Microarray analysis of mRNAs extracted from the Peyer's patch cells of mice given a diet including *Escherichia coli* and its specific bovine milk IgG

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Five-week-old mice were given a diet consisting of ovalbumin alone (OVA, control diet) or a mixture of OVA, *Escherichia coli*, and its specific bovine milk IgG (IgG/*E.coli*-added diet) as a protein source for 5 weeks, and mRNAs extracted from Peyer's patch cells of the mice were analyzed by means of DNA microarray. The gene expression of proteins relating to immunoglobulin production and development of immune diseases was reduced in mice given the IgG/*E.coli*-added diet compared with those given the control diet. In contrast, the gene expression of marker proteins on Th1, Th3, and negatively regulatory T cells was noticeably increased. On the other hand, Peyer's patch cells from mice that had not been given any *E. coli* or milk IgG were cultured with milk IgG, *E. coli*, or a mixture of *E. coli* and its specific milk IgG, and were subjected to a cell function analyzer. The numbers of $CD19^+$ cells and interleukin-4^+CD4^+ cells increased significantly when the cells were cultured with either milk IgG or *E. coli*, while the mixture of *E. coli* and its specific milk IgG hardly influenced the numbers of these cells. These results indicate that the result obtained by DNA microarray analysis is not due to free milk IgG or *E. coli* alone, but is attributable to a mixture of *E. coli* and its specific IgG, suggesting that a mixture of *E. coli* and its specific IgG in intestinal tracts would reduce the development of allergic symptoms and autoimmune diseases.

Mikroarray-Analyse von aus Zellen Peyer'scher Platten extrahierten mRNAs von Mäusen mit einer Ration unter Einschluss von *Escherichia coli* und dem spezifischen bovinen Milch-IgG

Fünf Wochen alte Mäuse erhielten als Eiweißquelle eine Ration aus Ovalbumin allein (OVA, Kontrolle) oder eine Mischung von OVA, *Escherichia coli* und dem spezifischen bovinen Milch-IgG (IgG/*E. coli*-Ration) über einen Zeitraum von 5 Wochen. Die aus den Zellen der Peyer'schen Platten der Mäuse extrahierten mRNAs wurden mithilfe eines DNA-Mikroarrays analysiert. Die Genexprimierung der Proteine hinsichtlich der Immunglobulinbildung und die Entwicklung von Immunkrankheiten war bei Mäusen reduziert, die die IgG/*E. coli*-Ration erhalten hatten, im Vergleich zu den Versuchstieren, denen die Kontrollration verabreicht worden war. Im Gegensatz hierzu war die Genexprimierung der Markerproteine auf Th 1, Th 3 und negativ-regulatorischen T-Zellen deutlich erhöht. Zellen der Peyer'schen von Mäusen, die keine *E. coli* und kein Milch-IgG erhalten hatten, wurden mit Milch-IgG, *E. coli* oder einer Mischung von *E. coli* und dem spezifischen Milch-IgG kultiviert und mit einem Zellfunktionsanalysator untersucht. Die Anzahl der $CD19^+$- und der Interleukin-4^+CD4^+-Zellen erhöhte sich signifikant bei Kultur der Zellen entweder mit Milch-IgG oder *E. coli*, während die Mischung von *E. coli* und dem spezifischen Milch-IgG die Anzahl dieser Zellen kaum beeinflusste. Diese Befunde zeigen, dass die Ergebnisse der DNA-Mikroarray-Analyse nicht auf freies Milch-IgG oder *E. coli* allein zurückgehen, sondern einer Mischung von *E. coli* und dem spezifischen IgG zuzurechnen sind. Die Befunde weisen darauf hin, dass eine Mischung von *E. coli* und dem spezifischen IgG im Intestinaltrakt de Entwicklung allergischer Symptome und Autoimmunerkrankungen reduzieren dürfte.

16 Allergies (modulation in mice), **autoimmune diseases** (modulation in mice)

16 Allergien (Modulation bei Mäusen), **Autoimmunerkrankungen** (Modulation bei Mäusen)

1. Introduction

In previous papers, OHNUKI *et al.* (1) reported that the oral ingestion of bovine milk IgG suppressed immunoglobulin production in mice, while MIZUTANI *et al.* (2) observed that bovine milk IgG stimulated the production of immunoglobulin in mouse spleen cell cultures. In another paper, OHNUKI and OTANI (3) demonstrated that the different effects of milk IgG on immunoglobulin production observed following oral ingestion and in spleen cell cultures might be due to differences in the receptors for IgG present on accessory cells, i.e., the antigen-free milk IgG binds to accessory cells through the Fcγ receptor type I (FcγRI) and increases immunoglobulin production while the complex of *Escherichia coli* and its specific milk IgG binds to accessory cells through Fcγ receptor type IIb (FcγRIIb) and suppresses immunoglobulin production. It is unclear, however, how the oral ingestion of a mixture of *E. coli* and its specific milk IgG influences gene expression relating to the production of immunoglobulin.

In this paper, mice were given a diet consisting of ovalbumin (OVA), *E. coli*, and its specific bovine milk IgG as a protein source, and mRNAs extracted from Peyer's patch cells of the mice were compared with those of mice given a diet consisting of OVA alone by means of DNA microarray.

2. Materials and methods

This experiment was conducted in accordance with the guidelines for the Regulation of Animal Experimentation at Shinshu University and according to Law No. 105 and Notification No. 6 of the Japanese government.

2.1 Materials

Defined fetal bovine serum (FBS) was obtained from Equitech-Bio Inc. (Kerrville, TX, USA). RPMI-

1640 medium was purchased from Nissui Pharmaceutical (Tokyo). Biolegend (San Diego, CA) supplied phycoerythrin (PE)-labeled anti-mouse interleukin (IL)-4 monoclonal antibodies (mAb, clone 11B11), PE-labeled anti-mouse interferon (IFN)-γ mAb (clone XMG1.2), PE-labeled anti-mouse CD49b mAb (clone DX5), biotin-labeled anti-mouse CD4 mAb (clone RM4-5), biotin-labeled anti-mouse CD11b mAb (clone M1/70), biotin-labeled anti-mouse CD19 mAb (clone MB19-1), and phycoerythrin/cyanine 5 (PE/Cy5)-labeled streptavidin. Brefeldin A (BFA), ionomycin, and phorbol 12-myristate 13-acetate (PMA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). IntraPrep Permeabilization Reagent was bought from Beckman Coulter Inc., Tokyo. A defined protein-free purified diet (PM15765) was obtained from Purina Mills (St. Louis, MO, USA). OVA was obtained from Wako Pure Chemical Industries (Osaka, Japan). Penicillin G potassium and streptomycin sulfate were obtained from Meiji Seika, Tokyo. Guava Viacount Reagent was purchased from Guava Technologies (Hayward, CA). Other chemicals were of the highest analytical grade commercially available.

2.2 Bovine milk IgG and E. coli

Bovine milk IgG was prepared from immune milk (Kanematsu Wellness Corporation, Tokyo, Japan) according to a procedure described previously (3). The strain of *E. coli* was NBRC 3301, and *E. coli*-specific bovine milk IgG was prepared according to the method described in a previous paper (3).

2.3 Feeding procedure

Table 1: Composition of test diets

	Control diet (%)	IgG/*E.coli*-added diet (%)
IgG	0.000	0.010
E. coli	0.000	0.040
OVA	25.000	24.950
Dextrin	32.738	32.738
Sucrose	27.112	27.112
RP mineral*	3.750	3.750
Corn oil	3.750	3.750
Lard	3.750	3.750
Cellulose	2.250	2.250
RP vitamin**	1.500	1.500
Choline	0.150	0.150
Total	100.000	100.000

*RP mineral mix#10: calcium, 0.60%; phosphorous, 0.40%; potassium, 0.40%; magnesium, 0.07%; sodium, 0.21%; chlorine, 0.24%; fluorine, 5.00ppm; iron, 60.00ppm; zinc, 21.00ppm; manganese, 65.00ppm; copper, 15.00ppm; cobalt, 3.20ppm; iodine, 0.57ppm; chromium, 3.00ppm; molybdenum, 0.82ppm; selenium, 0.23ppm.

**RP vitamin mix: vitamin K, 10.4ppm; thiamine hydrochloride, 20.6ppm; riboflavin, 20.0ppm; niacin, 90.0ppm; pantothenic acid, 55.0ppm; choline chloride, 1400.0ppm; folic acid, 4.0ppm; pyridoxine, 16.5ppm; biotin, 0.4ppm; vitamin B12, 20.1μg/Kg; vitamin A, 22.1 IU/g; vitamin D3, 2.2 IU/g; vitamin E, 50.0 IU/kg.

Four-week-old male C3H/HeN mice (Japan SLC, Hamamatsu, Japan) were assigned to test regimens and given a commercial mouse pellet (MF, Oriental Yeast Company, Tokyo, Japan) for 1 week. They were then given 75% PM15765 supplemented with 25% OVA (control diet) or a mixture of 24.45% OVA, 0.01% *E. coli*-specific IgG, and 0.04% *E. coli* NBRC 3301 (IgG/*E.coli*-added diet) for 5 weeks. The detailed composition of each diet is shown in Table 1. The diets were continuously available in columnar form from stainless-steel feeders. Water was provided ad libitum from drinking bottles. The mice were maintained at 23[H.O1]±2 °C under 12 h-light/12 h-dark cycles.

2.4 Cell cultures

Peyer's patch cells were prepared from mice given the control or IgG/*E.coli*-added diet and from 6-week-old male C3H/HeN mice (Japan SLC) that had not been given any *E. coli* or milk IgG according to the procedure described previously (3). The Peyer's patch cells from mice given the control or IgG/*E.coli*-added diets were subjected to DNA microarray analysis. The Peyer's patch cells from the 6-week-old mice were cultured with milk IgG (0, 50 or 100 μg/ml), *E. coli* NBRC 3301 (0, 10^4 cells/ml or 10^5 cells/ml), or a mixture of IgG (0, 50 or 100 μg/ml) and *E. coli* NBRC 3301 (0, 10^4 or 10^5 cells/ml) at 37 °C in RPMI-1640 medium containing 5% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). After 72 h cultivation, the cells were subjected to cell function analysis.

2.5 Cell function analysis

The cultured Peyer's patch cells were incubated with mouse Fc block for 15 min at 4□ before being reacted with PE-labeled anti-mouse mAbs specific to CD49b, or with biotin-labeled anti-mouse mAbs specific to CD19 or CD11b for 15 min at 4□. Cells already reacted with the biotin-labeled antibody were further incubated with PE/Cy5-labeled streptavidin for 15 min at 4□. Finally, all cells were analyzed using a Guava Personal Cell Function Analyzer (Guava PCA, Guava Technologies).

In the case of $CD4^+$cells having intracellular cytokines, on the other hand, spleen cells were incubated at 37°C in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml BFA, 2 μg/ml ionomycin and 20 ng/ml PMA for 4 h. The marker antigen of the cells was reacted with biotin-labeled anti-mouse mAbs specific to CD4 (clone RM4-5) for 15 min at 4°C, and then incubated with PE/Cy5-labeled streptavidin for 15 min at 4°C. Intracellular cytokines were performed by permeabilization of PE-labeled anti-mouse cytokine mAbs specific to IL-4 or IFN-γ. Briefly, the cells were fixed in IntraPrep regent 1. After 15 min, the cells were washed and permeabilized by incubation with IntraPrep regent 2. The cells containing cytokines were visualized after incubation with PE-labeled anti-mouse mAbs specific to IL-4 or IFN-γ and were analyzed by means of Guava PCA.

2.6 DNA Microarray analysis

The genome-wide gene expression of Peyer's patch cells was examined using Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA), which contains 45101 probe sets for approximately 34000 mouse genes. Briefly, total RNA was extracted from Peyer's patch cells using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). GeneChip analysis was performed according to the GeneChip Eukaryotic Target Preparation & Hybridization Manual (Affymetrix). Data analysis was performed with GeneChip Operating software 1.4. Data are shown as relative expression levels. Relative expression levels were calculated as follows: Relative expression level = Fluorescence

intensity based on mRNAs extracted from mice given the IgG/*E.coli* diet / Fluorescence intensity based on mRNAs extracted from mice given the control diet.

2.7 Statistical analysis

Cell numbers were expressed as the means ± standard deviation (n=4). The significance of differences was tested with the Student's *t*-test.

3. Results and discussion

3.1 Gene expression of proteins relating to the immune system in Peyer's patch cells of mice given the E.coli-added diet

Mice were given the control or IgG/*E.coli*-added diet for 5 weeks. No significant difference was observed in body weight between mice given different diets (data not shown). This indicates that there is little nutritional difference between the control and the IgG/*E.coli*-added diets.

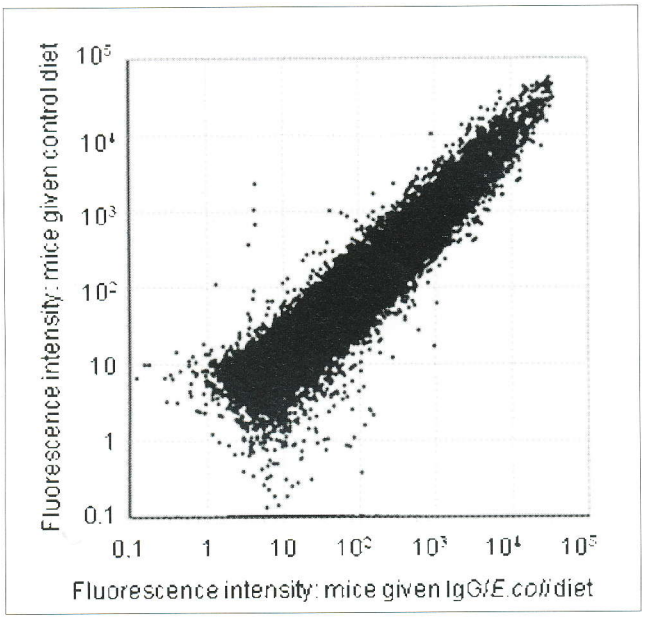

Fig. 1: Distribution of fluorescence spots derived from mRNA extracted from mouse Peyer's patch cells by DNA microarray.

Figure 1 shows the distribution of fluorescence spots derived from 33696 kinds of mRNA extracted from mouse Peyer's patch cells by DNA microarray. The fluorescence intensity of 3585 spots was at least 2-fold higher in the IgG/*E.coli*-added diet group than in the control diet group, while that of 4373 spots was less than 0.5-fold lower in the former than in the latter. Of the genes that showed a difference in expression between the control diet and IgG/*E.coli*-added diet groups, the expression levels of those genes relating to immunoglobulin production and autoimmune disease are shown in Fig. 2. Cd10, Cd122, Il18r, Tgfb1, and Pdlim2 increased more than 1.5-fold in mice given the IgG/*E.coli*-added diet in comparison with those given the control diet. In contrast, Adamdec1, Cd80, Cd28, Il4, Il10, Fcer1a, Fcer1b, Fcer1g, Slc22a4, and Fcgr3a decreased by less than 0.75-fold in the mice given the IgG/*E.coli* diet compared with those given the control diet.

The proteins transcribed from Adamdec1, Cd10,

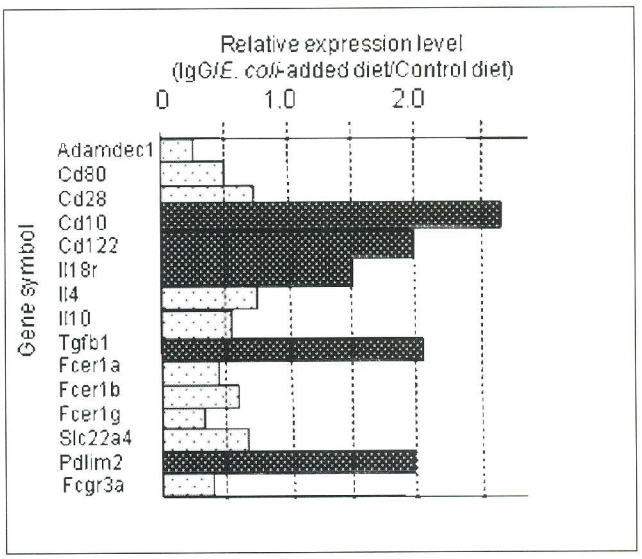

Fig. 2: Expression levels of genes relating to immunoglobulin production and autoimmune diseases in Peyer's patch cells of mice given the control or IgG/*E. coli*-added diet.

Cd80, Cd28, and Cd122 are decysin, CD10, CD80, CD28, and CD122, respectively. Decysin is one of the marker proteins on mature dendritic cells and is absent on immature dendritic cells (4), while CD10 is one of the marker proteins present on immature B cells and is absent on mature B cells and plasma cells (5). Dendritic cells are professional antigen-presenting cells (6). CD80 and CD28 are co-factors on antigen-presenting cells and T cells, respectively. These co-factors were demonstrated to be essential for the differentiation of Th0 cells into Th2 cells, which stimulate antibody responses (7). In addition, CD122 is one of marker proteins present on negatively regulatory T cells (8). These facts and the result shown in Fig. 2 indicate that the oral ingestion of E. coli and its specific milk IgG suppresses immunoglobulin production.

The protein transcribed from Il18r, Il4, Il10, and Tgfb1 are the IL-18 receptor, IL-4, IL-10, and TGF-β[H.O2]1, respectively. IL-4 and IL-10 are typical cell markers of Th2 cells (9), while the IL-18 receptor and TGF-β[H.O3]1 are typical cell markers of Th1 and Th3 cells, respectively (10, 11). These facts and the result shown in Fig. 2 indicate that the oral ingestion of E. coli and its specific milk IgG preferably induce the differentiation of Th0 cells into Th1 and Th3 cells rather than Th2 cells, suggesting that the oral ingestion of E. coli and its specific milk IgG suppresses the humoral immune response, or immunoglobulin production, and stimulates the cellular immune response.

On the other hand, the high-affinity receptor for IgE, otherwise known as Fcε[H.O4]receptor I (Fcε[H.O5]RI) that is expressed on mast cells is a crucial component in allergic responses (12). The proteins produced by transcription of Fcer1a, Fcer1b, and Fcer1g are the α[H.O6]-chain of Fcε[H.O7]RI (Fcε[H.O8]RI-α[H.O9]), the β-chain of FcεRI (Fcε [H.O10] RI-β [H.O11]), and the γ[H.O12]-chain of Fcε[H.O13]RI (Fcε[H.O14]RI-γ[H.O15]), respectively, and these proteins combine to form the IgE receptor. Moreover, the proteins produced by transcripotion of Fcgr3a, Pdlim2, and Slc22a4 are Fc receptor-like 3 (FCRL3), PDLIM2, and SLC22A4,

respectively. FCRL3 is associated with a susceptibility to several autoim-

Table 2: Numbers of immunocompetent cells in Peyer's patch cells cultured with or without *E. coli* and its specific IgG (Cell number ($x10^4/10^6$)

Cell	Without IgG and *E. coli*	IgG (50mg/ml)	IgG (100mg/ml)	*E.coli* (10^4 cells/ml)	*E.coli* (10^5 cells/ml)	IgG (50mg/ml) and *E.coli* (10^4 cells/ml)	IgG (100mg/ml) and *E.coli* (10^5 cells/ml)
CD19	49.0±0.4	55.0±0.9**	56.9±0.8**	56.4±0.7**	58.6±1.2**	48.4±1.1	50.6±1.5
CD4	29.9±1.3	33.3±0.6**	33.0±1.3**	31.0±0.8	30.8±0.7	30.9±1.0	29.3±0.7
CD11b	4.5±0.6	4.5±0.9	5.1±0.3	5.0±0.8	5.4±0.8	5.1±0.6	5.6±0.5
CD49b	3.6±0.2	3.3±0.4	3.5±0.5	3.4±0.5	3.4±0.3	4.0±0.3	3.5±0.4
IFN-γ^+CD4	2.1±0.5	2.3±0.3	2.1±0.2	1.8±0.3	2.1±0.5	1.9±0.2	2.2±0.2
IL-4^+CD4	1.2±0.2	2.5±0.5**	2.7±0.4**	1.8±0.3	2.2±0.1**	1.7±0.3	1.1±0.2

Values significantly differ from the culture without IgG and *E. coli* at **p<0.01.

mune diseases including rheumatoid arthritis, and is highly expressed on B cells of individuals with rheumatoid arthritis (13).

Similarly, SLC22A4 is the organic cation/carnitine transporter 1 and is associated with chronic inflammatory diseases, such as rheumatoid arthritis and Crohn's diseases. SLC22A4 is expressed in the synovial tissues of individuals with rheumatoid arthritis (14). Activation of transcription factor NF-κ[H.O16]B in the innate immune system is tightly regulated to prevent excessive inflammatory responses (15). TANAKA et al. (15) demonstrated that PDLIM2 negatively regulates NF-κ[H.O17]B activity through intranuclear sequestration and subsequent degradation. These facts and the result shown in Fig. 2 indicate that the oral ingestion of E. coli and its specific milk IgG suppress the development of allergic syndromes and autoimmune diseases through several mechanisms.

3.2 *Effects of E. coli and its anti-milk IgG on the number of immunocompetent cells in mouse Peyer's patch cell cultures*

Peyer's patch cells from mice that had not been given any E. coli or milk IgG were cultured with E. coli alone, its specific milk IgG alone, or mixtures of E. coli and its specific milk IgG for 72 h, and the numbers of several kinds of immunocompetent cells were determined. As shown in Table 2, both milk IgG alone and E. coli alone significantly increased the number of CD19+cells and IL-4+CD4+cells in Peyer's patch cell cultures, while the mixture of *E. coli* and its specific milk IgG hardly influenced the either cell number. CD19 is a typical cell surface antigen on B cells, which ifferentiate into plasma cells (16), and IL-4+CD4+cells are Th2 cells that stimulate immunoglobulin production (10). These results indicate that both the milk IgG alone and *E. coli* alone stimulate antibody responses but that a mixture of these does not. Thus, the immunomodulatory action observed after the oral administration of the mixture of *E. coli* and its specific milk IgG is concluded to be due to the action of the mixture of *E. coli* and its specific milk IgG, probably a complex of *E. coli* and IgG, and is not attributable to *E. coli* or milk IgG alone.

In conclusion, the DNA microarray analysis data in this paper demonstrate that a diet including *E.coli* and its specific milk IgG suppresses not only the production of immunoglobulin but also the development of allergic syndromes and autoimmune diseases through several mechanisms. We propose that antigens and their IgG complexes in intestinal tracts negatively modulate immunoglobulin production and suppress the development of allergic and autoimmune diseases. It is suggested that a mixture of edible microorganisms such as *Saccharomyces cerevisiae* and *S. carlsbergensis* and their specific milk IgGs would be useful as an anti-allergic food ingredient.

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