μCi/well of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added and incubation was continued for another 18 h under the same conditions. Then the cells were harvested by an automatic cell harvester, and their radioactivities were counted by a liquid scintillation counter. All experiments were carried out in triplicate.

Tumor cells. Meth A fibrosarcoma syngeneic to the BALB/c mouse was maintained in the peritoneal cavity of CDF_1 mice. Cells freshly removed from the peritoneal cavity were washed twice with RPMI 1640 medium and subcutaneously inoculated (1×10^5) into the right hind-footpads of the mice 8 weeks after starting the treatments with VE. The footpad thickness was measured by a caliper and expressed in mm as an indicator of tumor diameter.

Statistics. Two-way analysis of variance was applied for the comparison of serum VE levels, *in vitro* lymphocyte proliferation to Con A, PHA, or LPS, and tumor sizes among the groups treated with VE. Significant differences were calculated by the multiple comparison of Scheffe's method for serum VE levels and tumor size, and by Turkey's method for *in vitro* lymphocyte proliferation. Survival times were compared by Kruskal Wallis' method, and Scheffe's method was used to test for significance of differences [17].

RESULTS

Serum VE level

Serum VE was measured before and 2, 4, 6, 8, 11, and 14 weeks during the VE treatment. The mean value of serum VE before the treatment was 2.11 ± 0.55 μg/ml. Figure 1a shows the changes in the estimated values of VE with the intake of the VE diets. The serum VE value rapidly decreased after 4 weeks on the VE-deficient diet and was below 0.1 μg/ml after 6 weeks. This low level of serum VE was maintained during the remainder of the observation period ($p < 0.01$). Serum VE values in the group given the control diet were similar to the pretreatment values. In the group given the VE-sufficient diet, the values were 1.5 to 2.4 times higher than those of the group fed the control diet ($p < 0.01$) (Fig. 1a).

In the other experiment, the serum VE values of the group injected i.p. every other day with 40 mg of VE/kg of body weight were about 3 times higher than

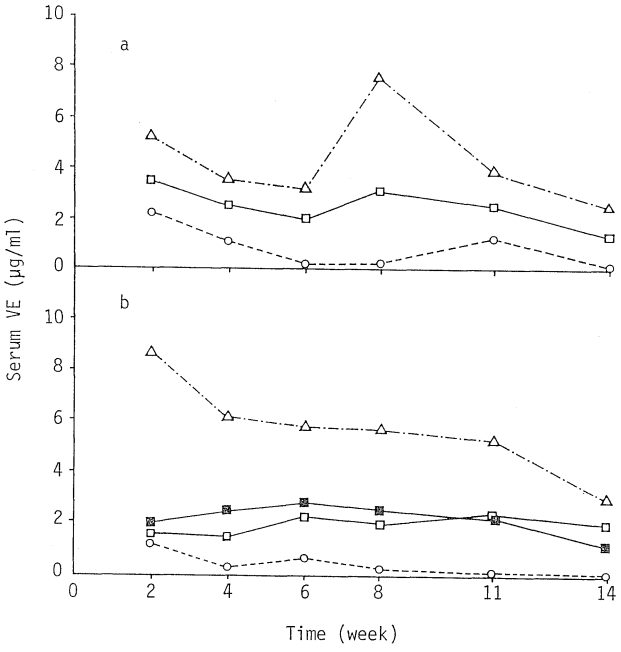

Fig. 1. Serum VE levels after the VE treatment. a: △, VE-sufficient diet; □, control diet; ○, VE-deficient diet. b: △, i.p. injection of VE; □, control diet; ○, VE-deficient diet; ▩, i.p. injection of vehicle.

those of the group given the control diet during most of the observation period ($p<0.01$), while the levels in the group injected i.p. with solvent only were almost the same as those of the group given the control diet (Fig. 1b).

In vitro lymphocyte proliferation

In vitro lymphocyte proliferation to Con A, PHA, or LPS was determined in the groups given the VE-deficient diet, the control diet, or the VE-sufficient diet at the same time serum VE values were measured. There was no significant difference in *in vitro* lymphocyte proliferation to Con A among the 3 groups (Fig. 2a). However, proliferation to PHA was augmented by the VE-sufficient diet compared with that obtained by the VE-deficient diet ($p<0.01$). These responses in the group given the VE-deficient diet were suppressed compared with those of the group given the control diet ($p<0.01$), while there was no significant difference between the responses in the groups receiving the VE-sufficient and control diets (Fig. 2b). *In vitro* lymphocyte proliferation to LPS showed higher responses in the group receiving the VE-sufficient diet than in that given the VE-deficient diet ($p<0.05$). The responses in the group given the VE-deficient diet were suppressed compared with those of the group given the control diet ($p<0.01$). On the other hand, there was no significant difference between the group receiving the VE-sufficient diet and that given the control diet (Fig. 2c).

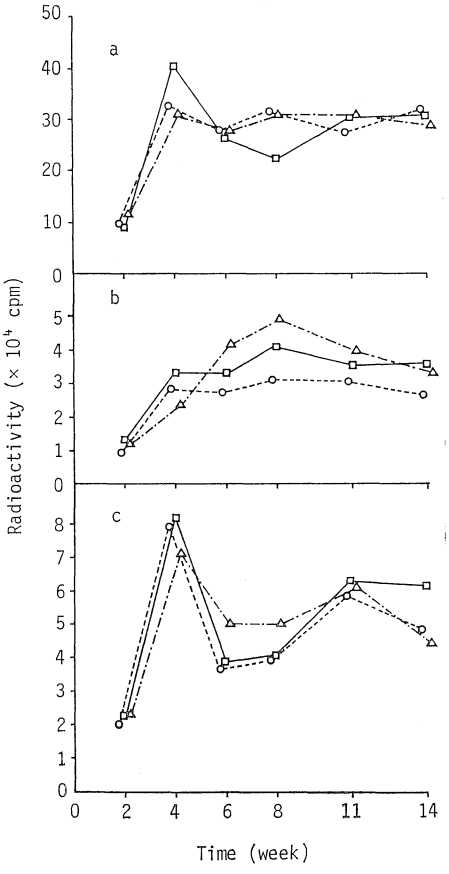

Fig. 2. *In vitro* lymphocyte proliferation during the VE treatment to mitogens. a, To Con A; b, to PHA; c, to LPS. Symbols are the same as those in Fig. 1a.

Tumor growth

Meth A fibrosarcoma cells (1×10^5 cells/footpad) were inoculated into the right hind-footpad of CDF_1 mice 8 weeks after the start of treatment with VE (i.e., at 12-weeks of age). In the group given the VE-deficient or VE-sufficient diet, tumors showed linear growth 2 weeks after the tumor inoculation and grew faster than those in the group given the control diet ($p<0.01$). There was no significant difference between the group given the VE-deficient diet and the group receiving the VE-sufficient diet (Fig. 3a). Tumors in the group injected i.p. with VE were larger 6 weeks after the tumor inoculation than that those of other two groups ($p<0.01$). Although data are not shown here, the control group injected i.p. with vehicle only showed almost the same tumor growth as the group given the control diet.

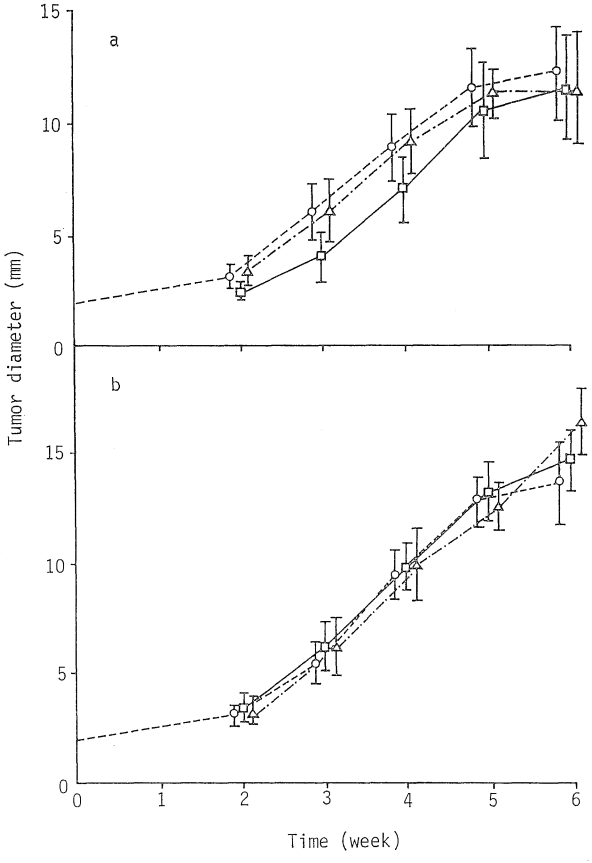

Fig. 3. Tumor growth during the VE treatment. VE was supplemented by diet (a), or by i.p. injection (b). Symbols are the same as those in Fig. 1 a and b, respectively. Mean± SD is given.

Survivals

Survival was not prolonged by the VE-sufficient diet (Fig. 4a), nor by the i.p. injection of VE (Fig. 4b). The VE-deficient state shortened survival of tumor-bearing mice compared with the group receiving the control diet in one of the experiments ($p<0.05$) (Fig. 4a). There was no significant difference in survival between the group given the VE-sufficient diet or i.p. injected VE and the group given the VE-deficient diet (Fig. 4 a and b).

DISCUSSION

Serum VE levels in the group given the VE-deficient diet decreased to below 0.1 μg/ml 4 to 6 weeks after the start of the treatment. In contrast, the group given the VE-sufficient diet showed 1.5 to 2.4 times higher levels of serum VE than the

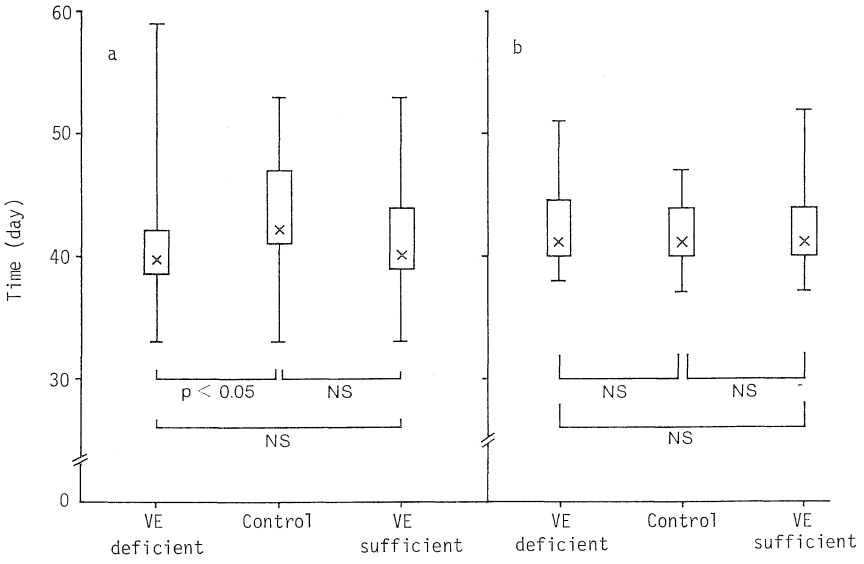

Fig. 4. Survival time of tumor-bearing mice during the VE treatment. VE was supplemented by diet (a), or by i.p. injection (b). Error bars show the maximal and the minimal survival times; squares, the mean±SD; ×, the median in each group.

group receiving the control diet during the treatment. Intraperitoneal injection of VE maintained serum VE at a level about 3 times higher than the control during the treatment.

Yasunaga *et al.* [15] showed that *in vitro* lymphocyte proliferation to Con A, PHA, or LPS was enhanced when the serum VE levels of the donor animal were kept about 2 times higher than the control level. Enhancement of these responses was also reported by other investigators [18]. In our study, *in vitro* lymphocyte proliferation to Con A, PHA, or LPS was enhanced 4 weeks after the start of treatment, regardless of the diet type. Such enhancement is due to the maturation of immunity in mice, which occurs at 8 weeks of age. Therefore only the responses obtained after the 4th week of the experiment are valuable for comparison among the groups. There was no significant difference in the response to Con A among these 3 diet groups. On the other hand, *in vitro* lymphocyte proliferation to PHA and LPS was suppressed in the group given the VE-deficient diet, while the responses to the same mitogens were not enhanced by the VE-sufficient diet with respect to the control diet.

Tumor growth and survival of CDF_1 mice inoculated with 1×10^5 Meth A cells at 8 weeks after start of the VE treatment were examined to determine the effect of the VE-sufficient or the VE-deficient state. The tumors in the groups given the VE-deficient or the VE-sufficient diet grew faster than those in the group given the control diet. The VE-sufficient state attained by i.p. injection did not suppress tumor growth. Supplementation of VE by diet or i.p. injection failed to prolong

the survival time of tumor-bearing mice. However, survival was shortened in the group given the VE-deficient diet in one of the experiments. Although VE can prevent chemical carcinogenesis in experimental models [1–4] our data suggest that a serum VE at about 2 to 3 times higher levels than normal does not affect established tumor cells or their growth; namely, the survival of tumor-bearing hosts is not prolonged. Recent studies show that the risk of cancer is higher in a population with a low concentration of serum VE than in one with a higher concentration [19, 20]. The VE-deficient state was rather disadvantageous for immune responses monitored by *in vitro* lymphocyte proliferation to mitogens, and even for the growth of established tumor cells and survival of tumor-bearing animals. Further examination is recommended to determine if the VE-deficient state is a risk factor in a tumor-bearing host. The effective dosage of and route of administration with other kinds of VE derivatives should also be examined in addition to the dl-α-tocopheryl nicotinate and d-α-tocopherol used, because VE-sufficient state was not always effective in these experiments compared with the control one.

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