Isolation of Tryptophan as an Inhibitor of Ovalbumin Permeation and Analysis of Its Suppressive Effect on Oral Sensitization

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Tryptophan was isolated from rat feces as an active compound against ovalbumin permeation in an *in vitro* Caco-2 cell model. Tryptophan dose-dependently inhibited ovalbumin permeation with accompanying increase in transepithelial electric resistance, and its inhibitory activity reached a plateau at 10 mM. Brown Norway rats were sensitized by intragastric administration of ovalbumin together with or without tryptophan. Antibody levels specific to ovalbumin in the sera and proliferative responses of spleen mononuclear cells to ovalbumin were significantly lower in rats administered ovalbumin plus tryptophan than those administered ovalbumin alone. These results suggest that tryptophan suppresses oral sensitization to ovalbumin, probably via suppression of ovalbumin absorption from the intestinal tract.

Key words: allergen absorption; tryptophan; Caco-2 cells; ovalbumin

Food allergy has been thought to be an excessive immune reaction against allergens absorbed from the intestinal tract.[1] The intestinal epithelium is a physical barrier and plays an important role in excluding the entry of allergens. However, Walker *et al.*[2] reported that even in a healthy person, while a small amount of protein had left allergenicity, it advanced through circulation through the body. Moreover, enterocytes have the capacity of antigen presentation via class II major histocompatibility complex and are capable of direct and humoral contact with immunocytes.[3] Thus increased uptake of antigen could lead to an excessive inflammatory response.[4] Indeed, it has been reported that antigen permeability through the intestinal tract is increased in allergic patients[5,6] and Crohn's disease patients.[7] In the light of these facts, it is conceivable that an increase of barrier function of intestinal epithelium elicits prevention of food allergy. In our previous study regarding this subject, we identified peptides from hypoallergenic wheat flour[8] and Edam cheese,[9] and polyphenolic compounds from some spices.[10]

To search for allergen absorption inhibitors, we selected a human colon carcinoma cell line, Caco-2, as an intestinal model. These cells have microvilli and polarized morphology when grown on permeable filters.[11] Ovalbumin (OVA), a major allergen of egg white, was used as a model of allergens. We hypothesized that endogenous barrier increaser exists in the luminal content. In this paper, we describe isolation and characterization of an active compound that inhibits OVA permeation through the Caco-2 cell monolayer, and evaluation of its allergy preventive effect by using a rat oral sensitization model.

Materials and Methods

Animals. Male Wistar rats were purchased from Japan SLC (Hamamatsu, Japan) and male Brown Norway rats from Japan Charles River (Tokyo, Japan), at 5 weeks of age. They were housed in individual cages in a temperature-controlled ($23 \pm 2°$C) room with a dark period from 1900 to 0500, and were allowed free access to water and to a purified diet prepared according to AIN93G.[12] All animal experiments were approved by the Hokkaido University Animal Use Committee, and animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Measurement of OVA permeability. Caco-2 cells were obtained from the American Type Culture Collection (VA, USA) and were used between 30 and 50 passages. Cells were seeded at a density of 2×10^5 cells/cm^2 in a transwell with polycarbonate membranes having 0.4 μm pores size and 12 mm in diameter (Corning, NY, USA). Dulbecco's modified Eagle's medium (Invitrogen Japan, Tokyo, Japan) containing 20% fetal calf serum (Dainippon Pharmaceutical, Osaka, Japan), 1% nonessential

amino acids (Invitorogen Japan), 100 IU/ml penicillin (Wako Pure Chemicals, Osaka, Japan), 100 μg/ml streptomycin (Wako Pure Chemicals), and 50 μg/ml gentamicin (Wako Pure Chemicals) was used as a growth medium.[8] The monolayers of Caco-2 cells were used for the activity measurement after culture for 14–20 days. One day before the measurements, the monolayers were cultured in the presence of a test sample or authentic sample of amino acid in the growth medium. Transepithelial electric resistance (TEER) was measured using Millicell-ERS (Millipore, MA, USA) after culture with a test sample. After being washed twice with Hank's balanced salt solution (HBSS; Invitrogen Japan), HBSS (0.5 ml) containing both sample and OVA (Sigma-Aldrich, Tokyo, Japan, 0.5 mg) were poured into the apical side and HBSS (1.5 ml) containing only the sample into the basolateral side. The monolayers were incubated at 37°C for 30 min. At the end of the incubation period, the solution in the basolateral side was taken, and permeated OVA was measured by ELISA. Inhibitory activity is expressed as $(Pc - Ps)/Pc \times 100$ (%), where Pc and Ps are permeated OVA in the absence and presence of the sample, respectively.

Measurement of OVA concentration. Each well of a 96-well microtiter plate (Becton Dickinson, NJ, USA) was coated overnight at 4°C with the solution in the basolateral side diluted with 50 mM carbonate buffer (pH 9.6, 100 μl/well) and then blocked with 2% bovine serum albumin (BSA, fraction V, Serological Proteins, IL, USA) in phosphate-buffered saline (PBS) at 37°C for 2 hrs. Rabbit anti OVA (Bethyl Laboratories, TX, USA) in PBS containing 0.2% BSA and 0.02% Tween-20 (PBS-BT, 100 μl/well) was added and the mixtures were incubated at 37°C for 2 hrs. Horseradish peroxidase (HRP)-conjugated goat anti rabbit IgG (Zymed, CA, USA) in PBS-BT (100 μl/well) was added and the mixtures were incubated at 37°C for 2 hrs. Between each step, the wells were washed five times with PBS containing 0.02% Tween-20 (PBS-T). Plates were developed at room temperature after the addition of *o*-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.016%) in citrate-phosphate buffer (pH 5.0). Finally, 2M H_2SO_4 was added, and the absorbance at 490 nm was measured with a microplate reader (Model 550; Bio-Rad, CA, USA). The detection limit in this ELISA was 5 ng/ml.

Isolation of an active compound. Feces were collected from Wistar rats 5–6 days after consuming AIN93G diet. The feces (10 g) were mixed with 20% methanol (100 ml) and then centrifuged at 5000 × *g* for 20 min to obtain a supernatant. The supernatant was evaporated under a vacuum. The residue was dissolved in 0.1% trifluoroacetic acid (TFA, 10 ml) and the solution was treated with an ODS column (Sep-Pak C18; Japan Waters, Tokyo, Japan). The column was washed with 0.1% TFA (10 ml) and then eluted with 80% methanol in 0.1% TFA.

The ODS adsorbed and 80% methanol eluted fraction was separated by the first HPLC run on a reversed phase column (Shodex RSpak RP18-415, 4.6 × 150 mm; Shodex, Tokyo, Japan). Linear gradient elution was done starting with 10% methanol in 0.1% TFA and ending with 80% methanol in 0.1% TFA at a flow rate of 1 ml/min at 20°C. Detection was at the wavelength of 220 nm. The eluate collected near and at each peak was dried and then its activity was measured.

The active peak obtained by the first HPLC run was separated by the second HPLC run on a reversed phase column (Shodex RSpak DE-613, 4.6 × 150 mm; Shodex) with 30% methanol in 10 mM ammonium acetate (isogradic) in elution, other conditions being the same as in the first run.

The active peak obtained by the second run was desalted in a similar manner in the second run except for elution solvent of 30% methanol in water.

Instrumental analyses. An FAB-mass spectrum was taken in a negative ion mode with a JEOL JMS-AX500 instrument. NMR spectra were recorded on a Bruker AMX500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz).

Measurement of tryptophan concentration. Five Wistar rats were anesthetized by an intraperitoneal injection of a mixed solution of ketamine hydrochloride (70 mg/kg body wt, Wako Pure Chemicals) and xylasine hydrochloride (8 mg/kg body wt, ICN Biomedicals, OH, USA). Following a laparotomy, the luminal contents of jejunum, ileum, cecum and colon were obtained. After measuring the weight of the contents, PBS (5 ml) was added and the mixture was boiled for 5 min to inactivate enzymes. They were stored at −20°C until the measurement of their tryptophan concentrations. The luminal content from each part of the intestinal tract was diluted with 100-times volume of PBS and the suspension was centrifuged at 10,000 × *g* for 20 min under cooling. The supernatant was chromatographed in the same manner as in the first HPLC run except for the elution solvent of 15% methanol in 0.1% TFA. A fluorescence detector (FP-920; Japan Spectroscopic Co., Tokyo, Japan) was used with excitation at 290 nm to observe a tryptophan-derived emission spectrum at 350 nm. Retention time of tryptophan was 6.6 min. Tryptophan concentration was expressed as μmol/kg content.

Oral immunization assay. Male Brown Norway rats were used for this assay. After 14 days of consuming the AIN-93G diet, 12 rats were started to be immunized by intragastric administration of 1 ml of 0.1% OVA in saline once a day. In addition, half of the rats were administered 10 mg tryptophan together with OVA. Blood samples were obtained from the tail vein once a week and their antibodies measured. After 10 weeks, all

rats were anesthetized by an intraperitoneal injection of mixed solution of ketamine hydrochloride and xylasine hydrochloride. Following a laparotomy, rats were euthanized by bleeding from the abdominal aorta, and blood samples were collected for antibody measurement. The spleen was excised to obtain the mononuclear cells.

Antibody measurement. Serum levels of IgG1, IgG2a, and IgE specific to OVA were measured by ELISA. All assays were done in 96-well microtiter plates (Becton Dickinson). For the detection of OVA-specific IgG1 and IgG2a, plates were coated overnight at 4°C with 200 μg/ml of OVA in 50 mM carbonate buffer pH 9.6. Plates were blocked with 1% BSA in PBS at 37°C for 2 hrs. Test sera diluted with PBS-BT were then added and incubated at 37°C for 1 hr. After the incubation, HRP-conjugated mouse anti-rat IgG1 (MARG1-2, Zymed) or mouse anti-rat IgG2a (MARG2a-1, Zymed) in PBS-BT was added and incubated at 37°C for 1 hr. Between each pair of steps, the wells were washed five times with PBS-T. Color development and measurement were as described for the measurement of OVA concentration.

To detect OVA-specific IgE, plates were coated overnight at 4°C with mouse anti-rat IgE (MARE-1, Zymed) in 50 mM carbonate buffer pH 9.6. After blocking, diluted serum samples were added and incubated overnight at 4°C. After the incubation, digoxigenin (DIG)-labeled OVA in PBS-BT was added and incubated at 37°C for 1 hr. The coupling of OVA with DIG was done using a DIG protein labeling kit (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's protocol. HRP-conjugated anti-DIG Fab fragment (Roche Diagnostics) in PBS-BT was added and incubated at 37°C for 2 hrs. Between each pair of steps, the wells were washed five times with PBS-T. A chemiluminescent signal was obtained on X-ray film by using Super Signal ELISA Pico Chemiluminescent Substrate (Pierce, IL, USA) and measured by NIH Image. The absorbance units of the diluted test samples were confirmed empirically as tripled concentrations.

Mononuclear cell culture and proliferation assay. Single-cell suspensions of splenocytes were prepared as mononuclear cells on Nycoprep (Axis Shield, Oslo, Norway). Mononuclear cells were then cultured at 1×10^5 cells per well in 96-well plates with a standard culture medium at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The standard culture medium consisted of RPMI1640 (Invitrogen Japan) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, 50 μM 2-mercaptoethanol, and 10% fetal calf serum. Cells were incubated in the presence with either 10 μg/ml phytohemagglutinin-P (PHA-P, Wako Pure Chemicals) or 100 μg/ml or 1000 μg/ml of OVA for 72 hrs. The DNA synthesis rate was measured as an index of cell proliferation by using Cell proliferation ELISA BrdU (Roche Diagnostics).

Statistical analysis. Results were expressed as means ± SEM. Significance was evaluated by unpaired t-test at $p < 0.05$. The statistical calculations were done using StatView 5.0 computer software (SAS Institute, NC, USA).

Results

Isolation of an active compound from rat feces

Twenty percent methanol extract of rat feces was put onto an ODS column, and the active compound was desorbed with 80% methanol. The eluate was put on the first HPLC run in a reversed-phase column. The eluate collected near and at each peak was put through activity measurement. One asterisked peak, which was eluted at a methanol concentration of 35%, was active (Fig. 1A). The resulting active fraction was put through the second HPLC run on a reversed-phase column to obtain an active fraction that is again indicated with an asterisk (Fig. 1B). The active fraction was desalted to obtain a white powder (about 1 mg from 10 g of rat feces).
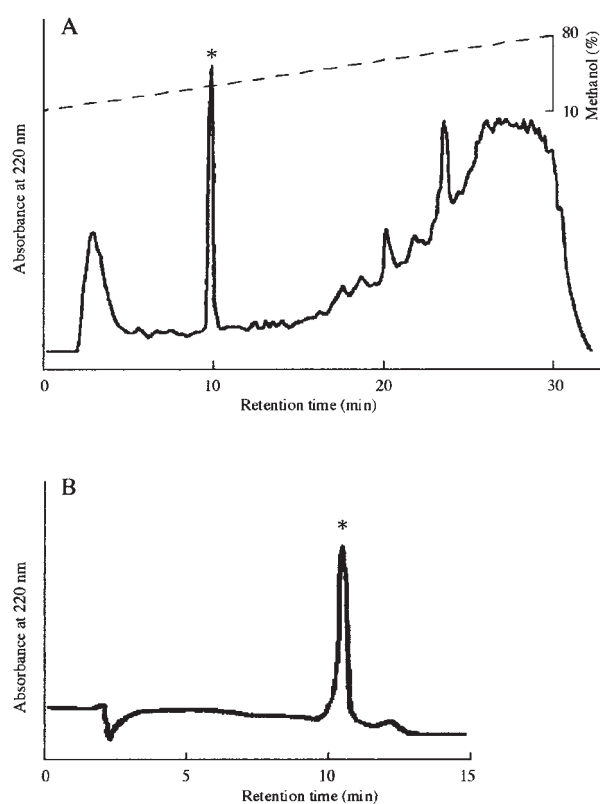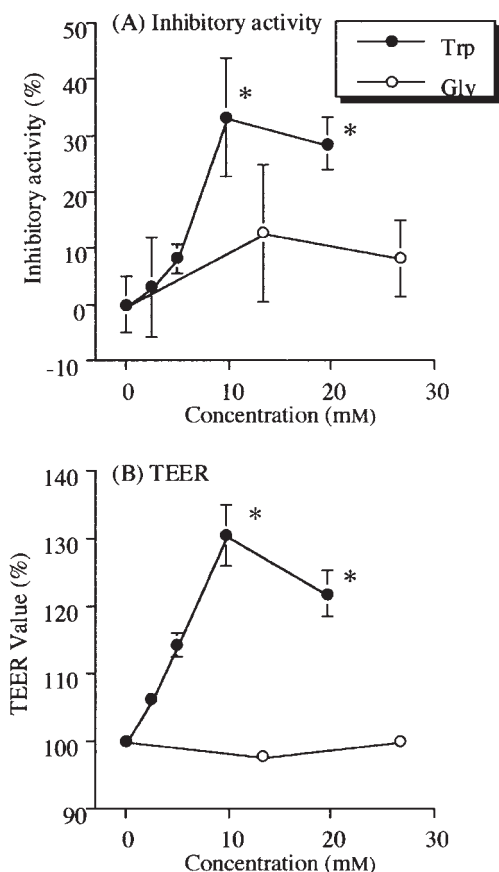

Fig. 1. Reversed-phase HPLC Patterns of an Extract from Rat Feces. A, ODS-adsorbed and 80% methanol-eluted fraction of rat feces. B, Active peak of the first HPLC run (asterisked in Fig. 1-A). In each chromatogram, an asterisked peak shows the inhibitory activity, the vertical axis being drawn to an arbitrary scale.

Identification of active compound

The active principle showed five aromatic protons characteristic of the 3-indolyl skeleton as well as side-chain methine and methylene protons coupled to each other in the ^{1}H NMR spectrum. The ^{1}H and ^{13}C NMR spectra coincided well with those of authentic tryptophan. The negative FAB-mass spectrum gave a pseudo-molecular ion at m/z 203, which corresponded to $[\text{M} - \text{H}]^-$ ion of tryptophan. Thus, the active compound was identified as tryptophan.
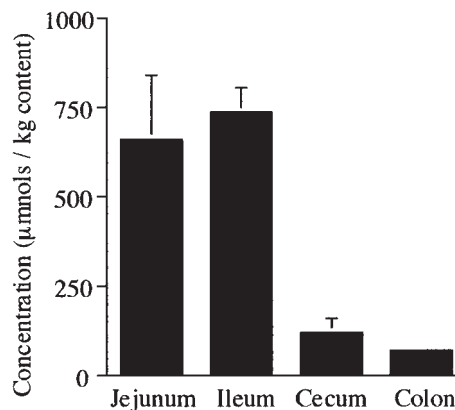
Inhibitory activity of tryptophan on allergen permeation

Tryptophan inhibited OVA permeation in a dose-dependent manner (Fig. 2A). TEER values were also dose-dependently increased during the culture with tryptophan (Fig. 2B). Glycine showed neither inhibitory activity against OVA permeation nor increase in the TEER value (Fig. 2A, 2B).
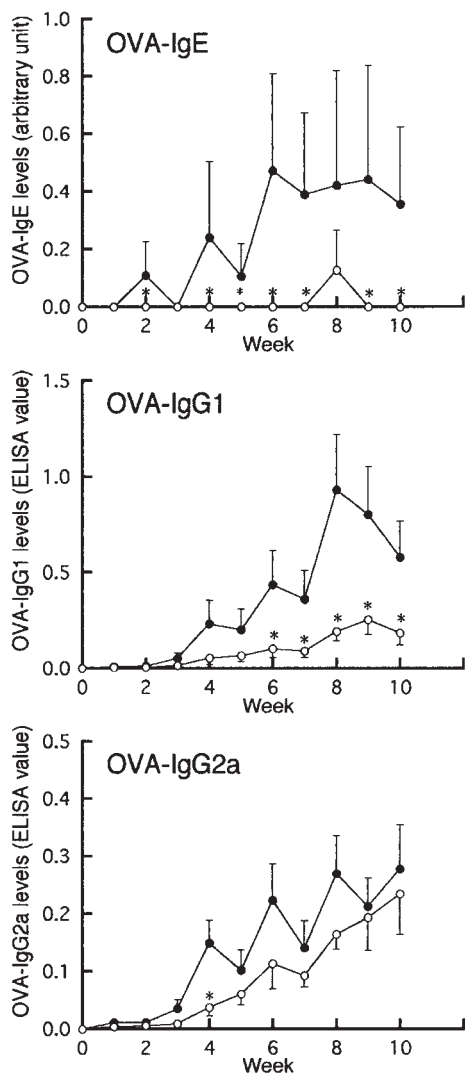

Fig. 3. Tryptophan Concentrations of Rat Intestinal Contents.

Luminal contents of jejunum, ileum, cecum, and colon were collected from Wistar rats and their tryptophan concentrations were measured by HPLC. Values are given as means ± SEM, n = 5.

Tryptophan concentration of rat intestinal content

Tryptophan concentrations of luminal contents from jejunum, ileum, and colon were measured by HPLC. The luminal tryptophan concentration of jejunum and that of ileum was 662.3 ± 178.8 and $733.8 \pm 69.2\ \mu\text{mol/kg}$, respectively. Detectable levels of tryptophan were also seen in luminal contents of the cecum and colon (Fig. 3).

Antibody production in orally immunized rats

No significant differences in body weight were observed between the rats administered OVA plus tryptophan and those administered OVA alone (data not shown). Figure 4 shows the time course of changes in serum OVA-specific IgE, IgG1, and IgG2a levels in rats administered OVA plus tryptophan or OVA alone (control). There was no detectable OVA-specific antibody in rats before oral immunization with OVA. Detectable levels of OVA-specific antibodies were not produced in rats administered saline nor in rats administered tryptophan (data not shown). Four weeks after starting OVA immunization, rats in the control group produced detectable levels of OVA-specific IgE. On the other hand, OVA-specific IgE levels in rats administered with OVA plus tryptophan were under the detection limit during the experimental period except for 8 weeks, and were significantly lower than those in rats of the control group. Detectable levels of OVA-specific IgG1 were produced both in tryptophan-administered rats and in the control rats 3 weeks after OVA immunization or later. The serum levels of OVA-specific IgG1 in tryptophan-administered rats were significantly lower than those in the control rats after 6–10 weeks. Detectable levels of OVA-specific IgG2a were produced both in tryptophan-administered rats and in the control rats 2 weeks after OVA gavage or later. The serum levels of OVA-specific IgG2a in tryptophan-administered rats tended to be lower than those in control rats.

Fig. 2. Effects of Tryptophan and Glycine on OVA Permeability through Caco-2 Monolayers (A), and TEER Values of Caco-2 Monolayers (B).

Inhibitory activity is expressed as $(Pc - Ps)/Pc \times 100\ (\%)$, where Pc and Ps are permeated OVA in the absence and presence of the sample, respectively. TEER values are shown relative to the average value in the absence of amino acid as 100. Values are given as means ± SEM, n = 3. *, Significantly different ($p < 0.05$) from the values in the absence of amino acid.
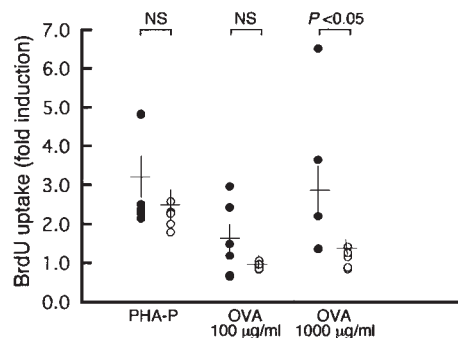
Fig. 4. Time Course of Changes in Serum OVA-Specific IgE, IgG1 and IgG2a Levels in Rats Administered with OVA Alone (closed circles) and OVA Plus Tryptophan (open circles).

Values are given as means ± SEM, n = 6. *, Significantly different ($p < 0.05$) from the antibody level in rats administered with OVA alone.

Fig. 5. Proliferative Responses to PHA-P or OVA of Spleen Mononuclear Cells from Rats Administered with OVA Alone (closed circles) and OVA Plus Tryptophan (open circles).

Cell proliferation was shown as fold induction of BrdU uptake by adding PHA-P or OVA. Individual data and means (horizontal bars) ± SEM (vertical bars) of 6 animals are shown.

Discussion

We searched for inhibitors of OVA permeation through the intestinal tract from rat feces by using Caco-2 cell monolayers as a model for the intestinal tract. Tryptophan was isolated from rat feces as an active compound. It is widely accepted that macromolecules, including allergens, permeate through the intestinal epithelium via paracellular and transcellular pathways. Soderholm *et al.*[7] reported that OVA passed both transcellularly and paracellularly. The TEER value is a hallmark of barrier function of the epithelium, and tightly correlates with paracellular permeability.[13] Tryptophan dose-dependently inhibited OVA permeation and its inhibitory activity reached a plateau at 10 mM (Fig. 2A). Similarly, a dose-dependent increase in the TEER value was seen when Caco-2 cells were cultured with tryptophan, and the increase reached a plateau at 10 mM (Fig. 2B). Thus it is plausible that tryptophan suppresses OVA absorption by inhibiting paracellular permeation. If this is the case, it would also be possible for tryptophan to inhibit paracellular permeation of other macromolecules. Further experiments about the effects of tryptophan on the permeability of other molecules would be helpful in elucidating the mechanism for action of tryptophan. Caco-2 cells were cultured in the presence or absence of a test sample in growth medium the tryptophan concentration of which is 0.07 mM. However, this concentration is probably negligible, because 2.5 mM of tryptophan affected neither OVA permeability nor changes in TEER value (Fig. 2). To find if amino acids generally inhibit allergen permeation, we measured the inhibitory activity of glycine. Glycine showed neither inhibitory activity against OVA permeation nor TEER-increasing activity. Moreover, ethyl esters of non-aromatic amino acids did not have the inhibitory activity (Kobayashi *et al.*, submitted for publication). Thus it is not plausible that inhibition of allergen absorption is a general property of amino acids. This is the first report on the

Proliferative response of spleen mononuclear cells from orally immunized rats

Figure 5 shows the proliferative responses of spleen mononuclear cells stimulated with PHA-P or OVA. No significant differences in proliferative responses of mononuclear cells to PHA-P were observed between tryptophan-administered rats and the control rats. Although mononuclear cells from the control rats dose-dependently proliferated by OVA stimulation, those from rats administered with OVA plus tryptophan rarely proliferated even in the presence of 1000 μg/ml OVA. Mononuclear cells from tryptophan-administered rats showed significantly weaker responses than those from the control rats when they were stimulated with 1000 μg/ml of OVA.

inhibitory activity of tryptophan against allergen permeation, although it has been reported that tryptophan changed fluorescent labeled dextran permeability via the paracellular pathway.[14] We reported that Trp-Ser-Asn-Ser-Gly-Asn-Phe-Val-Gly-Gly-Lys inhibited OVA permeation at a concentration of 10^{-7} M in Caco-2 model, and that a tryptophan residue without a free carboxyl group would be required for its strong activity.[8] The fact that tryptophan showed much weaker activity than the undecapeptide would support our previous speculation about the free carboxyl group of tryptophan residue. It would be possible that active peptides with tryptophan at the N-terminal are produced from dietary protein and inhibit the allergen permeation in the small intestinal tract, while the inhibitory activity of rat feces observed in this study is thought to be derived mainly from tryptophan.

Luminal tryptophan concentrations were measured to find whether the concentration was enough to inhibit allergen permeation in the intestinal tract. Luminal tryptophan concentrations of jejunum and ileum, from which OVA would be absorbed, were 662.3 ± 178.8 and $733.8 \pm 69.2\,\mu$mol/kg, respectively (Fig. 3). Although these values do not correctly reflect the local tryptophan concentrations in the vicinity of the intestinal epithelium, it is not conceivable that luminal tryptophan is enough to inhibit allergen permeation. This fact indicates that tryptophan has a low likelihood of acting as an endogenous barrier enhancer, and also indicates that orally administered tryptophan can increase its concentration in the vicinity of the epithelium enough to have inhibitory activity against allergen absorption.

Brown Norway rats are used as a model for measuring the allergenicities of food proteins because it has been reported that antibody production is induced by oral administration of the proteins.[15] We found whether oral administration of tryptophan suppressed allergen sensitization by using this oral sensitization model. The results demonstrated that orally administered tryptophan suppressed an increase in serum OVA-specific antibodies (Fig. 4) and proliferative responses of spleen mononuclear cells to OVA (Fig. 5). It is unlikely that tryptophan nonspecifically suppresses immune reactions, because administration of tryptophan failed to change the proliferative response to PHA-P. Thus these observations clearly demonstrated that tryptophan suppresses immune responses specific to orally administered OVA. In this experiment, the daily dosage of tryptophan was set at 10 mg. We are planning to measure the minimum efficient dose of tryptophan by the oral sensitization model. Concerning the mechanism, two explanations would be possible. One is that tryptophan suppresses oral sensitization against OVA via suppression of OVA absorption from the intestinal tract. Because tryptophan was shown to inhibit OVA permeation in the Caco-2 cell model in this study, it would be expected that tryptophan also suppresses OVA uptake in rat intestine *in vivo*. Naturally, the limited

amount of the antigen in the body could be hard to sensitize. The other is that a decreased uptake of OVA in the intestine by tryptophan may induce oral tolerance. According to Strokes *et al.*,[16] oral tolerance induction is related to a gradual and continuous absorption of the antigen. In addition, Akiyama *et al.*[17] reported that intragastric administration of OVA induced OVA-specific antibody production, and that oral administration via drinking induced oral tolerance with a resulting failure to produce detectable levels of OVA-specific antibodies in BALB/c mice. Thus limited and/or delayed uptake of oral antigen would induce oral tolerance rather than sensitization. Therefore it seems likely that oral administration of tryptophan induces oral tolerance by inhibiting abrupt antigen absorption from the intestinal tract.

Different patterns of cytokine release are characteristic of certain subgroups of T helper cells, termed Th1 and Th2; the former secrete IL-2 and IFN-g, while the latter secrete IL-3, IL-4, and IL-5, according to the observations in mice.[18] It has been reported that Th1 mediates IgG2a and IgG2b production by B cells, while Th2 mediates IgE and IgG1 production.[19] IL-4 has been shown to be essential for inducing IgE synthesis.[20] We showed in this report that the rise in OVA-specific IgE concentration was suppressed by administration of tryptophan throughout the experimental period, and that the suppressive effect of tryptophan on IgG2a production was weaker than that on IgE and IgG1 production. Thus it seems possible that oral administration of tryptophan changes the Th1/Th2 balance to Th1-predominant status. In our laboratory, a more sophisticated study is currently under way to clarify the precise mechanisms of the allergy-preventive effect of tryptophan.

In conclusion, we isolated tryptophan from rat feces as an active compound by using a Caco-2 model. We also found that administration of tryptophan suppressed oral sensitization against OVA in Brown Norway rats. These results indicate that tryptophan could be effective in prevention of food allergy.

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