Dietary Effect of Tocopherols and Tocotrienols on the Immune Function of Spleen and Mesenteric Lymph Node Lymphocytes in Brown Norway Rats

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The immunoregulatory effects of dietary α-tocopherol (Toc) and tocotrienols (T-3) on humoral and cell-mediated immunity and cytokine productions were examined in Brown Norway rats. We found that the IgA and IgG productivity of spleen and mesenteric lymph node (MLN) lymphocytes was significantly enhanced in the rats fed on Toc or T-3, irrespective of concanavalin A (Con A) stimulation of the lymphocytes. On the contrary, the IgE productivity of the lymphocytes from the rats fed on Toc or T-3 was less without Con A stimulation, but was greater in the presence of Con A, especially in the T-3 group. Toc or T-3 feeding significantly decreased the proportion of CD4+ T cells and the ratio of CD4+ /CD8+ in both spleen and MLN lymphocytes of the rats fed on Toc or T-3. The interferon-γ productivity of MLN lymphocytes was higher in the rats fed on Toc or T-3 than in those fed on a control diet in the presence of Con A, while that of spleen lymphocytes was lower in the rats fed on Toc or T-3. In addition, T-3 feeding decreased the productivity of tumor necrosis factor-α of spleen lymphocytes, while it enhanced the productivity of MLN lymphocytes. These results suggest that oral administration of Toc and T-3 affects the proliferation and function of spleen and MLN lymphocytes.

Key words: α-tocopherol; tocotrienols; immunoglobulin production; IFN-γ; TNF-α

It has been reported that various food components exert a variety of biological effects. For example, vitamin E compounds such α-tocopherol (Toc) and tocotrienols (T-3) are well recognized for their effective inhibition of lipid peroxidation in foods and in vivo.1-6 It has also been reported that oral administration of Toc or T-3 significantly modulated immune functions6-8 such as the production of immunoglobulin (Ig) and cytokine, and changes in the productions of CD4+ and CD8+ T cells.2-14 Interferon-γ (IFN-γ) is a cytokine which activates macrophages, and plays an important role in the manifestation of T cell-mediated inflammatory response.12,14 On the other hand, tumor necrosis factor-α (TNF-α) is an inflammatory mediator which frequently increases during inflammation.

The enhancement of IgE production and the inhibition of other Ig production in rat mesenteric lymph node (MLN) lymphocytes have been reported for polyunsaturated fatty acids10 and hydrogen peroxide.16 Toc suppressed the enhancement of IgE production induced by polyunsaturated fatty acid.10 In the previous studies, we have reported the Ig production-regulating activity of Toc in feeding experiments with Brown Norway7 and Sprague Dawley rats.10 However, the biological functions of T-3 have received less attention, because their occurrence is limited except in palm oil. T-3 has recently been reported to exert diverse functions which are different from those of Toc.19-21 Thus, it is important to examine the effect of Toc and T-3 on immune functions.

Concanavalin A (Con A) is a mitogen which specifically stimulates the proliferation of suppressor T cells, and enhances the production of IgE, and of cytokines such as IL-4, IL-5, IFN-γ and TNF-α.12,14,22,23 In previous studies, we have shown the effects of Toc and T-3 on lipid metabolism, leukotriene production, and some immune indices in Brown Norway rats.19 However, information on the immunoregulatory activity of dietary Toc and T-3 on spleen and MLN lymphocytes is still deficient. In the present study, we elucidate the immunoregulatory effect of Toc and T-3 on Ig production, T cell population, and IFN-γ and TNF-α production in Brown Norway rats in the presence or absence of Con A.

Materials and Methods

Materials. D-α-tocopherol (Toc) was a product from Eisai Co. (Tokyo, Japan), and a mixture of tocotrienols (T-3) was provided by the Palm Oil Research Institute of Malaysia (PORIM, Kuala Lumpur) which was composed of 13.1% of α-tocotrienol, 57.9% of β-tocotrienol, 20.5% of δ-tocotrienol, and 8.5% of others mainly involving γ-tocopherol. Sesamin of 99.5% purity as total lignans, which was an equiweight mixture of sesamin and episesamin, was presented by Suntory Ltd. (Osaka, Japan). Borage oil was purchased from BioRegional Food and Science Corp. Co. (Saskatoon, Canada).

Con A was purchased from Vector Lab. (Burlingame, CA, U.S.A.) and dissolved in phosphate buffered saline (PBS, pH 7.5). Enzyme-linked immunosorbent assay (ELISA) kits for measuring IFN-γ and TNF-α were purchased from Biosource International Co. (Camarillo, CA, U.S.A.).

Fetal bovine serum (FBS) was from GIBCO (Grand...
Island, NY, U.S.A.). Tween 20 (0.05%) in PBS was used for rising, and Block Ace (Dainihon Pharmaceutical Co., Osaka, Japan) for blocking and antibody dilution\textsuperscript{20} in the ELISA evaluation of rat IgGs.

**Animals and diets.** Four-week-old male Brown Norway rats (Seac Yoshitomi, Fukuoka, Japan) housed individually in stainless steel mesh cages were acclimated for 3 days on a non-purified diet (NMF, Oriental Yeast Co., Tokyo, Japan) in an air-conditioned room (20–23°C, light cycle 08:00–20:00). The rats weighing an average of 81 g were divided into 3 groups of 6 animals each and given free access to the experimental diets and water. The diet composition followed the recommendation of the American Institute of Nutrition (AIN76),\textsuperscript{25} and was fortified with 5% borago oil and 0.2% sesamin. One group of rats was fed on the control diet, and the other 2 groups of rats were fed on the containing 0.2% Toc or 0.2% T-3. Body weight and food intake were recorded every other day. Blood was obtained from the tail vein on days 1, 8 and 15, and serum was separated and stored at −20°C for analysis. After 3 weeks of feeding, the rats were killed by withdrawing blood from the abdominal aorta under diethyl ether anesthesia. The liver, spleen, epididymal adipose tissue, and kidneys were immediately excised into ice-cold saline, blotted, and weighed. The spleen and MLN lymphocytes were isolated from 4 rats of each group and cultured in an RPMI1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FBS.\textsuperscript{20}

**Measurement of the serum peroxide and prostanoid \textit{E}\textsubscript{2} levels.** The level of lipid oxidation products in the serum was measured as thiobarbituric acid (TBA) reactive substances.\textsuperscript{20} Serum prostanoid \textit{E}\textsubscript{2} (PGE\textsubscript{2}) was extracted by the method of Green \textit{et al.}\textsuperscript{27} and measured with an enzymatic immunoassay kit (PGE\textsubscript{2} Monoclonal ELISA Kit, Cayman Chemical, Ann Arbor, MI, U.S.A.).\textsuperscript{20}

**Analysis of \textit{T} cell subsets.** Spleen and MLN lymphocytes (1 × 10\textsuperscript{6} cells/ml) were stained either with \textit{R} phycocerythrin (RPE)-labeled mouse anti-rat CD4\textsuperscript{+} or with fluorescein isothiocyanate (FITC)-labeled mouse anti-rat CD8\textsuperscript{+} monoclonal antibodies (Serotec Ltd., Kidlington, Oxford, U.K.) for 30 min at 4°C. The lymphocytes were collected by centrifugation at 160 × g for 5 min and rinsed three times with PBS containing 10% FBS. The washed lymphocytes were fixed with 2% paraformaldehyde, and approximately 1 × 10\textsuperscript{6} cells were analyzed by a flow cytometer (Epics Profile II; Coulter Electronics Ltd., Beds, U.K.).

**Measurement of immunoglobulins and cytokines.** Spleen and MLN lymphocytes were inoculated at 2 × 10\textsuperscript{6} cells/ml into 24-well micrometer plate (Falcon; Becton and Dickinson Co., NJ, U.S.A.) and cultured with or without 5 µg/ml of Con A for 4 hr to measure the IgE level and for 24 hr to measure the IgA, IgG and IgM levels. The Ig levels in the culture supernatant were measured by ELISA as described previously.\textsuperscript{20} The IFN-γ and TNF-α contents were measured with commercial ELISA kits (ASY-18 and KRC 3012 for IFN-γ and TNF-α, respectively; Biosource International Products, Camarillo, CA, U.S.A.).

**Statistical analyses.** Data were usually analyzed by Duncan's new multiple-range test\textsuperscript{20} to determine the exact nature of the differences among groups. Differences within a set of cultures for each Ig were analyzed by Student's \textit{t} test.

**Results**

**Growth and serum lipid level**

As shown in Table 1, there was no significant difference in the food intake, food efficiency and body weight gain. On the other hand, the relative liver weight (g/100 g body weight) was significantly higher in the Toc group than in the T-3 groups, though there was no significant difference from the control group.

**Serum TBA values and PGE\textsubscript{2} level**

There was no significant difference in serum TBA values on day 1, but the administration of Toc or T-3 significantly reduced the serum TBA level on days 8, 15 and 22 when compared to the control group (Fig. 1A).

In the control group, the TBA level was highest on day 8, and then decreased to the level of day 1 by day 22, though there was no significant difference during the feeding period. On the contrary, the serum PGE\textsubscript{2} level was not influenced by dietary manipulation or the feeding period, as shown in Fig. 1B.

**Immunoglobulin productivity of spleen and mesenteric lymph node lymphocytes**

The IgA, IgG and IgM levels in spleen lymphocytes after 24 hr of incubation were higher in the rats fed on Toc and T-3 diet than in those fed on the control diet in the absence of Con A. However, the IgA, IgG and IgM levels were higher in cells isolated from the rats fed on T-3 in the presence of Con A (Fig. 2). In the control and Toc-fed rats, the effect of Con A on the IgA, IgG and IgM production was moderate. In addition, the IgE productivity of the splenocytes was lower in the rats fed

### Table 1. Effect of Antioxidant Feeding on the Food Intake, Growth, and Tissue Weight of BN Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Food intake (g/day)</th>
<th>Food efficiency</th>
<th>Body weight gain (g)</th>
<th>Tissue weight (g)/100 (g) Body Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Gain</td>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Control</td>
<td>81 ± 2</td>
<td>80 ± 3</td>
<td>11.2 ± 0.2</td>
<td>0.3 ± 0.0</td>
<td>4.5 ± 0.1\textsuperscript{ab}</td>
</tr>
<tr>
<td>Toc</td>
<td>80 ± 2</td>
<td>77 ± 3</td>
<td>10.9 ± 0.2</td>
<td>0.3 ± 0.0</td>
<td>4.7 ± 0.1\textsuperscript{a}</td>
</tr>
<tr>
<td>T-3</td>
<td>81 ± 2</td>
<td>77 ± 3</td>
<td>11.3 ± 0.2</td>
<td>0.3 ± 0.0</td>
<td>4.4 ± 0.0\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Each value is the mean ± SE of 6 rats. \textsuperscript{a,b}Values without the same superscript letter are significantly different at \(p < 0.05\). Toc, α-tocopherol; T-3, Tocotrienols.
Immunoregulatory Effects of Tocopherol and Tocotrienols

**Fig. 1.** Effect of Dietary Antioxidant on the Serum TBA (A) and Prostaglandin E\(_2\) (B) Levels in Brown Norway Rats.

Each data value is the mean±SE (n=6) and values without the same superscript letter are significantly different at p<0.05 by Duncan’s multiple-range test. Toc, \(\alpha\)-tocopherol; T-3, tocotrienols.

**Fig. 2.** Effect of Dietary Antioxidant on the Immunoglobulin Productivity of Spleen Lymphocytes in Brown Norway Rats.

Spleen lymphocytes were incubated at 37°C for 4 hr for the IgE determination, and for 24 hr for the IgA, IgG, and IgM determinations, with or without Con A. Each data value is the mean±SE of 4 rats. Values without the same superscript letter are significantly different at p<0.05 by Duncan’s multiple-range test. The differences within a set of cultures for each Ig (with or without Con A) were analyzed by Student’s t-test, *p<0.05, **p<0.01 and ***p<0.001. Toc, \(\alpha\)-tocopherol; T-3, tocotrienols.

on Toc or T-3 than in the rats fed on the control diet in the absence of Con A. There was no significant difference between the groups fed on Toc or T-3. In the presence of Con A, an increase of IgE level was induced in the Toc and T-3 groups, and a decrease of IgE level in the control group.
The IgA and IgG productivity in MLN was significantly higher in the rats fed on T-3 than in the rats fed on the other diets in the absence of Con A (Fig. 3). In the presence of Con A, the levels of IgA, IgG and IgM were higher in the Toc and T-3 groups than in the control group. The enhancing effect was more marked in the T-3 group than in the Toc group. In the control group, Con A significantly reduced the level of IgA. The effect on the IgE level was similar to that observed in spleen lymphocytes. IgE productivity was significantly lower in the rats fed on Toc or T-3 than in the control group in the absence of Con A. In the presence of Con A, IgE production was markedly enhanced in the Toc and T-3 groups, but completely suppressed in the control group.

Spleen and mesenteric lymph node T cell analysis

To clarify the Ig production-regulating mechanism for these dietary components, the effect on the T cell population was examined. In spleen T lymphocytes, the proportion of CD4+ T cells was significantly lower in the Toc and T-3 groups than in the control group, being lowest in the Toc group (Table 2). The proportion of CD8+ T cells was higher in the Toc group than in the T-3 group, but the difference was not significant against the level of the control group. In combination, the CD4+/CD8+ ratio was significantly lower in the Toc and T-3 groups than in the control group, especially in the Toc group.

In MLN, the proportion of CD4+ T cells was significantly lower in the Toc and T-3 groups than in the control group, but there was no difference in the proportion of CD8+ cells. In combination, the CD4+/CD8+ ratio was significantly lower in the Toc and T-3 groups than in the control group.

Cytokine production by spleen and mesenteric lymph node lymphocytes

When spleen and MLN lymphocytes isolated from the rats fed on Toc or T-3 were cultured for 24 hr with or without Con A, IFN-γ production was detected only in the presence of Con A as shown in Table 3. In spleen cells, the IFN-γ level was lower in the rats fed on T-3 than in those fed on the control and Toc diets. In contrast, IFN-γ production of MLN lymphocytes was sig-

![Fig. 3. Effect of Dietary Antioxidant on the Immunoglobulin Productivity of Mesenteric Lymph Node Lymphocytes in Brown Norway Rats. MLN lymphocytes were incubated, and the data were analyzed as described in the legend to Fig. 2. Toc, α-tocopherol; T-3, tocotrienols.](image-url)
Table 3. Effects of Antioxidant Feeding on the IFN-γ and TNF-α Concentrations of Spleen and Mesenteric Lymph Node Lymphocytes in BN Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>IFN-γ (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Con A (−)</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>N.D.</td>
<td>119 ± 7a</td>
</tr>
<tr>
<td>Toc</td>
<td>N.D.</td>
<td>139 ± 5b</td>
</tr>
<tr>
<td>T-3</td>
<td>N.D.</td>
<td>151 ± 9c</td>
</tr>
<tr>
<td>Con</td>
<td>330 ± 15d</td>
<td>936 ± 14e</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>Con A (−)</td>
</tr>
<tr>
<td>MLN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>N.D.</td>
<td>4 ± 2a</td>
</tr>
<tr>
<td>Toc</td>
<td>N.D.</td>
<td>23 ± 2b</td>
</tr>
<tr>
<td>T-3</td>
<td>N.D.</td>
<td>6 ± 1c</td>
</tr>
<tr>
<td>Con</td>
<td>272 ± 24d</td>
<td>670 ± 26e</td>
</tr>
</tbody>
</table>

Each value is the mean ±SE of 4 rats per group. *Values without the same superscript letter are significantly different at p<0.05 by Duncan's multiple-range test. Toc, α-tocopherol; T-3, tocotrienols; N.D. not detected. The lymphocytes were incubated without and with Con A at 37°C, and the concentrations of IFN-γ and TNF-α (after 24 hr of incubation) were measured by ELISA.

significantly higher in the Toc and T-3 groups than in the control group, being highest in the T-3 group. A small amount of TNF-α was produced by spleen and MLN lymphocytes even in the absence of Con A. In splenocytes, the TNF-α level was significantly higher in the T-3 group than in the control group, while it was higher in the Toc group in MLN lymphocytes. In the presence of Con A, TNF-α production of spleen lymphocytes was markedly stimulated: the level was lower in the Toc and T-3 groups than in the control group, being lowest in the T-3 group. In MLN, the TNF-α level was higher in the Toc and T-3 groups than in the control group, being highest in the T-3 group.

Discussion

Immune diseases such as allergies are often accompanied with increases in IgE production, chemical mediator release, proportion of CD4+ T cells and cytokine secretion. In the present study, the dietary effects of Toc and T-3 on these immune indices were examined by using a strain of immunosensitive rats. In the previous study, we showed that T-3 feeding gave a significantly higher CD4+/CD8+ ratio than Toc feeding in MLN lymphocytes. In addition, combined use of sesamin and T-3 caused a significant reduction in the serum IgE level and increased the IgA and IgG productivity of MLN lymphocytes. In the present study, we found that the IgA and IgG productivity of spleen and MLN lymphocytes was significantly enhanced in the T-3 group with or without Con A stimulation. On the other hand, IgE productivity of these lymphocytes was lower in the rats fed on Toc or T-3 in the absence of Con A, but was higher in the presence of Con A, especially in the rats fed on T-3.

We have also reported that Toc and sesamin feeding suppressed 4-series eicosanoid synthesis from arachidonic acid. We show here that both Toc and T-3 significantly decreased lipid peroxidation as indicated by the level of serum TBA. However, there was no effect on the serum PGE2 level. These results suggest that dietary Toc and T-3 can express antioxidative activity in vivo, but do not affect cyclooxygenase activity by themselves.

Mosmann et al. have recently reported that CD4+ T cells were further divided into at least two types of cells in their cytokine productivity: Th1 cells which produced IFN-γ and IL-2, and Th2 cells which produced IL-4 and IL-5. These cytokine have been reported to regulate Ig synthesis class specifically. Since dietary Toc and T-3 significantly affected the T cell population of spleen and MLN lymphocytes, we examined here the productivity of IFN-γ and TNF-α by these lymphocytes. TNF-α is a cytokine produced by both Th1 and Th2 cells which is related to cell-mediated immunity. IFN-γ produced by Th1 cells and suppresses development of Th2 cells in favor of Th1 cell growth. In the present study, we show that dietary Toc and T-3 enhanced the IFN-γ productivity of MLN lymphocytes, but suppressed that in splenocytes. Similar results were obtained for the productivity of TNF-α in these lymphocytes. These results indicate that dietary antioxidants influenced the cytokine productivity of lymphocytes in a tissue-specific manner.

It has been reported that Toc was mainly absorbed by mucosal cells in lymph, and that the absorption of T-3 was much slower than Toc in hamsters (averaging 1:180). However, T-3 exerted as strong antioxidative activity as that of Toc in the present study. These results suggest that T-3 was absorbed as fast as Toc in rats, or that T-3 was a more effective antioxidant than Toc in vivo. Studies on the absorption and metabolism of T-3 are important to confirm these suggestions.

In the present study, we show that dietary Toc and T-3 exerted antioxidative activity and modified the Ig and cytokine productivity of spleen and MLN lymphocytes. IgA can be detected in various secretory fluids such as tears, saliva and digestive fluid and inhibits the absorption of such xenobiotics as bacteria, viruses and allergens. IgG is a major class of serum antibody and plays an important role in the elimination of xenobiotics and the establishment of eternal immunity. In addition, IgG suppresses type I allergy through competition with IgE. The enhancement of IgA and IgG production by dietary Toc and T-3 may improve our immune condition, especially in preventing the contraction of infectious diseases and alleviating allergic symptoms. We also show that Toc and T-3 feeding affected to the population of T cells and the productivity of some cytokines. These results suggest that these antioxidants regulate Ig production class specifically by regulating the T cell activities. To clarify the immunoregulatory mechanism for dietary antioxidants, further studies on T cell differentiation are essential.

Acknowledgments

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References


