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ORIGINAL ARTICLE

Increased Number of CCR4-positive Cells in the Duodenum of Ovalbumin-induced Food Allergy Model NC/jic Mice and Antiallergic Activity of Fructooligosaccharides

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ABSTRACT

Background: Fructooligosaccharides (FOS) in prebiotic foods can alter intestinal immune responses. The combination of probiotics with oligosaccharides has been reported to alter intestinal flora and suggested to be beneficial against food allergy in humans.

Methods: All male Nc/jic mice used in this 8-week study were 6 weeks of age and were allotted to the following three groups: (1) the nonsensitization group; (2) the ovalbumin (OVA) sensitization +5% fructose-containing control food administration group; and (3) the OVA sensitization +5% FOS-containing food administration group. Duodenal tissues were collected and then immunohistochemically stained with monoclonal antibodies to CCR4 and CCR5. The number of mast cells and the villus edema formation rate in the duodenum were determined by image analysis.

Results: The number of CCR4-positive cells increased significantly in Group 2 as compared with Group 1 and tended to decrease in Group 3 as compared with Group 2. Relatively few CCR5-positive cells were observed in the duodenum. FOS tended to reduce the number of CCR4-positive cells but significantly reduced the number of mast cells and the edema formation rate in the duodenum.

Conclusions: This study demonstrated a correlation between the number of CCR4-positive cells and villus edema formation rate. Therefore, FOS, which we inferred to show antiallergic activity for food allergy in this study and which has already been established to be safe for use as food in humans, can be considered to be potentially useful for the prevention of food allergy in pediatric patients with allergy.

KEY WORDS

antiallergic activity, CCR4- and CCR5-positive cells, food allergy, fructooligosaccharides, prebiotic food

INTRODUCTION

The number of pediatric patients with food allergy has increased in recent years. However, the standard therapeutic modality for the disease is still not established, and treatments, including food restriction, fail to be sufficiently effective for the disease at present.[1] Since a long period of time is required to treat the disease in children, food restriction elicits concern about the issue of child growth. Therefore, the development of new therapeutic modalities and preventive procedures for food allergy constitutes a challenge in clinical practice. In the United States, patients with food allergies have been reported to account for 8% and 1 to 2% of pediatric patients $\leq$3 years of age and the total population, respectively.[2] In Japan, patients

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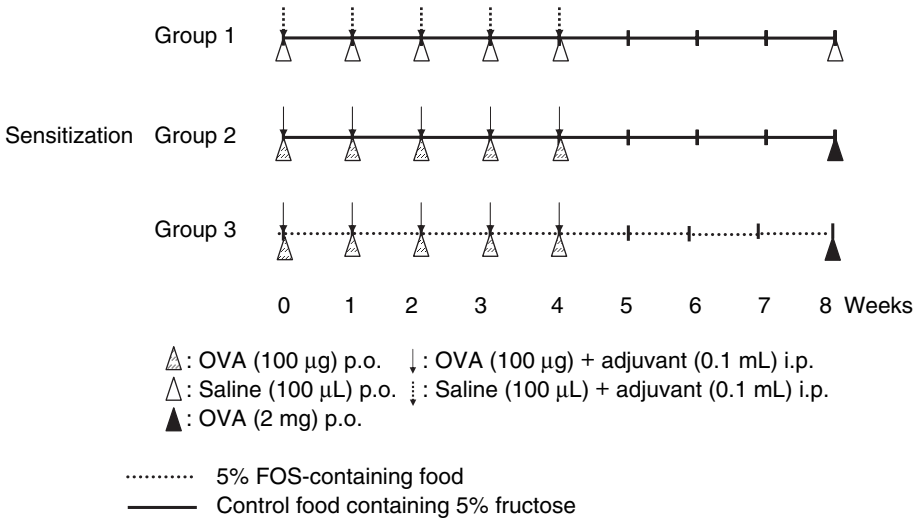


Fig. 1 Composition of the study groups and administration schedule. The study groups comprised three groups. Group 1 (OVA nonsensitization group) consisted of 10 intact mice which were not sensitized with OVA and which were allowed to take control food for 8 weeks. Group 2 (control group) consisted of six mice which were sensitized with OVA and were then allowed to take control food for 8 weeks. Group 3 (FOS administration group) consisted of seven mice which were sensitized with OVA and were then allowed to take 5% FOS-containing food for 8 weeks. To sensitize mice, 100 μg of ovalbumin were given by oral gavage, along with five consecutive intraperitoneal administrations of a mixed solution (100 μg of OVA and 0.1 mL of adjuvant) every other week. Nonsensitized control mice were treated similarly except using the same volume of saline instead of the OVA solution. At the end of the study, OVA (2 mg/0.1 mL) was given by oral gavage to mice in order to conduct antigen challenge.

with immediate food allergy account for 12.6% of pediatric patients with allergy less than 6 years of age, and their number has tended to increase in recent years.[3] In 2005, the food allergy guidelines were implemented also in Japan. Treatment of food allergy consists principally in the removal or restriction of food.

In recent years, the understanding of the relationship between the intestinal flora and allergy has advanced rapidly. The disruption of intestinal barrier function is considered one of the mechanisms by which food allergy develops. Clinical studies have reported that lactobacillus preparations improved the incidence of atopic dermatitis and dermatological symptoms[4-8] and ameliorated food allergy.[9] However, prebiotic foods possess advantages in that they are free of side effects and are administrable to children, including younger children and pediatric patients with milk allergy, for a long period of time. Probiotics have been reported to improve small intestinal barrier function. Therefore, we hypothesized that the administration of fructooligosaccharides (FOS) alone alters intestinal bacterial flora and repairs intestinal barrier function, resulting in the improvement of symptoms of food allergy.

We prepared a mouse model of food allergy in the present study to examine the mechanism of action of FOS, using the numbers of CCR4- and CCR5-positive cells and the villus edema formation rate in the duodenum as variables for examining their potential roles in the prevention and treatment of food allergy. We used CCR5- and CCR4-positive cells in Th1 and Th2 immune responses, respectively, because they are considered to be present independently in Th1 and Th2 cells. Th2 cells (CCR4 positivity cells) in blood have been reported to increase under allergy environments.[10-12]

Here we report the results of an *in vivo* study.

METHODS

ANIMALS AND THE COMPOSITION OF STUDY GROUPS

At the onset of the study, all male NC/jic mice, 6 weeks of age and 24.2 ± 0.7 grams in body weight (CLEA Japan, Tokyo, Japan), were housed under special pathogen-free (SPF) environments.

Prior to the onset of this 8-week study, the animals were allotted to the following three study groups: Group 1 (n = 10) in which they were not sensitized with ovalbumin (OVA) and were then allowed to freely take control food (CE-2; CLEA Japan) containing 5% fructose; Group 2 (n = 6) in which they were

sensitized with OVA and were then allowed to freely take control food containing 5% fructose; and Group 3 (n = 7) in which they were sensitized with OVA and were then allowed to freely take food containing 5% FOS.

METHOD OF SENSITIZATION

A mouse model of food allergy was prepared according to the method of Sakamoto *et al.*.[13] Briefly, a gastric tube was used to give 100 μg of ovalbumin (OVA; Sigma, St., Louise, MS, USA) by oral gavage, along with 5 consecutive intraperitoneal administrations of a mixed solution consisting of 100 μg of OVA and 0.1 mL of adjuvant (Imject Alum®; Pierce, Rockford, IL, USA) every other week. OVA was dissolved in saline before use. Nonsensitized control mice were treated similarly except using the same volume of saline instead of the OVA solution. At the end of the study, OVA (2 mg/0.1 mL) was given by oral gavage to mice in order to conduct antigen challenge (Fig. 1). At 3 hours after antigen challenge, mice were sacrificed to remove organs.

The present study was approved by the Animal Research Ethics Committee at Showa University.

DETERMINATION OF SERUM TOTAL IgE ANTIBODY LEVELS AND SERUM OVA-SPECIFIC IgE ANTIBODY LEVELS

A kit (IgE, Mouse, ELISA Quantitation Kit®; Bethyl Laboratory, Montgomery, TX, USA) was used to determine serum total IgE antibody levels.

Serum OVA-specific IgE antibody levels were determined by ELISA according to the method of Hirano *et al.*.[14] Namely, a microplate reader was used to determine the level in optical density at 450 nm (OD450).

We verified sensitization by serum total IgE antibody levels and serum OVA-specific IgE levels. The mean +2 SD of serum total IgE antibody levels or serum OVA-specific IgE levels in Group 1 (n = 10) was used as the cutoff value.

Immunohistochemical Staining

Immunohistochemical staining (streptavidin-biotin method) was conducted to examine the tissue distributions of Th1- and Th2-positive cells. The following antibodies were used as primary antibodies: antimouse CCR4 rabbit IgG1 [Antiserum (purified) to CCR4 (mouse)®, the 100-fold dilution of the concentrate solution; Alexis Biochemicals, San Diego, CA, USA] to detect CCR4; and antimouse CCR5 goat IgG1 [CKR-5 (M-20)®, the 100-fold dilution of the concentrate solution; Santa Cruz Biotechnology, Santa Cruz, CA, USA] to detect CCR5. HISTOMOUSE-PULUS KITS®, manufactured by ZYMED (streptavidin-biotin amplification), was used according to the LAB-SA method. Sections blocked with intrinsic peroxidase, avidin, and biotin, as well as washed with the 0.01 M phospate buffer solution were incubated with the first antibodies at room temperature for 60 minutes. Subsequently, biotinylated second antibody and labeling enzyme were incubated for 30 and 20 minutes, respectively. A substrate (DAB) was used for coloring operation over 3 minutes.

Counting of positive cells was performed in the following procedures: (1) selection of the observation site from 3 sites; (2) observation of photomicrographs of damage-free villi; (3) determination of a site where negative control had neither nonspecific binding (NSB) nor background; and (4) observation of round nucleated cells 8 to 12 μm in diameter.

HISTOPATHOLOGICAL ANALYSIS OF THE DUODENUM

A 15 mm long segment of the duodenum, located 50 mm from the pylorus of the stomach, was removed and was then fixed in the 10% buffered formalin solution for 12 hours. H&E stain and toluidine blue (pH 7.4) stains were applied.

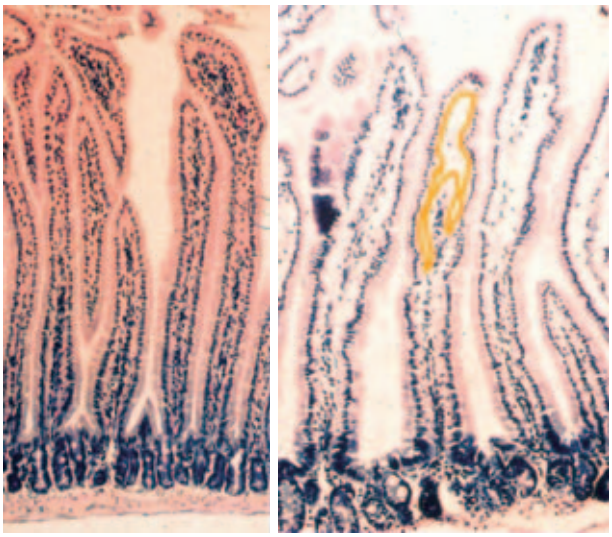
MicroGrid® was used to count mast cells in the duodenum at 3 sites where tissue remained intact. The mean of the counts was used to evaluate the cells. All histological examinations were conducted in a blinded fashion.

CALCULATION OF DUODENAL VILLUS EDEMA FORMATION RATES

Graphic software (WinRoof, version 5・0; Mitani Syoji, Tokyo, Japan) was used to calculate the edema formation rate of the duodenal villi. To determine the sites of calculation, 3 duodenal villi, which were free of tissue damage and contained the layer of muscularis mucosae, were selected from H&E-stained samples. All mice were coded and conducted in a blinded fashion. The mean area thereof was used to evaluate the rate. The total area per villus and the area occupied by edema were calculated automatically, and the ratio thereof was considered as the edema formation rate per villus. In order to avoid making the coefficient of variation for repeated measurements the difference between operating personnel, calculations were carried out by automatic analysis using WinRoof (Fig. 2). The mean +2 SD of the edema formation rate per villus in Group 1 (n = 10) was used as the cutoff value.

STATISTICAL ANALYSES

Values were expressed as the mean ± SE. JMP version 4.00 J (SAS Institute Inc., Cary, NC, USA) was used to conduct statistical analyses. The Wilcoxon rank-sum test and the Kruskal-Wallis rank test were conducted for rank sum and comparisons among the three study groups, respectively. The Spearman rank correlation coefficient was used to make correlation analyses. A value of $P < 0.05$ was considered statisti-



OVA nonsensitization group　　OVA sensitization group

Fig. 2 The image analysis procedure in the duodenum. The total area per villus and the area occupied by edema were calculated, and the ratio thereof was considered the edema formation rate per villus.

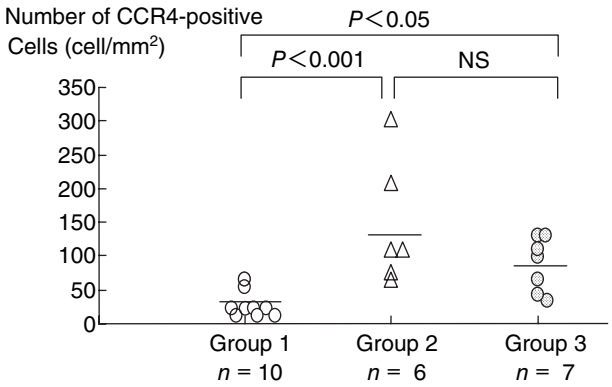


Fig. 3 Number (cell/mm²) of CCR4-positive cells in the duodenal villi of mice in the study groups. Group 2 (control group) showed a significant increase ($P < 0.001$) as compared with Group 1 (OVA nonsensitization group) with respect to the number (cell/mm²) of CCR4-positive cells. Group 3 (FOS administration group) tended to show a difference as compared with Group 2 (control group) which was determined in accordance with the criterion for CCR4-positive cell counting.

cally significant.

RESULTS

SERUM TOTAL IgE LEVELS AND SERUM OVA-SPECIFIC IgE LEVELS

The mean + 2 SD of serum OVA-specific IgE levels in Group 1 (n = 10) was used as the cutoff value (OD 450 : 0.08). The cutoff value of serum total IgE was 4031.4 ng/mL. Consequently, all animals in Groups 2 and 3 provided serum OVA-specific IgE levels which exceeded the cutoff value. Therefore, the sensitization rate was assessed to be 100%.

Serum total IgE levels (mean ± SE) were as follows: 1,131.2 ± 1499.7 ng/mL (n = 10) in Group 1 (OVA nonsensitization group); 6,525.9 ± 1,936.1 ng/mL (n = 6) in Group 2 (Control group); and 14,449.9 ± 1,792.5 ng/mL (n = 7) in Group 3 (FOS administration group). Furthermore, serum OVA-specific IgE levels (mean ± SE) were as follows: 0.05 ± 0.05 OD 450 (n = 10) in Group 1; 0.44 ± 0.07 OD450 (n = 5) in Group 2); and 0.67 ± 0.06 OD450 (n = 7) in Group 3. Serum total IgE levels and serum OVA-specific IgE levels increased significantly in Groups 2 and 3 ($P < 0.05$ and $P < 0.001$, respectively) as compared with Group 1. There was a significant correlation (r = 0.78, $p < 0.0001$) between serum OVA-specific IgE levels and serum total IgE levels.

NUMBERS OF CCR4- AND CCR5-POSITIVE CELLS

The numbers of CCR4-positive cells (cell/mm²; mean ± SE) were as follows: 30.5 ± 16.9 (n = 10) in Group 1; 145.2 ± 21.9 (n = 6) in Group 2; and 87.1 ± 20.2 (n = 7) in Group 3 (Fig. 3). The numbers thus increased significantly ($P < 0.001$) in Group 2 as compared with Group 1. On the other hand, Group 3 showed no statistically significant difference in the numbers as compared with Group 2 but showed a decrease in the number of CCR4-positive cells. A large number of CCR4-positive cells were observed in the mucous layer of the duodenal villi. Apart from lymphocytes, which are considered to be monocytes, dendritic cells were also observed among CCR4-positive cells.

CCR5-positive cells (cell/mm²) in the duodenal villi were observed only in one of 10 mice in Group 1 (CCR5/CCR4 = 0.15). Furthermore, a small number of CCR5-positive cells were observed in the submucosa (Fig. 4). No significant difference was found among Groups with respect to the number of CCR5-positive cells. On the other hand, a significant correlation (r = 0.61, p = 0.0027) between serum OVA-specific IgE level and the number of CCR4-positive cells was observed. Furthermore, a significant correlation (r = 0.49, p = 0.017) was found among serum total IgE level and number of CCR4-positive cells.

NUMBER OF MAST CELLS IN THE DUODENUM

The numbers (cell/mm²) of mast cells in the duodenum of mice in Groups 1, 2, and 3 are shown in Figure 5. The numbers (mean ± SE) were as follows: 86.6 ± 6.7 (n = 10) in Group 1; 163.4 ± 8.7 (n = 6) in Group 2; and 58.4 ± 8.0 (n = 7) in Group 3. Mast cells in the duodenum were observed in the perivascular region underneath the muscularis mucosae. The number of mast cells increased significantly ($P < 0.05$) in Group 2 as compared with Group 1. On the

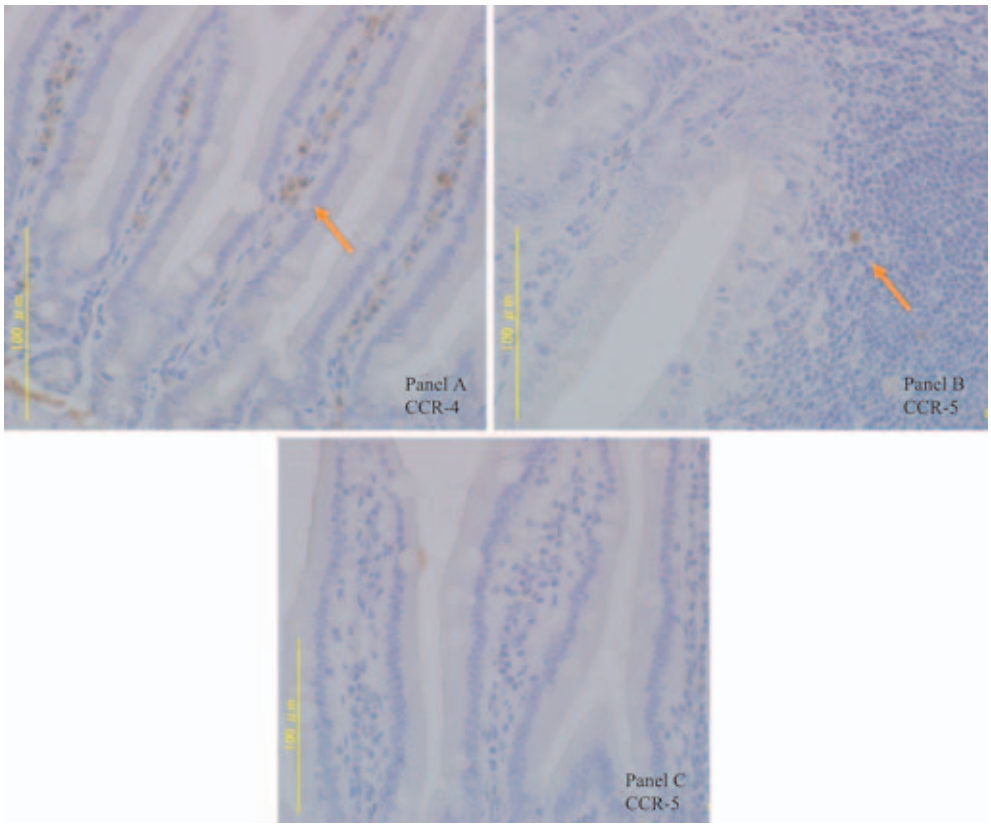


Fig. 4 Immunohistochemical photomicrographs of CCR4- and CCR5-positive cells. A 15 mm long section of the duodenum, located at 50 mm from the pylorus of the stomach, was removed and used to conduct immunohistological staining. Immunohistochemical photomicrographs of CCR4-positive cells (brown; arrow) (Panel A) and CCR5-positive cells (Panel B) in the duodenal villi. A number of CCR4-positive cells were observed in the mucus layer of the duodenal villi. A small number of CCR5-positive cells were observed in the lymphatic tissue (Panel C) (immunohistochemical staining, ×400).

other hand, the number of mast cells decreased significantly ($P < 0.001$) in Group 3 against Group 2. The number of mast cells and the number of CCR4-positive cells increased significantly ($P < 0.05$ and $P < 0.001$, respectively) in Group 2 as compared with Group 1. In Group 3, however, the number of mast cells decreased significantly ($P < 0.001$); no correlation ($r = 0.20$, $p = 0.36$) was found between the number of CCR4-positive cells and the number of mast cells.

HISTOPATHOLOGY

Regarding the abnormal value detection ratio, one (1/10) mouse in Group 1 showed edematous changes in the duodenal villi. On the other hand, 100% (6/6) of the mice in Group 2 showed severe edematous changes in their duodenal villi. However, 29% (2/7) of the animals in Group 3 showed a persistence of edematous changes and histochemical photomicrographs demonstrated duodenal villi which repaired to an extent close to those of animals in Group 1. In Group 2, the edematous changes occurred in mucosal tissue of the duodenal villi with inflammatory cell infiltration around the vessels.

DETERMINATION OF DUODENAL VILLUS EDEMA FORMATION RATES

The duodenal villus edema formation rates in Groups are shown in Figure 6. The edema formation rates per villus (mean ± SE) were as follows: 0.95 ± 0.53% ($n = 10$) in Group 1; 6.86 ± 0.69% ($n = 6$) in Group 2; and 1.93 ± 0.64% ($n = 7$) in Group 3 (mean ± SE). The cutoff value of the edema formation rate per villus in Group 1 was 1.99%/one villus. The rates increased significantly ($P < 0.001$) in Group 2 as compared with Group 1. The rates decreased significantly ($P < 0.05$) in Group 3 as compared with Group 2. A correlation ($r = 0.70$, $p = 0.0002$) was found between the number of CCR4-positive cells and the duodenal villus edema formation rate (Fig. 7). A correlation ($r = 0.48$, $p = 0.02$) was found between the number of mast cells and the duodenal villus edema formation rate (Fig.

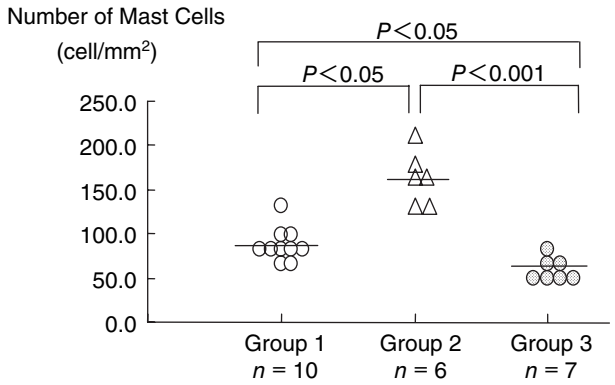


Fig. 5 Numbers of mast cells in duodenal villi of mice in the study groups. A number of toluidine blue-stained mast cells in the duodenum were observed in the perivascular area underneath the muscularis mucosae. Group 2 (control group) showed a significant increase ($P < 0.05$) in the number of mast cells as compared with Group 1 (OVA nonsensitization group). Group 3 (FOS administration group) showed a significant decrease ($P < 0.001$) in the number of mast cells as compared with Group 2 (control group).

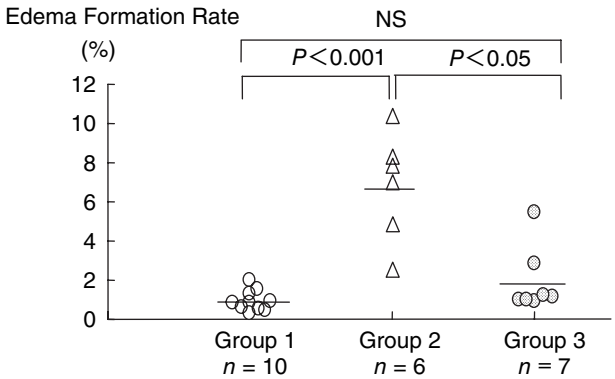


Fig. 6 Edema formation rates in the duodenal villi in the study groups. The edema formation rates per villus (mean ± SE) were as follows: 0.95 ± 0.53% ($n = 10$) in Group 1; 6.86 ± 0.69% ($n = 6$) in Group 2; and 1.93 ± 0.64% ($n = 7$) in Group 3 (mean ± SE). The rates increased significantly ($P < 0.001$) in Group 2 as compared with Group 1. The rates decrease significantly ($P < 0.05$) in Group 3 as compared with Group 2.

8).

DISCUSSION

The gastrointestinal tract of humans is constantly exposed to uncountable numbers of environmental microorganisms, food proteins, and other substances. Defense against these potential threats occurs through specific barrier mechanisms while maintaining interactions with the intestinal flora. When the mechanisms are disrupted due to repeated stimula-

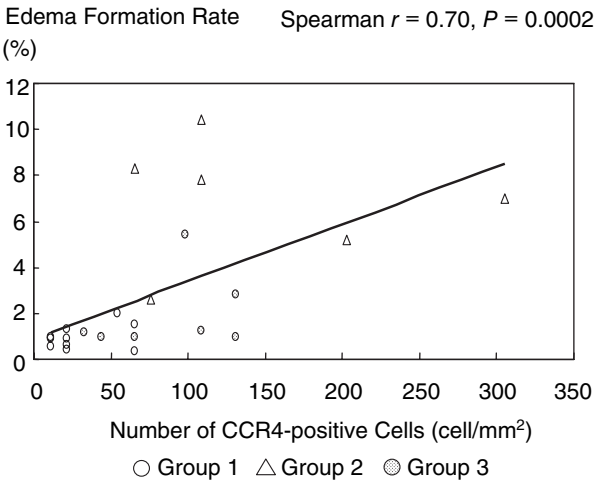


Fig. 7 Correlations between the number of CCR4-positive cells and the duodenal villus edema formation rate. A significant correlation ($r = 0.70$, $p = 0.0002$) was noted between the number of CCR4-positive cells and the edema formation rate. Group 2 (control group) showed significant increases ($P < 0.001$) in the edema formation rate and in the number of CCR4-positive cells as compared with Group 1 (OVA nonsensitization group). Group 3 (FOS administration group) showed significant decreases ($P < 0.05$) in the villus edema formation rate as compared with Group 2. However, Group 3 tended to show a decrease in the number of CCR4-positive cells as compared with Group 1.

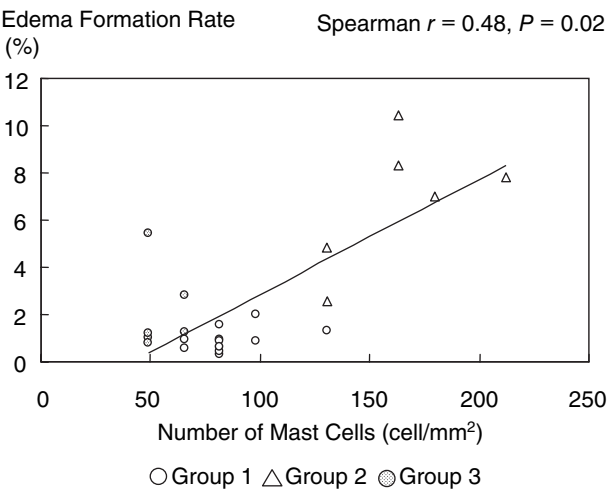


Fig. 8 Correlations between the number of mast cells and the duodenal villus edema formation rate. A significant correlation ($r = 0.48$, $p = 0.02$) was noted between the number of mast cells and the edema formation rate. Group 2 (control group) showed significant increases ($P < 0.001$, $P < 0.05$) in the edema formation rate and in the number of mast cells as compared with Group 1 (OVA nonsensitization group).

tion by antigens and from other events, orally incorporated food antigens are able to pass across the mechanisms, activate T cells via antigen-presenting cells, e.g., dendric cells, macrophages and B cells, and induce a Th1- or Th2-type immune response. Subsequently, activated lymphocytes are considered to migrate to the intestinal mucosa via mesenteric lymph nodes, where allergic symptoms develop.

We focused our attention on the Th2 cells in the duodenum in an attempt to examine their relationship with the mechanisms of the pathogenesis of allergy after challenge with the OVA antigen, as well as the effects of FOS on these cells. Regarding the intercellular specificity of the variables, the expression of CCR4 has been previously reported.[15-17] Therefore, the possibility that cells other than helper T cells were overcounted was admissible. We hence endeavored to make precise evaluations by establishing assessment criteria which were based on the typical morphological features of lymphocytes.

In our study we found that relatively few CCR5-positive cells were expressed in the duodenal villi, and expressed cells were mostly represented by CCR4-positive cells (Fig. 4). Similarly to a previous study on peripheral blood by flow cytometry,[10-12] our results demonstrated a predominance of Th2-positive cells in the small intestine. In consideration of the fact that the number of CCR4-positive cells increased significantly after challenge with the OVA antigen, findings from the present study furthermore indicated that the small intestine, similar to the skin, constitutes a target organ for T cells and that the small intestine provides an environment where Th2-positive cells predominate.

In the present study, the significant correlation of the number of CCR4 positive cells and the rate of duodenal villus edema suggests that the cellular type (delayed type) allergic reaction may have occurred. Furthermore, in Group 2, the increase in both OVA-specific IgE levels and the number of mast cells indicated that the IgE-dependent allergy reaction may have occurred. Tissue damage of the duodenum was represented only by edema and inflammatory cell infiltration, all of which were reversible lesions. The development of duodenal villus edema was considered attributable to the actions of substances which were released by mast cells and Th2-positive cells, e.g., chemical mediators and cytokines.

In consideration of the major findings, namely that the number of CCR4 positive cells increased, and the duodenal villi showed edematous changes, we can assume that the following events occurred: 1) Antigens, which succeeded in passing across defense barrier mechanisms that were debilitated by villus edema and penetrated the submucosal tissue of the small intestine; 2) Particular APCs, including M cells and DC cells, presented antigens to T cells at selected sites, such as Peyer patches where T cells were sensitized to antigens; 3) The number of memory T cells increased to recirculate in tissues where lymphocytes were present, including the lamina propria mucosae of the gastrointestinal tract; 4) T cells, which had been in an unactivated condition (resting state), in which the threshold for antigen response was lower than that of other T cells, were also activated as a surveillant by stimulation with antigens in the lamina propria mucosae; and 5) Activated T cells infiltrated tissues where allergic inflammation occurred, thus leading to an increase in the number of measured cells. Therefore, duodenal villi showed edematous changes. On the other hand, in the case that the antigen which penetrated is a microorganism, a Th1-type immune response is elicited by the activation of toll-like receptors.[18] Therefore, we consider that Th1-positive cells were not induced in the present study.

As the 2005 Japanese guidelines state,[1] current therapeutic modalities present limitations; namely, the actual condition depicts a lack of effective measures. The present study revealed that tissue damage does not progress if the intestinal barrier mechanism repairs or improves successfully in clinical settings.

In Japan, FOS, health foods with intestine-controlling activity whose function is allowed to be displayed commercially, have extensively been consumed by the Japanese population, ranging from infants to the elderly. No side effects have been reported from them over the last 12 years.

The pharmacological actions of FOS reported to date are as follows: colitis-alleviating activity of TNBS in rats by Christine C *et al.*[19]; colon cancer incidence-lowering activity of FOS in mice by Fabrile Pierre *et al.*[20]; increasing activity of FOS on the secretion of IgA from intestinal Peyer patches and their Th2 cell-inhibitory activity[21]; and increasing activity of FOS on the secretion of IgA from the intestines of infant mice.[22]

All studies have demonstrated the immunoregulatory activity of FOS in the intestines and reported that FOS may sustain and improve the barrier mechanisms of the intestines by controlling the intestinal flora and intestinal organic acid concentrations. FOS administration changes the intestinal environment of microflora by increasing anaerobic bacteria, such as Bifidobacterium and Lactobacillus and leads to upregulation of IgA secretion of the systemic immune response.[21] In addition, IgA adsorbs to macromolecule protein as allergen and prevents absorption in the small intestine. Furthermore, FOS increases short-chain fatty acid concentration, and decreases pH in the intestines. This environment does not promote proliferation of some pathogenic microbes, and improves barrier mechanisms of the intestines. In this way, the present study similarly showed variations in the intestinal flora and intestinal organic acid concentrations due to the administration of FOS (data not shown) and involves potential unreproduci-

bility because of the use of special experimental animals.

As compared with Group 1, Group 2 showed significant increases in all variables examined, *i.e.*, number of CCR4-positive cells, villus edema formation rate, number of mast cells, and serum OVA-specific IgE levels. These findings indicated that the increases in the numbers of CCR4-positive cells and mast cells contribute to the allergic reaction in the duodenum and that villus edema is provoked by allergic reaction in the duodenum. In other words, these findings suggest that the control of the allergic reaction in the duodenum is essential for improving food allergy. Furthermore, the fact that FOS tended to reduce the number of CCR4-positive cells indicates that its allergenicity is not potent. However, the fact that there was a significant decrease in the number of mast cells suggests the possibility of the antiallergic activity of FOS.

In conclusion, this study demonstrated a correlation between the number of CCR4-positive cells and villus edema formation rate. Therefore, FOS, whose antiallergic activity for food allergy was inferred in this study and whose safety as food in humans has already been established, can be considered to be potentially useful for the prevention of food allergy in pediatric patients, especially those with a milk allergy.

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