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Dietary nucleotides increase the mucosal IgA response and the secretion of transforming growth factor β from intestinal epithelial cells in mice

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Abstract

We have investigated the influence of dietary nucleotides on the intestinal immune system in ovalbumin (OVA)-specific T-cell receptor (TCR) transgenic mice (OVA-TCR Tg mice). When mice were supplied with water supplemented with 2% OVA *ad libitum*, the faecal OVA-specific immunoglobulin A (IgA) level significantly increased in those fed a nucleotide-supplemented diet (NT(+) diet) compared with those fed a nucleotide-free control diet (NT(–) diet). In the NT(+) diet-fed mice, secretion of transforming growth factor β (TGF-β), which is an isotype-specific switch factor for IgA, from intestinal epithelial cells (IECs) was significantly increased. Furthermore, an increased proportion of intestinal intraepithelial lymphocytes (IELs) bearing $\gamma\delta$ TCR (TCR$\gamma\delta^+$ IELs) and increased secretion from IECs of interleukin 7 (IL-7), which is essential for the development of TCR$\gamma\delta^+$ IELs, were also observed in OVA-TCR-Tg mice fed the NT(+) diet, as we previously demonstrated using BALB/c mice (Nagafuchi et al., Biosci. Biotechnol. Biochem. 64: 1459-65 (2000)). Considering that TCR$\gamma\delta^+$ T cells and TGF-β are important for an induction of the mucosal IgA response, our results suggest that dietary nucleotides augment the mucosal OVA-specific IgA response by increasing the secretion of TGF-β from IECs and the proportion of TCR$\gamma\delta^+$ IELs.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IECs, intestinal epithelial cells; IELs, intraepithelial lymphocytes; IFN, interferon; Ig, immunoglobulin; IL, interleukin; OVA, ovalbumin; TCR, T-cell receptor; TGF-β, transforming growth factor β.

Introduction

When proteins such as food antigens traverse the mucosal endothelial barrier, secretory immunoglobulin A (IgA) antibodies are secreted into the gastrointestinal lumen. These antibodies bind with specific antigens and thereby suppress the uptake of the antigens. In the intestines of infants, the concentration of secretory IgA is very low because of gastrointestinal immaturity. The gastrointestinal tracts of infants are more permeable to food antigens than those of adults. These antigens may induce the production of food-specific IgE antibodies. Since an excessive amount of food antigens entering the circulation may cause the development of food allergies, antigen-specific IgA is very important for preventing allergy development (Chowdhury, 1995; Metcalfe, 1992).

A recent study has shown that $\gamma\delta$ TCR-bearing T cells (TCR$\gamma\delta^+$ T cells) are important for development of the mucosal IgA response (Fujihashi et al., 1996). Intestinal intraepithelial lymphocytes (IELs) consist of both TCR$\alpha\beta^+$ T cells and TCR$\gamma\delta^+$ IELs

with phenotypic and functional features distinct from those of cells in peripheral lymphoid tissues. We previously showed that dietary nucleotides induced an increase in TCR$\gamma\delta^+$ T cells in BALB/c mice (Nagafuchi et al., 2000b). However, the influence of dietary nucleotides on the mucosal IgA response has not yet been determined.

Dietary nucleotides have been found to have various effects on immune responses such as enhancement of immune responses (Carver et al., 1991; Jyonouchi et al., 1993), protection from bacterial infections (Kulkarni et al., 1986), and skewed induction of Th1-type immune responses (Jyonouchi et al., 2001; Nagafuchi et al., 1997, 2000a; Sudo et al., 2000). Nucleotides have also been shown to have stimulating properties on IECs. Uauy et al. (1990) found increased mucosal protein, DNA, villus height, and disaccharidase activities in the intestines of weanling rats fed a diet supplemented with dietary nucleotides. It is also known that dietary nucleotides are involved in the development of IECs, especially under conditions of stress (Walker, 1996). Nunez et al. (1990) reported that the intestinal tissue content of DNA, and the activities of lactase, maltase, and sucrase were increased following chronic diarrhea in rats whose diets were supplemented with nucleotides. We also demonstrated in the previous study that dietary nucleotides enhanced the secretion of interleukin 7 (IL-7) from IECs in BALB/c mice (Nagafuchi et al., 2000b). On the other hand, cellular and molecular cross-talk between IELs and IECs is thought to regulate the differentiation of IELs and IECs (Beagley and Husband, 1998). It has been shown that activated TCR$\gamma\delta^+$ T cells obtained from skin and intestine express an epithelial-cell mitogen, keratinocyte growth factor (Boismenu and Havran, 1994). Conversely, IECs express IL-7, which plays a critical role in the development of TCR$\gamma\delta^+$ IELs (Maki et al., 1996; Laky et al., 2000). Nucleotides are considered to exert various effects on mucosal immune responses, at least in part by affecting the interaction between IELs and IECs.

In the present study, we have investigated the effects of dietary nucleotides on the mucosal OVA-specific IgA response in ovalbumin (OVA)-specific T-cell receptor transgenic mice (OVA-TCR Tg mice), in which most peripheral T cells express the transgenic TCR. Furthermore, we have examined the effects of dietary nucleotides on the subset composition of IELs, and cytokine secretion by IECs and IELs.

Table 1. Compositions of the experimental diets (%)

Ingredient	NT(–) diet	NT(+) diet
Whey protein isolate	22.0	22.0
Sucrose	5.0	5.0
Starch	60.0	59.6
Cellulose	3.0	3.0
Soybean oil	5.0	5.0
Vitamins[a]	1.0	1.0
Minerals[b]	4.0	4.0
Nucleotides[c]	–	0.4

[a] The composition was as follows (in mg/kg): vitamin A, 20 IU; 7-dehydrocholesterol, 2000 IU; α-tocopheryl acetate, 50; menadione, 5; choline chloride, 2000; ρ-aminobenzoic acid, 100; inositol, 100; niacin, 40; calcium pantothenate, 40; riboflavin, 8; thiamine HCl, 5; pyridoxine HCl, 5; folic acid, 2; D-biotin, 0.4; cyanocobalamine, 0.03.
[b] The composition was as follows (in mg/kg): NaCl, 5572; KI, 31.6; KH_2PO_4, 15560; $MgSO_4$, 2292; $CaCO_3$, 15256; $FeSO_4{\cdot}7H_2O$, 1080; $MnSO_4{\cdot}H_2O$, 160.4; $ZnSO_4{\cdot}7H_2O$, 0.053; $CuSO_4{\cdot}5H_2O$, 19.1; $CoCl_2{\cdot}6H_2O$, 0.92.
[c] The composition was as follows (in g/kg): Cytidine monophosphate, 1.62; guanosine monophosphate, 0.57; inosine monophosphate, 1.1; uridine monophosphate, 0.71.

Materials and methods

Mice and diets

Female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). OVA-TCR Tg mice (OVA23-3 mice) (Sato et al., 1994) were maintained by backcrossing them to BALB/c mice. All mice were maintained and used in accordance with the guidelines of the University of Tokyo. OVA-TCR Tg mice were kept on a nucleotide-free diet based on whey protein isolate (WPI) (NT(–) diet) or the NT(–) diet supplemented with nucleotides (NT(+) diet) from 3 weeks of age. The diet compositions are shown in Table 1. The supplemented nucleotides and WPI were obtained from Yamasa Co. (Choshi, Japan) and Davisco International Inc. (Le Sueur, MN, U.S.A.), respectively. The nucleotide composition comprised similar proportions of nucleotides to those in human milk at 12 weeks of lactation (Janas and Picciano, 1982). Nucleotide 5′-diphosphates were replaced by nucleotide 5′-monophosphates. Adenosine 5′-monophosphate was replaced by inosine 5′-monophosphate because adenosine increases the blood flow to several tissues (Berne et al., 1982).

Treatment of animals

Male or female OVA-TCR Tg mice at 3 weeks of age were randomly assigned to two groups, and then maintained on either the NT(–) diet or NT(+) diet. The volume of dietary nucleotides fed by one OVA-TCR Tg mouse for one day was increased from about 10 mg at 3 weeks of age to about 20 mg at 7 or 8 weeks of age. Both groups of mice received water supplemented with 2% OVA *ad libitum* during the experimental period. Faeces were collected from 6 to 8 weeks of age. To examine the IEL subsets, and the *in vitro* cytokine secretion by IELs and IECs, OVA-TCR Tg mice were sacrificed at 7 weeks of age.

Measurement of OVA-specific IgA antibodies in faecal samples

Collected faeces (20 mg) were suspended and homogenized in 1 ml of phosphate-buffered saline (PBS) containing 0.1 mg/ml trypsin inhibitor (Wako, Tokyo, Japan). After centrifugation at $500 \times g$ for 15 min, the supernatants were taken and stored at –25 °C until analysis of the OVA-specific IgA antibody titer. The faecal OVA-specific IgA level was determined by means of an enzyme-linked immunosorbent assay (ELISA). For ELISA to determine the OVA-specific IgA level, OVA (Seikagaku Co., Tokyo, Japan) and alkaline phosphatase-conjugated rabbit anti-mouse IgA (Zymed Lab., South San Francisco, CA, U.S.A.) were used. The OVA solution (0.1 mg/ml diluted in 0.11 M PBS, pH 7.2) was added to each well of microtitre plates (Nunc, Roskilde, Denmark), followed by incubation overnight at 4 °C. The wells were then washed with 0.11 M PBS, pH 7.2, containing 0.1% Tween 20 (PBS-0.1% Tween), and blocked with 0.11 M PBS containing 1.5% gelatin for 30 min at room temperature. The plates were washed again and then the faecal samples diluted with PBS-0.1% Tween containing 3% polyethylene glycol 6000 (Nacalai Tesque Inc., Kyoto, Japan) were added, followed by incubation for 2 h. After washing, the alkaline phosphatase-conjugated anti-mouse IgA, diluted with PBS-0.1% Tween containing 3% polyethylene glycol 6000, was added to each well, followed by standing for 2 h. The plates were washed and then the 4-nitrophenylphosphate substrate reagent (1 mg of 4-nitrophenylphosphate and 100 μg of $MgCl_2 \cdot 6H_2O$ in 1 ml of 0.1 M diethanolamine buffer, pH 9.8) was added to each well. The plates were then incubated for 1 h at room temperature and the reaction was terminated by adding 5 N NaOH. The absorbance was measured optically at 405 nm.

Preparation of IELs

IELs were isolated as described previously (Nagafuchi et al., 2000b; Nanno et al., 1994). In brief, small intestines were removed from mice. The small intestines free of the lumenal contents were turned inside-out with the aid of polyethylene tubing. Each inside-out intestine was cut into four segments, which were then transferred to a 50-ml conical tube containing 45 ml of Hanks' balanced salt solution (GIBCO, Grand Island, NY, U.S.A.) including 5% fetal calf serum (FCS). The tube was shaken at 37 °C for 45 min (horizontal position on an orbital shaker at 135 rpm). The resulting cell suspension was collected and passed through a glass-wool column to remove sticky cells. Subsequently, the cells were suspended in 30% (wt/vol) Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 25 min at $450 \times g$. IELs were obtained using Percoll as the separation medium for density gradient centrifugation ($450 \times g$, 25 min), and IELs were recovered at the 44 and 70% Percoll interface (>90% were TCR-positive).

Flow cytometry

Two-color or three-color analysis of IEL subsets was performed. The antibodies used for flow cytometry were biotin-labeled anti-mouse TCRβ (H57-597; BD PharMingen, San Diego, CA, U.S.A.), biotin-labeled anti-mouse CD4 (H129.19; BD PharMingen), biotin-labeled anti-mouse IL-7-receptor (IL-7R) (B12-1; BD PharMingen), biotin-labeled anti-mouse IL-2R (CD25) (7D4; BD PharMingen), fluorescein isothiocyanate (FITC)-labeled anti-mouse TCRδ (GL3; Cedar Lane Lab., Ontario, Canada), FITC-labeled anti-mouse CD4 (H129.19; BD PharMingen), phycoerythrin (PE)-labeled anti-CD8α (53-6.7; GIBCO), FITC-labeled anti-mouse CD8β (Y8.77; Seikagaku Co.), and PE-labeled anti-mouse TCRβ (H57-597; BD PharMingen) ones. We performed all incubations in the dark. Cells were incubated for 30 min on ice with a biotin-labeled antibody. The cells were then washed in Hanks' balanced salt solution and incubated for 30 min on ice with streptavidin-Red 670 (GIBCO), and FITC-labeled and PE-labeled antibodies. The cells were then washed by centrifugation. Stained cells were analyzed with a flowcytometer FACSort (BD Biosciences, San Jose, CA, U.S.A.). The data were analyzed with Lysis II software.

Preparation of antigen-presenting cells

Spleen cells were obtained from BALB/c mice. The organs were aseptically removed from the mice and gently teased. The spleen cells (1×10^8 cells) were incubated at 37 °C for 45 min in 1 ml of RPMI1640 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 50 μg/ml of mitomycin C (SIGMA, St. Louis, MO, U.S.A.). These cells were suspended in a 0.83% (w/v) ammonium chloride solution to lyse the red blood corpuscles. Then, the cell suspension was passed through stainless steel sieves to obtain a single cell suspension. The cells were washed twice with RPMI 1640 medium.

Culture of IELs for the cytokine assay

IELs (1.0×10^5 cells/well) from OVA-TCR Tg mice were resuspended in 0.2 ml of RPMI 1640 medium, which was supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (5×10^{-5} M), and 2 mM L-glutamine. Mitomycin C-treated spleen cells (5×10^5 cells), as antigen-presenting cells, were added to the IEL cultures. The cells were incubated in duplicate in 0.2 ml of RPMI 1640 medium with or without OVA at the concentration of 50 μM in 96-well plates for 2 d under a humidified atmosphere of 5% CO_2 at 37 °C. After incubation, the culture supernatants were collected by centrifugation and stored at –25 °C. These supernatants were used for measurement of the concentrations of interferon γ (IFN-γ) and IL-2.

Preparation of IECs

IECs were isolated from mice and cultured as described (Nagafuchi et al., 2000b; Perreault and Beaulieu, 1998). In brief, small intestines were opened longitudinally, washed with PBS, and then cut into 4 segments. The segments were placed in a 15-ml tube containing 10 ml of ice-cold MatriSperse (Collaborative Biomedical Products, Becton Dickinson Lab., Mississauga, Ontario, Canada). The intestine segments were incubated at 4 °C for 8 h without agitation. Then, each tube was gently shaken to separate the IECs. The resulting suspension was washed twice in PBS (4 °C, $300 \times g$, 8 min). These IECs (>94% were CD3ε- and immunoglobulin-negative cells) were resuspended in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (5×10^{-5} M), and 2 mM L-glutamine, and then plated on a 96-well plate. The freshly isolated IECs (2×10^5 cells/well) were cultured at 37 °C under 5% CO_2 without stimulation for 16 h.

Cytokine assays

The concentrations of IFN-γ, IL-2, IL-7 and TGF-β were determined by means of ELISA. The antibodies used for measurement of IFN-γ, IL-2 and IL-7 were rat monoclonal anti-mouse IFN-γ (6A2; BD PharMingen), rat monoclonal anti-mouse IL-2 (JES6-1A12; BD PharMingen), mouse monoclonal anti-human/mouse IL-7 (Genzyme, Cambridge, MA, U.S.A.), biotin-conjugated rat monoclonal anti-mouse IFN-γ (XMG1.2; BD PharMingen), biotin-conjugated rat monoclonal anti-mouse IL-2 (JES6-5H4; BD PharMingen), and goat polyclonal anti-mouse IL-7 (R and D, Minneapolis, MN, U.S.A.) ones. A 100 μl solution of anti-mouse IFN-γ antibodies (1 μg/ml) diluted with 0.05 M Tris buffer (pH 8.9), or anti-mouse IL-2 (1 μg/ml) or anti-mouse IL-7 (2 μg/ml) antibodies diluted with 0.1 M $NaHCO_3$ (pH 8.2) was added to the wells of microtitre-plates (Nunc), followed by incubation overnight at 4 °C. The wells were then washed with 0.11 M PBS, pH 7.2, containing 0.05% Tween 20 (PBS-0.05% Tween), and blocked with 0.11 M PBS (pH 7.2) containing 3% polyethylene glycol 6000 and 5% FCS for 1 h at room temperature. For IL-7 measurement, 0.11 M PBS containing 3% polyethylene glycol 6000 and 10% ovalbumin (Seikagaku Co.) was used as the blocking solution. The plates were washed again, and then the culture supernatants diluted with PBS containing 3% polyethylene glycol 6000 for IL-2 and IFN-γ measurement, or PBS containing 3% polyethylene glycol 6000 and 0.1% Tween 20 for IL-7 measurement were added, followed by incubation for 2 h. After washing, biotin-conjugated rat anti-mouse IFN-γ or biotin-conjugated rat anti-mouse IL-2 antibodies, diluted with PBS containing 3% polyethylene glycol 6000, were added to each well, followed by standing for 2 h. For IL-7 measurement, the plates were incubated with 1 μg/ml goat anti-mouse IL-7 antibodies diluted with PBS containing 3% polyethylene glycol 6000 and 0.1% Tween 20 for 2 h. After washing, biotin-conjugated anti-goat IgG antibodies diluted with PBS containing 3% polyethylene glycol 6000 and 0.1% Tween 20 were added to each well, followed by standing for 15 min. The plates were washed and then alkaline phosphatase-conjugated streptavidin (Zymed Lab.) diluted with PBS containing 3% polyethylene glycol 6000 was ad-

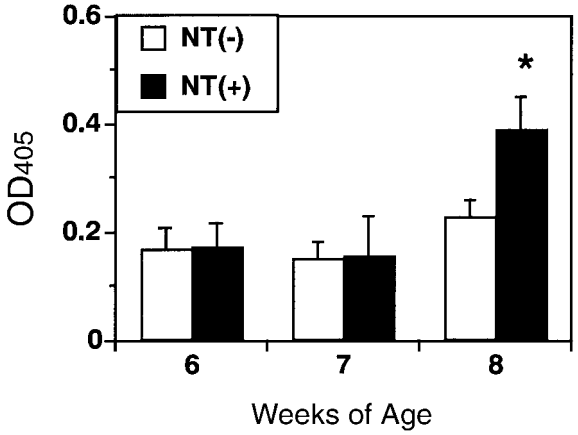

Figure 1. Effect of dietary nucleotides on the faecal level of OVA-specific immunoglobulin A (IgA) antibodies in OVA-TCR Tg mice. OVA-TCR Tg mice (3 weeks old) were fed *ad libitum* the NT(–) diet or the NT(+) diet plus water supplemented with 2% OVA (n = 10 for each group). Faeces were harvested from OVA-TCR Tg mice at 6, 7 and 8 weeks of age for determination of antibody levels. The faecal OVA-specific IgA levels for individual mice were determined by means of an enzyme-linked immunosorbent assay (ELISA). The results are presented as the means ± SE of data for individual mice per group. An asterisk (*) indicates a significant difference at $p < 0.05$ between the two groups.

ded to each well. After the plates had been washed, the 4-nitrophenylphosphate substrate reagent (0.1% 4-nitrophenylphosphate (Tokyo Kasei Co., Tokyo, Japan) solution in 0.1 M diethanolamine buffer, pH 9.8) was added to each well. The plates were then incubated for 1 h at room temperature and the reaction was terminated by adding 5 N NaOH. The absorbance was measured optically at 405 nm.

An ELISA kit (Promega, Madison, MI, U.S.A.) was used to determine the TGF-β contents. The TGF-β levels in the enterocyte supernatant fractions were determined by means of a standard curve obtained with recombinant TGF-β.

Statistics

The statistical significance of the data was determined by Student's *t*-test. A value at $p < 0.05$ was regarded as significant.

Results

Effect of dietary nucleotides on the OVA-specific IgA antibody response

In our previous study, it was shown that mucosal IgA response specific to OVA was induced by feeding OVA-TCR Tg mice *ad libitum* with a diet containing egg white proteins (Shida K et al., unpublished observations). We also found that the mucosal IgA response was similarly induced by supplying water supplemented with 2% OVA *ad libitum* to the mice. Thus, we first examined the effect of dietary nucleotides on the mucosal IgA response by feeding male OVA-TCR Tg mice with the NT(–) or NT(+) diet together with water supplemented with 2% OVA from 3 to 8 weeks of age (n = 10 for each group). The faecal OVA-specific IgA level was significantly increased in mice fed the NT(+) diet ($p < 0.05$) compared with in mice fed the NT(–) diet at 8 weeks of age (Figure 1). Similar results were obtained when female OVA-TCR Tg mice were used (data not shown).

Effects of dietary nucleotides on the composition of IEL subsets, and the expression of IL-2R and IL-7R in IELs

We next examined the effects of dietary nucleotides on the composition of IEL subsets in OVA-TCR Tg mice by flow cytometric determination of TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T cells. In our previous study, we showed that the proportion of TCR$\gamma\delta^+$ IELs in BALB/c mice fed the NT(+) diet was significantly higher than that in ones fed the nucleotide-free diet (Nagafuchi et al., 2000b). Similarly, in OVA-TCR Tg mice at 7 weeks of age, an increased proportion of TCR$\gamma\delta^+$ IELs in mice fed the NT(+) diet was observed compared with in ones fed the NT(–) diet, while the proportion of TCR$\alpha\beta^+$ IELs was decreased in mice fed the NT(+) diet (Figure 2A). The ratio of TCR$\alpha\beta^+$ IELs vs. TCR$\gamma\delta^+$ IELs in mice fed the NT(+) diet was significantly lower than that in ones fed the NT(–) diet (Figure 2B).

TCR$\alpha\beta^+$ T cells among IELs can be subdivided into five major subsets based on CD4 and CD8 expression: subsets with the phenotypes of $CD4^+CD8^-$, $CD4^+CD8\alpha\alpha^+$, $CD4^-CD8\alpha\beta^+$, $CD4^-CD8\alpha\alpha^+$, and $CD4^-CD8^-$ lymphocytes, respectively. In contrast, TCR$\gamma\delta^+$ IELs consist of two subsets with the phenotypes of $CD4^-CD8^-$ and $CD4^-CD8\alpha\alpha^+$ lymphocytes (Banderia et al., 1990; Guy-Grand et al., 1991; Lefrancois, 1991). Then we examined the

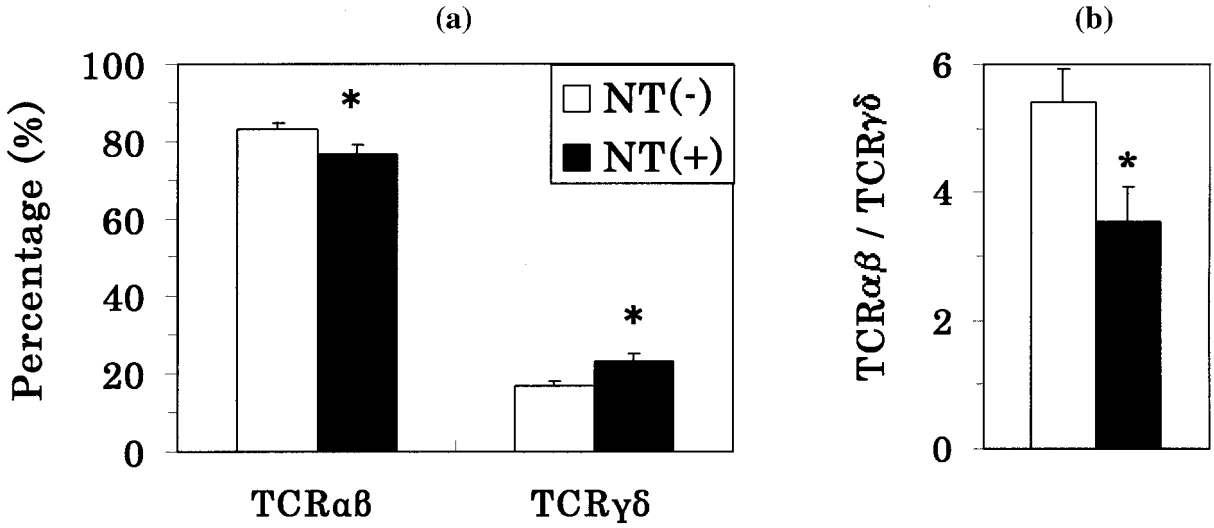

Figure 2. Effects of dietary nucleotides on the proportions of TCR$\alpha\beta^+$ T cells and TCR$\gamma\delta^+$ T cells among IELs from OVA-TCR Tg mice. OVA-TCR Tg mice (3 weeks old) were fed *ad libitum* the NT(−) diet (n = 10) or the NT(+) diet (n = 7) plus water supplemented with 2% OVA for 4 weeks. Stained cells were analyzed by flow cytometry to determine the proportions of TCR$\alpha\beta^+$ T cells and TCR$\gamma\delta^+$ T cells (A), and the ratio of TCR$\alpha\beta^+$ T cells vs. TCR$\gamma\delta^+$ T cells (B) among IELs from OVA-TCR Tg mice at 7 weeks of age. The percentages of TCR$\alpha\beta^+$ T cells and TCR$\gamma\delta^+$ T cells were calculated on the basis of the total percentage of TCR$\alpha\beta$ and TCR$\gamma\delta$-positive IELs. The results are presented as the means ± SE of data for individual mice per group. An asterisk (*) indicates a significant difference at $p < 0.05$ between the two groups.

influence of dietary nucleotides on IEL subsets in OVA-TCR Tg mice based on CD4 and CD8 expression. The proportion of TCR$\gamma\delta^+$CD8$\alpha\alpha^+$ T cells was significantly higher, and that of TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ T cells was significantly lower in the mice fed nucleotides. There was no significant difference between the two dietary groups in the proportions of the TCR$\alpha\beta^+$CD8$\alpha\beta^+$, TCR$\alpha\beta^+$CD4$^+$CD8$^-$, TCR$\alpha\beta^+$CD4$^+$CD8$^+$ and TCR$\gamma\delta^+$CD4$^-$CD8$^-$ subpopulations in IELs from OVA-TCR Tg mice. These results, which are similar to those obtained for BALB/c mice (Nagafuchi et al., 2000b), are summarized in Table 2.

Signals from IL-2R (Suzuki et al., 1997) and IL-7R (Maki et al., 1996; Fujihashi et al., 1997) play a critical role in the development of TCR$\gamma\delta^+$ IELs. Therefore, we examined the effects of dietary nucleotides on the expression of IL-2R and IL-7R in IELs. There was no difference in the population of IL-2R$^+$ cells among TCR$\alpha\beta^+$ IELs or TCR$\gamma\delta^+$ IELs between NT(–)-fed and NT(+)-fed mice. Similarly, there was no difference in the population of TCR$\alpha\beta^+$ IL-7R$^+$ IELs or TCR$\gamma\delta^+$ IL-7R$^+$ IELs between the two dietary groups (data not shown).

Effect of dietary nucleotides on cytokine secretion by IELs

We also examined the influence of dietary nucleotides on the cytokine secretion by OVA-stimulated IELs from OVA-TCR Tg mice at 7 weeks of age. IL-2 secretion by OVA-stimulated IELs from mice fed the NT(+) diet was significantly increased compared with that in mice fed the NT(–) diet (Figure 3). In contrast, no significant difference was observed in IFN-γ secretion by IELs, although IELs from mice fed the NT(+) diet showed a tendency to secrete a higher level of IFN-γ.

Effect of dietary nucleotides on cytokine secretion by IECs

TGF-β is known to be secreted by IECs and to be associated with the mucosal IgA response (Goodrich and McGee, 1998). Thus, we examined the influence of dietary nucleotides on TGF-β secretion by IECs. Increased TGF-β secretion was observed in mice fed the NT(+) diet (n = 4) compared with in ones fed the NT(–) diet (n = 4) (Figure 4A).

It is known that IL-7 secretion by IECs induces the development of TCR$\gamma\delta^+$ IELs (Laky et al., 2000), and our previous study showed that dietary nucleotides enhance the secretion of IL-7 from IECs (Nagafuchi et al., 2000b). Thus, we examined whether increased

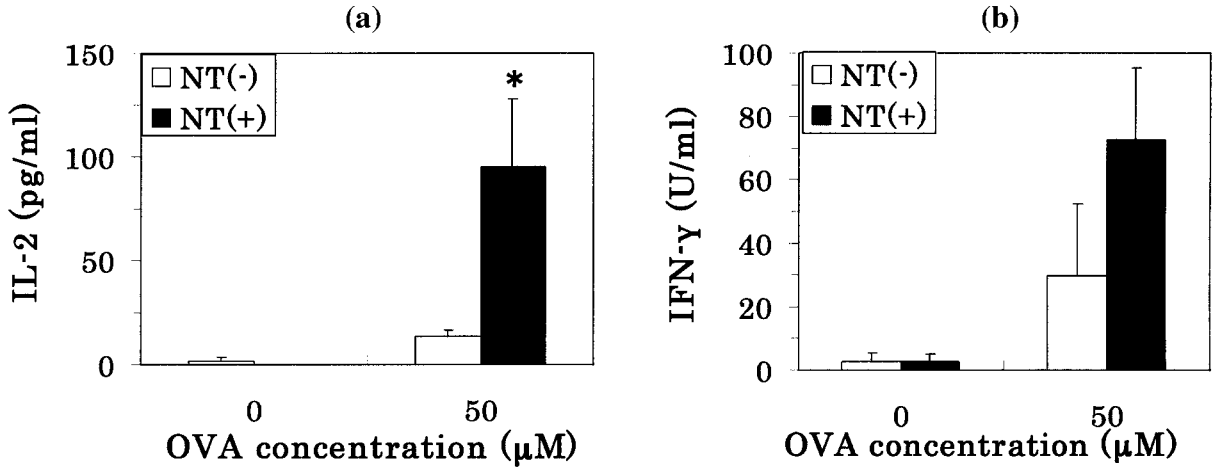

Figure 3. Effects of dietary nucleotides on secretion of interleukin 2 (IL-2) (A) and interferon γ (IFN-γ) (B) by IELs from OVA-TCR Tg mice. OVA-TCR Tg mice (3 weeks old) were fed *ad libitum* the NT(–) diet (n = 4) or the NT(+) diet (n = 3) plus water supplemented with 2% OVA *ad libitum* for 4 weeks. Cell cultures were carried out with or without OVA at the concentration of 50 μM for 2 days. The data shown are the results of a representative of two independent experiments. The results are presented as the means ± SE of data for individual mice per group.

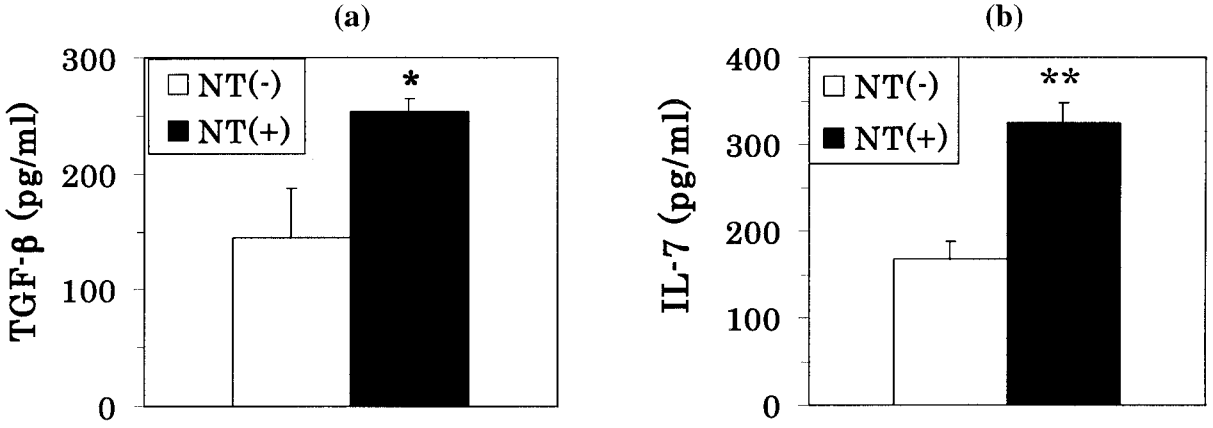

Figure 4. Secretion of transforming growth factor β (TGF-β) (A) and interleukin 7 (IL-7) (B) by small intestinal epithelial cells from OVA-TCR Tg mice fed the NT(–) or NT(+) diet. OVA-TCR Tg mice (3 weeks old) were fed *ad libitum* the NT(–) diet or the NT(+) diet plus water supplemented with 2% OVA for 4 weeks. The concentrations of IL-7 and TGF-β in culture supernatants of epithelial cells (2×10^5 cells/well) from individual mice (n = 10 for IL-7 and n = 4 for TGF-β) cultured for 16 h were measured. The data shown are the results of a representative of two independent experiments. The results are presented as the means ± SE of data for individual mice per group. An asterisk (*) and two asterisks (**) indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively, between the two dietary groups.

secretion of IL-7 from IECs induced by dietary nucleotides was observed in OVA-TCR Tg mice as well. Our results showed that IL-7 secretion was also significantly increased in OVA-TCR Tg mice fed the NT(+) diet (n = 10) compared with in ones fed the NT(–) diet (n = 10) (Figure 4B).

Discussion

In the present study, we demonstrated that the mucosal OVA-specific IgA response was significantly increased by oral administration of dietary nucleotides in OVA-TCR Tg mice fed OVA. Since the concentration of secretory IgA in the intestines of infants is very low and the gastrointestinal tracts of infants are more permeable to food antigens than those of adults because of gastrointestinal immaturity, infants are more susceptible to food allergies. Considering

Table 2. Profiles of CD4, CD8$\alpha\alpha$, and CD8$\alpha\beta$ expression by TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ IELs from OVA TCR-Tg mice fed the NT(–) or NT(+) diet plus water supplemented with 2% OVA (%)

	NT(–) diet	NT(+) diet
TCR$\alpha\beta^+$ IEL		
$CD4^+CD8^{+a}$	0.6 ± 0.1	0.4 ± 0.0
$CD4^+CD8^{-a}$	7.2 ± 0.7	6.3 ± 0.6
$CD4^-CD8\alpha\beta^{+b}$	6.3 ± 0.9	5.3 ± 0.7
$CD4^-CD8\alpha\alpha^{+b}$	20.2 ± 0.8	15.8 ± 1.0*
$CD4^-CD8^{-a}$	48.3 ± 1.9	48.4 ± 1.8
TCR$\gamma\delta^+$ IEL		
$CD4^-CD8\alpha\alpha^{+c}$	13.0 ± 1.2	18.2 ± 1.8*
$CD4^-CD8^{-c}$	3.4 ± 0.4	4.4 ± 0.5

Data are shown as means ± SE of the percentages of IELs from individual mice (n = 10 for NT(–) diet group, and n = 7 for NT(+) diet group), as determined by flow cytometry analysis. The percentages of $CD4^+CD8^+$, $CD4^+CD8^-$, $CD8\alpha\beta^+$, $CD8\alpha\alpha^+$, and $CD4^-CD8^-$ T cells were calculated on the basis of the total percentage of TCR$\alpha\beta$- and TCR$\gamma\delta$-positive IELs.

[a–c] The IELs were stained with combinations of biotin-labeled anti-mouse TCRβ, FITC-labeled anti-mouse CD4, and PE-labeled anti-mouse CD8α antibodies (for a), biotin-labeled anti-mouse TCRβ, FITC-labeled anti-mouse CD8β, and PE labeled anti-mouse CD8α antibodies (for b), and biotin-labeled anti-mouse CD4, FITC-labeled anti-mouse TCRδ, and PE-labeled anti-mouse CD8α antibodies (for c) for three-color analysis. To detect biotin-labeled antibodies, streptavidin-Red 670 was used. IELs were analyzed by flow cytometry as described under the Materials and Methods Section.

* A significant difference at $p < 0.05$ between the two groups.

that human milk contains a much higher quantity of nucleotides than cow's milk (Barness, 1994) and that the occurrence of food allergies is higher in formula-fed infants than in breast-fed ones, the abundance of nucleotides in human milk could be one reason for the advantage of exclusive breast feeding in preventing infants from developing food allergies (Vandenplas et al., 1988) through the enhancement of the intestinal IgA response.

TCR$\gamma\delta^+$ T cells are known to play an important role in IgA antibody production. Fujihashi et al. (1996) reported that the frequency of intestinal IgA plasma cells as well as the IgA levels in serum, bile, saliva, and faecal samples were markedly reduced in TCRδ-deficient mice. The TCRδ-deficient mice produced much lower levels of IgA antibodies when immunized orally with a vaccine of tetanus toxoid plus cholera toxin as an adjuvant (Fujihashi et al., 1996). Considering our finding that the proportion of the TCR$\gamma\delta^+$ IEL subset increased significantly in OVA-TCR Tg mice fed nucleotides, it is possible that dietary nucleotides up-regulate the mucosal IgA response by increasing the proportion of the TCR$\gamma\delta^+$ IEL subset.

We showed in the previous study that dietary nucleotides enhance the secretion of IL-7 from IECs in BALB/c mice (Nagafuchi et al., 2000b). In the present study, increased secretion of IL-7 was also observed for IECs from OVA-TCR Tg mice fed the NT(+) diet. Since it is known that IL-7 secretion by IECs is indispensable for TCR$\gamma\delta^+$ T cell development (Fujihashi et al., 1997), our results suggest that dietary nucleotides may increase the proportion of the TCR$\gamma\delta^+$ T cell subpopulation through enhancement of IL-7 secretion by IECs in OVA-TCR Tg mice. On the other hand, IL-2 also plays a critical role in TCR$\gamma\delta^+$ T cell development (Suzuki et al., 1997; Nikhat et al., 1998). Our results showed that IL-2 secretion by OVA-stimulated IELs was increased in nucleotide-fed mice, suggesting that the enhancement of IL-2 secretion by IELs may also cause the development of TCR$\gamma\delta^+$ IELs in OVA-TCR Tg mice.

Several cytokines secreted by T cells and other cells are also known to influence mucosal IgA synthesis. TGF-β acts as an isotype-specific switch factor for IgA (Coffman et al., 1989). TGF-β secretion by T cells in Peyer's patches has been thought to induce mucosal IgA synthesis (Holmgren et al., 1992). Moreover, recent *in vitro* studies demonstrated that TGF-β secretion by IECs also enhances IgA production. When lipopolysaccharide-stimulated B cells were cultured with the supernatants of an unstimulated rat intestinal epithelial cell line (IEC-6), IgA production was increased. Treatment of the IEC-6 supernatant with TGF-β-specific antibodies abolished the enhancement of IgA production, revealing that TGF-β was responsible for the enhanced IgA production (Goodrich and McGee, 1998). In this study, we observed that dietary nucleotides enhance TGF-β secretion by IECs. Thus, the increase in the mucosal OVA-specific IgA response on the feeding of dietary nucleotides may be also caused by the enhancement of TGF-β secretion.

IL-2 is also associated with the mucosal IgA response (Lebman et al., 1990). The addition of IL-2 together with TGF-β results in a 5- to 10-fold increase in IgA production compared to when only TGF-β is added (Lebman et al., 1990). It is suggested that dietary nucleotides induce IL-2 secretion in mice and humans. IL-2 secretion following concanavalin A stimulation of irradiated splenic lymphocytes was increased in an RNA-repleted group of mice compared with in an

nucleotide-free group of mice (Van Buren et al., 1985). In addition, IL-2 secretion by stimulated mononuclear cells was higher in the nucleotide-supplemented group than the nucleotide-free group in humans (Carver et al., 1991). Our results also showed that dietary nucleotides enhance IL-2 secretion by OVA-stimulated IELs. Considering that IL-2 enhances IgA production (Lebman et al., 1990), it is possible that dietary nucleotides may up-regulate the OVA-specific IgA antibody response through the enhancement of IL-2 secretion by IELs.

In conclusion, we demonstrated here that the feeding of dietary nucleotides enhance the mucosal OVA-specific IgA response and the proportion of TCR$\gamma\delta^+$ IELs. Furthermore, we showed that dietary nucleotides augment the secretion of TGF-β and IL-7 from IECs, and the secretion of IL-2 from IELs. Taken together, our findings suggest that dietary nucleotides may increase the mucosal IgA response by increasing the population of the TCR$\gamma\delta^+$ IEL subset, and by enhancing the secretion of TGF-β and IL-2 by IECs and IELs, respectively.

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