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Chronic allergy to dietary ovalbumin induces lymphocyte migration to rat small intestinal mucosa that is inhibited by MAdCAM-1

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Ogawa, Toshiko, Soichiro Miura, Yoshikazu Tsuzuki, Takashi Ogino, Ken Teramoto, Toshiaki Inamura, Chikako Watanabe, Ryota Hokari, Hiroshi Nagata, and Hiromasa Ishii. Chronic allergy to dietary ovalbumin induces lymphocyte migration to rat small intestinal mucosa that is inhibited by MAdCAM-1. *Am J Physiol Gastrointest Liver Physiol* 286: G702–G710, 2004. First published December 11, 2003; 10.1152/ajpgi.00183.2003.—Few models have described a chronic food allergy with morphological changes in the intestinal mucosa. Here we established an ovalbumin (OVA)-induced, cell-mediated, allergic rat model and examined lymphocyte migration in the gut. Brown Norway rats were intraperitoneally sensitized to OVA and then given 10 mg OVA/day by gastric intubation for 6 wk. Lymphocyte subsets and adhesion molecules were examined immunohistochemically, and the migration of T lymphocytes to microvessels of Peyer's patches and villus mucosa was observed by using an intravital microscope. Serum OVA-specific IgG and IgE levels were increased in animals repeatedly exposed to OVA. Significant villus atrophy and increased crypt depth was accompanied by increased infiltration of T lymphocytes in the small intestinal mucosa of the group given OVA. Expression of rat mast cell protease II and of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) was also increased in these groups. The administration of anti-MAdCAM-1 antibody significantly attenuated the OVA-induced changes in the mucosal architecture and in CD4 T lymphocyte infiltration. Intravital observation demonstrated that in rats with a chronic allergy, T lymphocytes significantly accumulated in villus microvessels as well as in Peyer's patches via a MAdCAM-1-dependent process. Our model of chronic food allergy revealed that lymphocyte migration was increased with MAdCAM-1 upregulation.

Brown Norway rats; rat mast cell protease II; delayed type hypersensitivity; Peyer's patch; intercellular adhesion molecule-1

THE CLINICAL MANIFESTATIONS of allergic reactions from food intolerance may be localized to the gut, including abdominal discomfort, nausea, vomiting, and diarrhea. Type I or IgE-mediated allergic reactions are involved in early-phase symptoms of food allergy (6, 8). On the other hand, cell-mediated reactions are also involved in the late phase and symptoms are often prolonged, causing mucosal damage such as crypt hyperplasia, villus atrophy, and lymphocyte infiltration (11, 13, 27, 30). Because human research is restricted, animal models of food allergies would be of significant value. Several efforts have been made to develop rodent models of food allergy (10, 14, 32). However, few models have been validated for studying the effects of chronic antigen exposure or for relevance to

clinical situations involving both IgE- and cell-mediated reactions (21, 22, 28). Although several groups (10) have investigated the effect of repeated oral challenge in rats with IgE-mediated hypersensitivity, lymphocyte infiltration was not obvious in the intestinal mucosa of these animals. Only a recent report by Yang et al. (36) has demonstrated that the oral antigen challenge of sensitized Sprague-Dawley rats induces sustained epithelial dysfunction with inflammatory cell recruitment. The Brown Norway (BN) rat is a high immunoglobulin (particularly IgE) responder (18), and Knippels et al. (19–21) reported that BN is the most suitable for inducing specific-IgE responses compared with Wistar, Hooded Lister, and PVG strains. Results from other studies (2, 20, 31) have also indicated that the BN rat is the most suitable strain for sensitization studies. However, a BN rat model of chronic food allergy that shows histological changes in the intestinal mucosa has not been developed.

Expression of adhesion molecules is enhanced in inflammatory mucosa, resulting in the migration of inflammatory cells to this lesion. Studies of other atopic diseases and animal models suggest that adhesion molecules and mucosal lymphocytes are implicated in the pathogenesis of food allergy (12). Several clinical reports (7, 33) have also indicated that the expression of adhesion molecules is increased in the duodenal mucosa of adult patients with food allergies or of infants with cow's milk-sensitive enteropathy. However, only a few studies have addressed lymphocyte trafficking in food allergies, and the mechanisms of directly "trafficking" cells have only scarcely been explored (11, 28). The $\alpha_4\beta_7$ heterodimer is the principal homing receptor that mediates the tissue-specific binding of lymphocytes to venules in the murine gut-associated lymphoid system by interacting with endothelial mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (3, 4, 15). It has also been shown that there is not simply one adhesion molecule with an exclusive function but rather that several have overlapping functions. L-selectin and CD11a/CD18, for example, play a role in the interaction between lymphocytes and high endothelial venules in the gut wall (1, 3). Therefore, whether lymphocyte trafficking is altered in food allergies, and if so which molecular interactions are responsible, should be determined.

The aim of this study was to establish an ovalbumin (OVA)-induced, chronic allergic model with histological changes in rats and to use the model to examine whether T cell homing is altered in the small intestine of rats with a food allergy. We monitored lymphocyte migration in Peyer's patches and in the

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small intestinal mucosa, using an intravital microscope, and our results indicated that vascular adhesion molecules, especially MAdCAM-1, participate in chronic T-cell migration to the intestine of rats with an OVA-induced allergy.

MATERIALS AND METHODS

Sensitization and chronic antigen exposure (Animal Model). Male BN rats (4–6 wk old at study initiation) obtained from Charles River (Boston, MA) were maintained under specific pathogen-free conditions and were given ad libitum access to food and water. The animals were sensitized by an intraperitoneal injection of 0.1 mg of OVA (Fluka Chemie, Buchs, Switzerland) with 5 mg of aluminum hydroxide (0.5 ml of alum solution) as an adjuvant (9). Fourteen days after sensitization, the rats were orally exposed to OVA. Two protocols were applied for chronic antigen exposure. The animals were exposed to OVA either ad libitum via the drinking water (10 mg/ml) continuously for 6 wk (free-drinking group) or by daily gavage for 6 wk using a 18-gauge stainless steel animal-feeding needle (10 mg OVA/ml tap-water, 1 ml/animal) (daily gavage group). Two protocols were applied as experimental control. One group (OVA-sensitized control) underwent daily gastric intubation with 1 ml of tap water after intraperitoneal sensitization with OVA. Another group (OVA-fed control) was injected with material prepared in the same manner without antigen and orally exposed to OVA by daily gavage. The drinking water containing OVA was refreshed twice daily to avoid metabolic breakdown. Body weight and food intake were monitored daily. Animals were used for experimentation 6 wk after oral OVA exposure. The Keio University School of Medicine Animal Research Committee approved all procedures involving animals.

Assay of anti-OVA antibody and rat mast cell protease II concentrations. Blood samples collected from the abdominal aorta at death were coagulated at room temperature, then centrifuged for 20 min at 2,000 *g* at 4°C to obtain sera. Serum IgG and IgE specific for OVA were determined by ELISA as described by Knippels et al. (21). To detect OVA-specific-IgG, 96-well microtiter plates (Becton Dickinson) coated with OVA were incubated with PBS containing BSA and Tween 20, washed, and then incubated for 1 h at 37°C with serially diluted rat serum in PBS/BSA-Tween 20. Peroxidase-conjugated goat

Table 1. Villus height, crypt depth, and eosinophil counts determined by light microscopy in jejunum and ileum of small intestine of rats with chronic food allergy

	Villus Height, μm	Crypt Depth, μm	Eosinophil Counts, per 10 crypt- villus units
Jejunum			
Control (sensitized)	608.5 ± 28.1	196.0 ± 5.4	7.5 ± 2.9
Control (fed)	544.0 ± 18.6	178.3 ± 18.3	12.8 ± 4.6
Free drinking	346.0 ± 67.6*#	224.0 ± 30.9*#	52.5 ± 6.5*#
Daily gavage	148.7 ± 21.3*#	332.3 ± 63.2*#	72.5 ± 9.6*#
Ileum			
Control (sensitized)	343.0 ± 29.2	92.3 ± 9.5	5.8 ± 3.0
Control (fed)	297.3 ± 25.4	66.0 ± 11.3	9.8 ± 3.5
Free drinking	280.9 ± 21.4*	137.7 ± 21.5*#	32.5 ± 6.5*#
Daily gavage	231.2 ± 23.7*#	253.1 ± 36.2*#	35.0 ± 5.8*#

Data are expressed as means ± SE; *n* = 6 animals per group. Two weeks after sensitization with ovalbumin (OVA), animals were exposed to OVA either ad libitum via drinking water (10 mg/ml) or continuously during 6 wk (free-drinking group) or by daily gavage, using a feeding needle (10 mg OVA/ml tap-water, 1 ml/animal) for 6 wk (daily-gavage group). Control (OVA-sensitized) animals underwent daily gastric intubation with 1 ml tap water after intraperitoneal sensitization with OVA. OVA-fed control animals were injected with material similarly prepared but without antigen and were orally exposed to OVA by daily gavage. **P* < 0.05 compared with OVA-sensitized control. #*P* < 0.05 compared with OVA-fed control.

anti-rat IgG (hematoxylin and eosin) (KPL, Guildford, UK) in PBS/BSA-Tween 20 was added to the wells. After samples were washed, enzyme substrate 3,3',5,5'-tetramethylbenzidine (Sigma, St. Louis, MO) was added followed by H₂SO₄. Optical density was read spectrophotometrically at 450 nm using an ELISA plate reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA). To detect OVA-specific IgE, 96-well microtiter plates were coated with mouse anti-rat IgE (KPL). Rat serum samples were added to the plates followed by OVA-digoxigenin (DIG) conjugate (Roche Diagnostics). The plates were washed, and then peroxidase-conjugated sheep anti-DIG Fab fragment (Roche Diagnostics) in PBS/BSA-Tween 20 was added. Color was developed and measured as described for the OVA-specific IgG ELISA. Pooled sera from naive animals constituted the negative control (no OVA exposure). The reciprocal of the furthest serum dilution giving an extinction coefficient above the reference value was read as the titer. Serum concentrations of rat mast cell protease II (RMCPII) were assayed by using an enzyme-linked immunosorbent assay kit (Moredun Animal Health, Edinburgh, Scotland).

Histological evaluation and immunohistochemistry of the intestinal mucosa. Specimens for light microscopy were obtained from a location 10 cm toward the anal side from the pylorus (jejunum) and from a location 10 cm toward the oral side from the ileocecal valve (ileum). Samples were fixed with 10% formalin and stained with hematoxylin and eosin to evaluate morphological changes. Eosinophils were identified after Giemsa staining.

Another part of the intestine was fixed in periodate-lysine-paraformaldehyde for an immunohistochemical study by the labeled streptavidin biotin method. Intestinal tissues were vertically embedded in optimum cutting temperature compound (Sakura Finetek, Tokyo, Japan) before being frozen in dry ice and acetone. Cryostat sections (7 μm thick) were transferred to poly-L-lysine-coated slides and air-dried for 1 h at 20°C. After being washed for 5 min in PBS (pH 7.4) containing 1% Triton X, sections were incubated in 5% normal goat serum in PBS. Primary antibodies against CD4 (OX35), CD8 (OX8), B cell (OX12), IL-2 receptor (OX39), MAdCAM-1 (OST-2), VCAM-1 (MR106), and ICAM-1 (1A29) were obtained from BD PharMingen (San Diego, CA), and antibody against RMCPII was obtained from Biochem (London, UK). Sections were incubated with

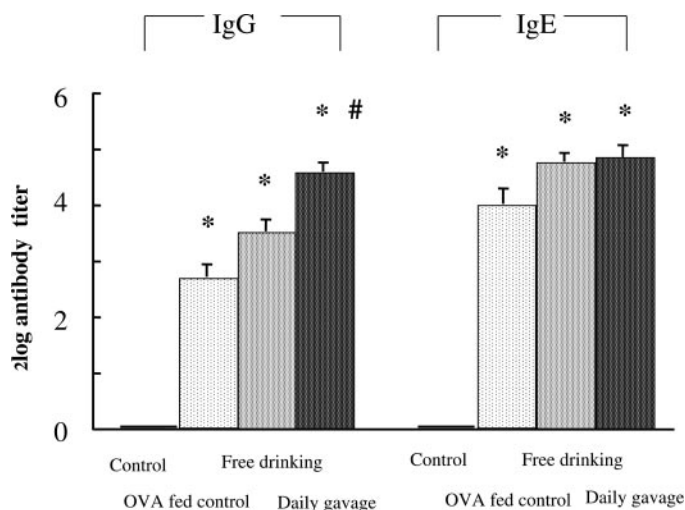
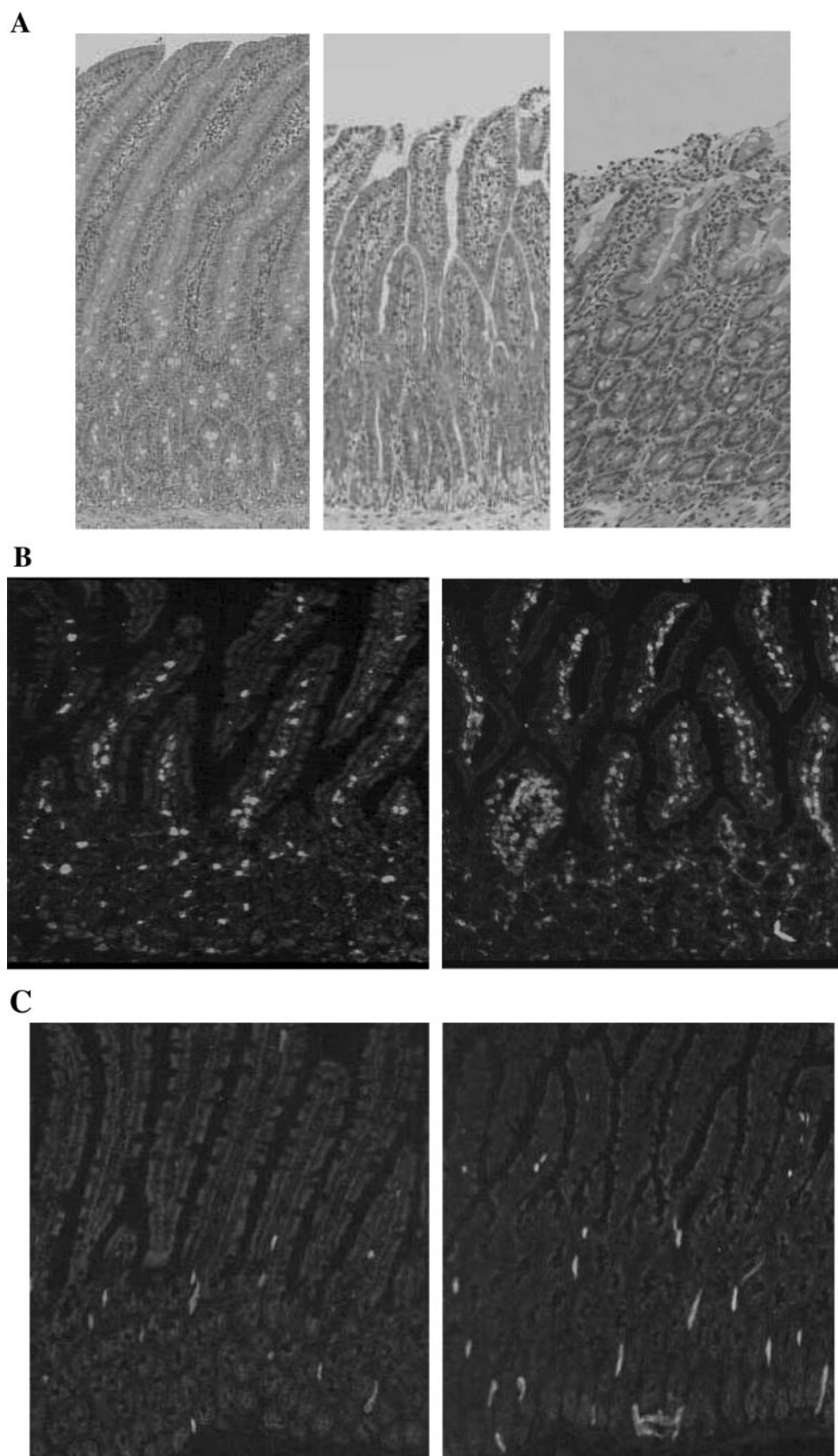


Fig. 1. Specific antibody titers in serum samples 6 wk after ovalbumin (OVA) exposure. Serum samples of naive animals were pooled and used as negative controls in ELISAs. OVA-specific IgE levels and IgG levels were determined in free-drinking and daily-gavage groups. Data are presented as 2 log IgG or IgE titers ± SE group. Control, OVA-sensitized control animals. **P* < 0.05 compared with OVA-sensitized control animals; #*P* < 0.05 compared with OVA-fed control animals or free-drinking groups. Values are means ± SE of 6 animals.



Fig. 2. *A*: representative hematoxylin and eosin-stained jejunal mucosa. OVA-sensitized controls (*left*), rats chronically exposed to OVA by free drinking (*middle*), and rats chronically exposed to OVA by daily gavage (*right*) (magnification, $\times 100$). *B*: representative immunohistochemical staining of CD4⁺ cells in jejunal mucosa. Controls (OVA-sensitized control; *left*) and rats chronically exposed to OVA (daily gavage; *right*). Numbers of CD4⁺ cells are significantly increased in intestinal lamina propria of OVA group (magnification, $\times 100$). *C*: representative images of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression in villus microvessels of ileal mucosa determined by immunohistochemistry. Primary antibody for immunostaining was monoclonal MECA367 that reacts to MAdCAM-1 (*left*, OVA-sensitized controls; *right*, chronic OVA exposure by daily gavage). MAdCAM-1-positive microvessels are significantly increased in lamina propria and in submucosal of animals exposed to OVA (magnification, $\times 100$).



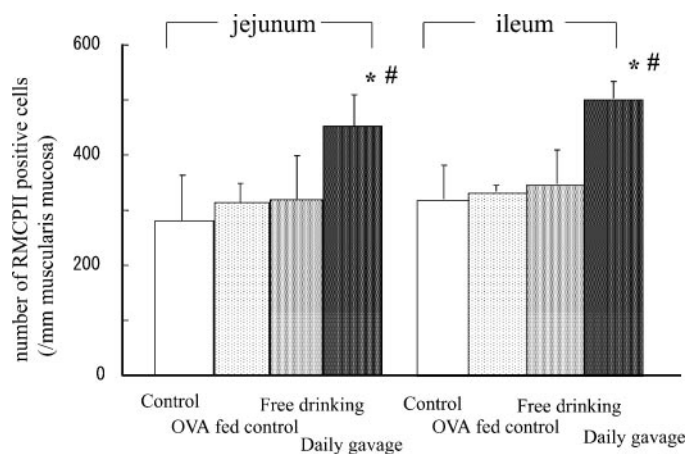


Fig. 3. Mucosal mast cells counted after immunohistochemical staining using anti-rat mast cell protease II (anti-RMCP II) antibody in small intestinal mucosa. Mucosal mast cells were examined in both jejunum and ileum of groups chronically exposed to OVA (free-drinking and daily-gavage) and compared with controls. * $P < 0.05$ compared with OVA-sensitized controls; # $P < 0.05$ compared with OVA-fed controls. Values are means $\pm$ SE of 6 animals.

the second antibody, biotinylated conjugated anti-rat IgG, for 1 h at room temperature, followed by FITC-conjugated streptavidin (Amersham, London, UK) for 30 min at room temperature. After each of these steps, the sections were rinsed with PBS containing 1% BSA. A coverslip was applied by using glycerol jelly, and the sections were observed under a fluorescence microscope (model BX60; Olympus, Tokyo, Japan). Vessels positive for adhesion molecules in the lamina propria were calculated by using an image analyzer and quantified as the area of positively stained vessels per millimeter of muscularis mucosa. The infiltrated cells are expressed as the number of CD4, CD8, B cells, IL-2 positive cells, or RMCP II-positive cells per millimeter of muscularis mucosa.

In another set of experiments, monoclonal blocking antibody against MAdCAM-1 (OST-2; 2 mg/kg) or nonblocking antibody to MAdCAM-1 (OST-20; 2 mg/kg) (17) in 0.2 ml saline was administered intraperitoneally twice a week during the OVA challenge period (6 wk) to the daily gavage group, and histological changes in the intestine were compared.

Collection and labeling of T lymphocytes from intestinal lymph. The rats were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg). The main mesenteric lymphatic duct was cannulated as described by Bollman et al. (5). Animals were then maintained in

Bollman cages, and isotonic saline was infused intravenously via the jugular vein at 2.4 ml/h to replenish fluids and electrolytes lost due to lymphatic drainage. Lymph samples were collected in ice-cold vials containing heparin (6 U/ml), FBS, and RPMI 1640 medium (pH 7.4; GIBCO, Grand Island, NY). Lymphocytes from the mesenteric lymph were washed three times with working medium [RPMI 1640 medium (pH 7.4) containing penicillin and streptomycin (GIBCO) and 0.1% BSA] before separation and labeling. The T cell-rich fraction of lymphocytes from mesenteric lymphatics was obtained by using a nylon wool column. The whole cell population of 1×10^8 lymphocytes in 20 ml of RPMI 1640 medium containing 1% FBS was incubated at 37°C for 1 h with 1 g of nylon wool (Kanto Kagaku, Tokyo, Japan) in a column, and the pass-through fraction was designated as the T cell-rich fraction. These manipulations did not significantly affect lymphocyte viability as assessed by trypan blue exclusion. The cells were washed and resuspended in RPMI 1640 medium containing 5% FCS on ice. Lymphocytes were labeled with 15.6 mM carboxyfluorescein diacetate succinimidyl ester (CFDASE) (Molecular Probes, Eugene, OR) in DMSO. Immediately before infusion to recipient rats, lymphocytes (1×10^8) in 20 ml RPMI 1640 medium were incubated with 20 μ l CFDASE for 30 min at 37°C.

Intravital observation of T lymphocyte migration at Peyer's patches. After an intraperitoneal injection of pentobarbital sodium (50 mg/kg), the abdomen was opened via a midline incision. Twelve centimeters of the ileal segment near the ileocecal valve was placed on a plastic plate for observation. The intestine was kept warm and moist by continuous superfusion with physiological saline warmed to 37°C. Two small incisions were made in the intestine, and warm Krebs-Ringer solution (pH 7.4) was instilled through a vinyl tube from the proximal end to maintain the luminal pressure at 15 cmH₂O. Suitable areas of the microcirculation in Peyer's patches were observed from the serosal side using an intravital microscope (model Diaphot TMD-2S; Nikon, Tokyo, Japan) equipped with a silicon-intensified target image tube camera with a contrast-enhancing unit (model C-2400-08; Hamamatsu Photonics, Shizuoka, Japan) (25). Lymphocytes (6×10^7 cells in 1 ml RPMI 1640 medium) were injected into the jugular vein of the recipient rats during ~3 min. The migration of CFSE-labeled T lymphocytes through the microvasculature of Peyer's patches was continuously recorded on S-VHS videotapes for up to 60 min after the injection. Epifluorescence was achieved by excitation at 470–490 nm and emission at 520 nm.

The lymphocyte behavior in postcapillary venules (PCV) was classified according to the nature of interaction with the vascular walls. Lymphocytes adhering to venular walls but with movement along them were defined as "rolling." The ratio of rolling lymphocytes per total cell flux was calculated. Lymphocytes adhering to the venular wall without movement for >30 s were defined as "sticking"

Table 2. Effect of chronic exposure to OVA on lymphocyte subpopulations in intestinal mucosa determined by immunohistochemistry

	CD4	CD8	OX12	IL-2R (OX39)
Jejunum				
Control (sensitized)	231.7 $\pm$ 55.3	291.7 $\pm$ 37.6	158.3 $\pm$ 28.6	257.5 $\pm$ 59.0
Control (fed)	266.7 $\pm$ 48.1	226.7 $\pm$ 48.1	100.5 $\pm$ 17.4	ND
Free drinking	390.0 $\pm$ 52.9*†	152.5 $\pm$ 37.7*†	277.5 $\pm$ 55.6*†	ND
Daily gavage	436.7 $\pm$ 52.8*†	150.0 $\pm$ 35.6*†	395.0 $\pm$ 42.0*†	197.5 $\pm$ 75
Ileum				
Control (sensitized)	223.3 $\pm$ 46.8	330.0 $\pm$ 21.6	118.3 $\pm$ 27.9	220.0 $\pm$ 63.2
Control (fed)	243.3 $\pm$ 14.53	320.0 $\pm$ 46.2	86.7 $\pm$ 17.63	ND
Free drinking	490.0 $\pm$ 21.4*†	227.5 $\pm$ 49.9*†	230.0 $\pm$ 25.8*†	ND
Daily gavage	706.7 $\pm$ 131.3*†	147.5 $\pm$ 34.0*†	386.7 $\pm$ 21.6*†	195.0 $\pm$ 50.0

Data are expressed as means $\pm$ SE as number of positive cells per millimeter muscularis mucosa; $n = 6$ animals per group. After sensitization with OVA, the animals were exposed to OVA either ad libitum (free-drinking group) or by daily gavage for 6 wk. Control (OVA-sensitized) animals underwent daily gastric intubation with 1 ml tap water after sensitization. OVA-fed control animals were injected without antigen and were orally exposed to OVA by daily gavage. * $P < 0.05$ compared with OVA-sensitized control. † $P < 0.05$ compared with OVA-fed control. ND, not determined.

Table 3. Expression of adhesion molecules in intestinal microvessels induced by chronic exposure to OVA determined by immunohistochemistry using antivascular adhesion molecules

	MAcCAM-1	ICAM-1	VCAM-1
Jejunum			
Control	1.9±0.6	9.8±1.7	5.8±2.5
Free drinking	18.3±6.5*	ND	ND
Daily gavage	23.7±6.0*	9.9±3.0	5.7±1.6
Ileum			
Control	2.5±0.7	10.5±2.9	6.7±3.7
Free drinking	21.0±3.6*	ND	ND
Daily gavage	24.7±3.2*	10.3±3.3	5.9±2.5

Data are expressed as means ± SE as area of positively stained vessels per millimeter of muscularis mucosa (micrometer squared per millimeter); $n = 6$ animals per group. After sensitization with OVA, animals were exposed to OVA either ad libitum (free-drinking group) or by daily gavage for 6 wk. Control (OVA-sensitized) animals underwent daily gastric intubation with 1 ml tap water after sensitization. * $P < 0.05$ compared with control. MAcCAM, mucosal addressin cell.

lymphocytes. The number of sticking lymphocytes was determined in 1-mm² observation fields that contained PCV of 25–50 μ m in diameter.

In some experiments, anti-MAcCAM-1 MAb (OST-2; 2 mg/kg) or anti-ICAM-1 (1A29; 2 mg/kg) in 0.2 ml saline was infused from the jugular vein 30 min before starting the infusion of T lymphocytes. The control was a nonblocking antibody to MAcCAM-1 (OST-20) or an isotype-matched IgG applied under the same conditions (17).

Intravital observation at villus mucosa. We examined the microcirculation of intestinal villi from the mucosal surface and observed lymphocyte migration in another set of experiments. A segment of the ileum was gently extended onto a plate, and a longitudinal incision was cut along its antimesenteric border by microcautery. The behavior of fluorescence-labeled lymphocytes in villus tips was visualized on a video monitor through a fluorescence microscope as described (26). The tip of each villus was observed as an oblique circle, and villus microvessels were observed. As in the observations of Peyer's patches, lymphocytes that remained in the same position for over 30 s were defined as sticking.

Statistics. All results are expressed as means ± SE. Differences among groups were evaluated by ANOVA and Fisher's post hoc test. Statistical significance was established at $P < 0.05$.

RESULTS

OVA-specific antibody titers and delayed-type hypersensitivity responses. Figure 1 shows specific antibody titers in serum samples 6 wk after OVA exposure. Anti-OVA antibodies were undetectable in pooled sera from naive animals (no OVA exposure). In OVA-sensitized control animals, specific IgE levels were undetectable and specific IgG levels were almost negligible, whereas there was an elevation of OVA-specific IgE and IgG levels in OVA-fed control animals. OVA-specific IgE levels were significantly elevated in both the free-drinking and in daily gavage groups at day 42. Antibody titers did not significantly differ between these two groups. OVA-specific IgG levels were also increased in both groups at day 42, whereas those of the daily gavage group were significantly higher than those of the free-drinking group.

Histological evaluation and immunohistochemical study of intestinal RMCPII, lymphocyte subpopulation, and adhesion molecules. Table 1 shows jejunal and ileal morphology determined by light microscopy. The villus height of the daily

gavage group was significantly reduced and crypt depth was increased with inflammatory cell infiltrations compared with the OVA-sensitized and OVA-fed controls at both sites (Fig. 2A). This was also true of the free-drinking group, although these changes were less significant compared with the daily gavage group. The numbers of eosinophils were increased in the mucosa of both regions of the intestine of rats exposed to OVA compared with OVA-sensitized and OVA-fed controls.

The serum concentrations of RMCPII were significantly increased in rats exposed to OVA compared with controls. The RMCPII concentrations in the daily gavage and free-drinking groups were ~2.3- and 1.1-fold greater, respectively, com-

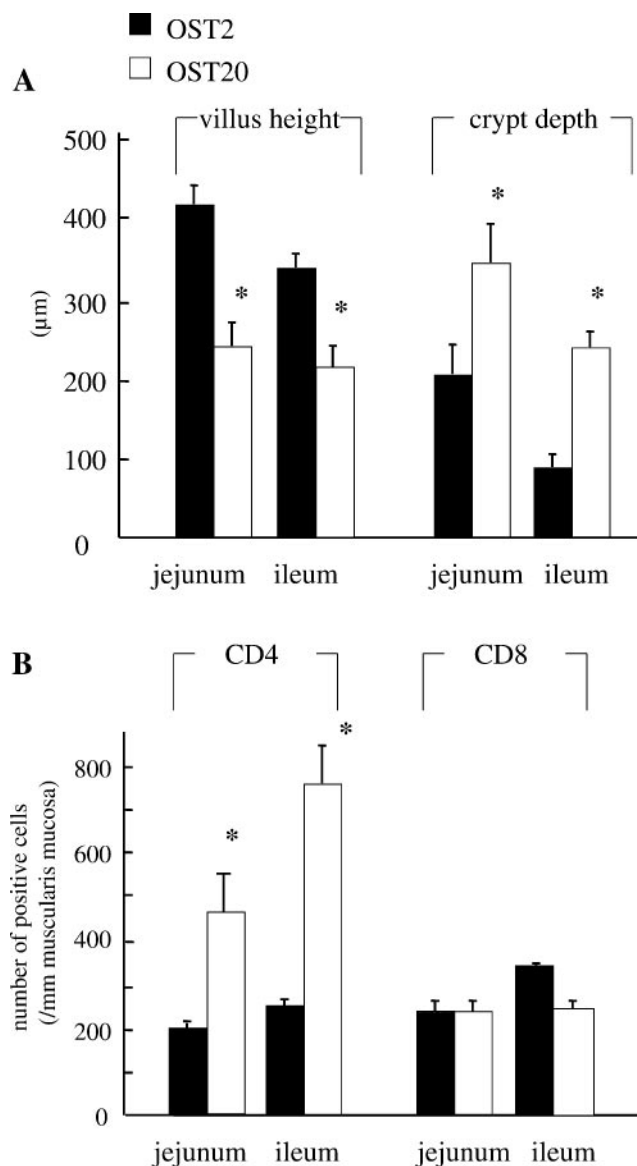


Fig. 4. Effect of functionally blocking MAcCAM-1 (OST-2) or nonblocking antibody (OST-20) on changes in morphology and infiltration of lymphocytes in small intestinal mucosa induced by chronic OVA exposure (daily gavage). A: villus height and crypt depth in jejunum and ileum of small intestine compared by light microscopy. B: T lymphocyte subpopulations (CD4 and CD8) in intestinal mucosa determined by immunohistochemistry are expressed as numbers of positive cells per millimeter of muscularis mucosa. * $P < 0.05$ compared with blocking antibody (OST-2). Values are means ± SE of 4 animals.



pared with OVA-sensitized controls ($P < 0.05$). Figure 3 shows counts of mucosal mast cells determined by using anti-RMCPII antibody in the small intestinal mucosa. The numbers of mucosal mast cells were significantly increased in the mucosa of both intestinal regions of daily gavage groups compared with OVA-sensitized or OVA-fed controls. However, mast cell numbers did not significantly differ between free-drinking and control groups.

We determined the effect of chronic OVA exposure on lymphocyte subpopulations in the intestinal mucosa by immunohistochemical means (Table 2). Figure 2B shows that the CD4 lymphocyte population was significantly increased in the daily gavage group exposed to OVA compared with either OVA-sensitized or OVA-fed controls both in the proximal and the distal small intestinal mucosa. The numbers of CD4 cells were also significantly increased in the free-drinking group, although to a lesser extent than in the daily gavage group, especially in the ileum. However, the numbers of CD8 lymphocytes were significantly decreased in both groups exposed to OVA compared with either of the controls at both sites. The numbers of OX12-positive B cells were significantly increased in the groups exposed to OVA compared with either of the controls. On the other hand, the numbers of IL-2 receptor-positive T cells did not significantly differ between control and daily gavage groups.

To determine whether the expression of adhesion molecules in intestinal microvessels is enhanced to induce lymphocyte

migration, we stained jejunal and ileal mucosa with antivascular adhesion molecules using an immunohistochemical technique. Figure 2C shows that MAdCAM-1 was mainly expressed in the endothelium of venules in the lamina propria of the intestinal mucosa just above the muscularis mucosa. However, daily gavage exposure to OVA induced a significant increase in MAdCAM-1 expression. This adhesion molecule was mainly induced in the lamina propria at the base of crypts, but its expression was also increased in some microvessels of the upper villus and in the submucosa. Table 3 shows a quantitative analysis of three adhesion molecules determined as the area of positively stained vessels per millimeter of muscularis mucosa in the small intestinal mucosa. MAdCAM-1 expression was significantly induced in both the proximal and the distal parts of the intestine of free-drinking or daily gavage groups compared with OVA-sensitized controls. On the other hand, the expression of ICAM-1 or VCAM-1 was less notable in the small intestine and did not significantly change after chronic OVA exposure.

We examined whether antibodies against MAdCAM-1 could affect the histological changes in the small intestinal mucosa of chronically allergic rats. Figure 4A shows that the functional blocking of MAdCAM-1 significantly attenuated the reduced villus height and the increased crypt depth induced by chronic OVA exposure compared with the control antibody groups at both sites. Figure 4B shows that anti-MAdCAM-1 blocking antibody, but not nonblocking antibody, significantly inhibited

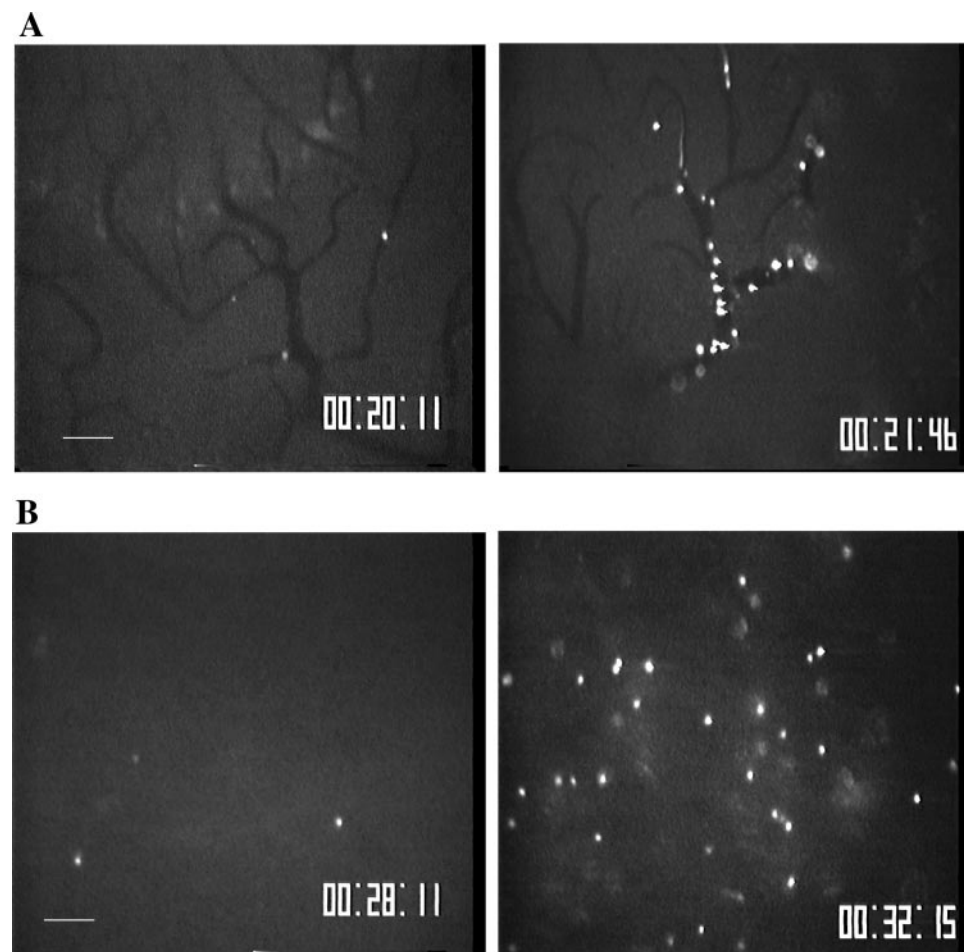


Fig. 5. A: representative photomicrographs showing distribution of CFSE-labeled T lymphocytes in postcapillary venules of Peyer's patches 20 min after infusion. Lymphocytes (6×10^7) were injected into OVA-sensitized controls (left). Lymphocytes were injected into animals chronically exposed to OVA (daily gavage; right). Bar, 100 μ m. B: microscopic images of distribution T lymphocytes sticking to microvessels of intestinal (ileal) mucosa 30 min after infusion. Images from control animals (left) and images from animals chronically exposed to OVA (daily gavage; right) are shown.

the increased numbers of CD4 cells in the intestinal mucosa of rats chronically exposed to OVA. However, the numbers of CD8 cells did not significantly differ between these two groups.

Lymphocyte migration to PCVs of Peyer's patches and villus mucosa. We examined whether T lymphocyte migration to the small intestinal mucosa is induced in chronic allergic animals exposed to OVA. Because cell-mediated immunity was more obvious in the daily gavage groups, we compared the effects in these animals with those in OVA-sensitized controls. We examined the *in vivo* migration of T lymphocytes to both lymphoid (Peyer's patches) and nonlymphoid (intestinal villus mucosa) areas of the small intestine. After an infusion of fluorescence-labeled T lymphocytes, the total numbers of lymphocytes that entered PCVs within 10 min of infusion into the jugular vein did not significantly differ between control and gavaged animals (58.5 ± 15.7 vs. 58.3 ± 13.3 per minute). Some lymphocytes showed characteristic rolling behavior on the surface of the endothelial wall in the PCVs of Peyer's patches, and the ratio (%) of rolling cells in daily gavage groups (61.8 ± 8.6) was much greater than that in controls (37.1 ± 8.9) 10 min after infusion. These findings indicated that lymphocyte-endothelial interaction was greater in the allergic rats. Some lymphocytes adhered to the endothelial walls of PCVs. The representative photomicrographs in Fig. 5A show that the number of T lymphocytes sticking in Peyer's patches 20 min after infusion was remarkably increased in allergic rats and reached about double and 1.7-fold the control numbers at 20 and 40 min, respectively (Fig. 6A).

We also visualized interactions between CFSE-labeled T lymphocytes and microvessels in the small intestinal mucosa. The total number of lymphocytes that entered intestinal microvessels did not significantly differ between control and chronic allergic rats at 10 min after infusion (35.3 ± 9.6 vs. 33.3 ± 7.6 /min). Figure 5B shows microscopic images of the distribution of T lymphocytes sticking in the intestinal (ileal) mucosa 30 min after infusion. A few T lymphocytes adhered to the intestinal microvessels of control rats, whereas in the daily gavage groups, adherence was significantly increased and reached ~3.7- and 2.7-fold the control numbers at 20 and 40 min, respectively (Fig. 6B).

We examined the effect of antibodies against adhesion molecules on T lymphocyte migration in the PCV of Peyer's patches and in the small intestinal mucosa of chronic allergic rats. Figure 6 shows that functionally blocking adhesion molecules inhibited the numbers of T lymphocytes that adhered to intestinal microvessels (PCV of Peyer's patches and villus microvessels) at 20 and 40 min. The number of T lymphocytes was significantly decreased by a prior infusion of anti-MAdCAM-1 to rats in both the PCV of Peyer's patches and the small intestinal mucosa. Nonblocking antibody to MAdCAM-1 (OST-20) did not affect the T lymphocyte sticking (data not shown). Nevertheless, the significant inhibitory effect of anti-MAdCAM-1 antibody on adherence of T lymphocytes to the PCV of Peyer's patches and villus microvessels (85.3 ± 6.0 and $89.2 \pm 8.0\%$ inhibition, respectively) was also observed in naive animals, suggesting that the effects are not restricted to the inflammatory state induced by feeding antigen. On the other hand, anti-ICAM-1 antibody did not significantly attenuate T lymphocyte interactions stimulated by exposure to OVA in either area.

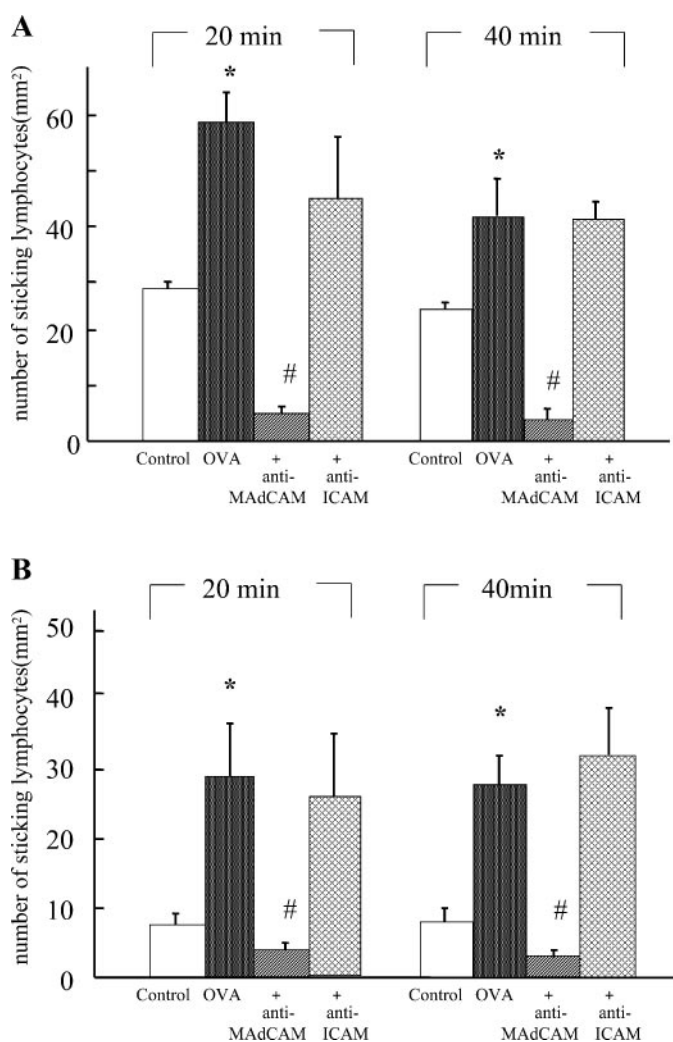


Fig. 6. A: numbers of lymphocytes sticking to postcapillary venules of Peyer's patches 20 and 40 min after infusion of T lymphocytes in OVA-sensitized controls and rats chronically exposed to OVA (daily gavage). T lymphocytes (6×10^7) separated from intestinal lymph were injected into recipient animals. Effects of blocking with MAdCAM-1 and ICAM-1 on sticking of T lymphocytes to postcapillary venules of Peyer's patches are shown. B: numbers of lymphocytes sticking to villus microvessels of ileal mucosa at 20 and 40 min after infusion of T lymphocytes in controls and rats chronically exposed to OVA (daily gavage). Effects of blocking MAdCAM-1 and ICAM-1 on sticking of T lymphocytes to villus microvessels are shown. * $P < 0.05$ compared with controls; # $P < 0.05$ compared with animals exposed to OVA. Values are means $\pm$ SE of 6 animals.

DISCUSSION

We describe a BN rat model of chronic food allergy that shows morphological changes and lymphocyte infiltration in the small intestine. We demonstrated, by using an intravital microscope, that the amount of T lymphocytes migrating to the intestinal mucosa exposed to OVA increased. Patients with chronic food allergies and cell-mediated reactions develop persistent diarrhea with dehydration, malnutrition, and sometimes failure to thrive. Biopsy specimens taken from such patients often show mucosal damage, such as villous atrophy and crypt hyperplasia, with significant infiltration of intraepithelial and lamina propria lymphocytes into the damaged mucosa (24, 27, 30). However, such mucosal damage has not

been well demonstrated in models of IgE-mediated intestinal anaphylaxis except for some infiltration of mast cells and edema (34). Lymphocyte infiltration into the intestinal mucosa is particularly limited in these conventional models, although Yang et al. (36) have recently shown that oral challenge with horseradish peroxidase results in mast cell activation and a late-phase reaction characterized by infiltration of mononuclear cells and ultrastructural damage to the intestinal epithelium.

Humans frequently develop allergic reactions to dietary proteins. The BN rat is a high immunoglobulin responder strain with a genetic predisposition to overproduce IgE in response to antigens. We reasoned that hyperstimulation of the IgE response would disturb the mucosal immune mechanisms to result in a cell-mediated reaction against exposure to oral antigens. Our model free-drinking and daily gavage groups both developed IgE-dependent intestinal allergies, because a specific IgE response was identified and RMCPII levels were increased in the serum of these animals. However, cell-mediated immunity was more obvious in the daily gavage group along with specific IgG, suggesting a Th1-like response. In our preliminary study of delayed-type hypersensitivity (DTH) determined by using the ear-swelling test, a significant DTH response developed in the daily gavage group and to a lesser extent in the free-drinking group (data not shown). These results are in accordance with significant lymphocyte infiltration of the intestinal mucosa in response to allergen challenge.

We speculate that if cell-mediated reactions are induced by the chronic phase of the IgE-mediated allergic response, then a T-cell-mediated process may largely contribute to the pathogenesis of mucosal morphological changes. Cell-mediated reactions on the intestinal mucosa have been documented in detail during the graft vs. host reaction (23). This hypothesis is supported by the finding in the mouse model of food-sensitive enteropathy, in which migrated lymphocytes in the intestinal mucosa can proliferate in response to stimulation with a specific antigen (29). The increased number of lymphocytes in the intestinal mucosa is thought to be the result of lymphocyte recruitment from the blood into the intestine. To investigate this possibility, we performed the *in vivo* migration study on T lymphocytes and uncovered direct evidence that T lymphocyte migration to both the intestinal Peyer's patches and villus mucosa was stimulated after chronic antigen exposure. We also demonstrated that these increases in T lymphocyte migration to small intestinal mucosa might be brought about via MAdCAM-1 on the microvascular endothelium, because prior exposure to this adhesion molecule significantly attenuated T-lymphocyte migration in both the PCV of Peyer's patches and in villus microvessels. The significant increase in MAdCAM-1 expression induced by chronic OVA exposure supports this notion. On the other hand, we did not find evidence that either ICAM-1 or VCAM-1 is involved in T lymphocyte migration in the small intestine. Several studies (7, 11, 33) on food allergy and animal models have suggested that the increased expression of ICAM-1 or VCAM-1 is related to the pathogenesis of leukocyte infiltration to the intestinal mucosa. We surmise that leukocyte subpopulations other than T cells, such as neutrophils, might play important roles via an ICAM-1-dependent mechanism in these situations.

Activated mast cells that produce sustained IgE responses might also contribute to the mechanism of enhanced lymphocyte migration. TNF- α produced from mast cells might

be an important factor for the upregulation of adhesion molecules including MAdCAM-1 (16, 35). To our knowledge, this is the first direct demonstration of lymphocyte migration to gut-associated lymphoid organs and intestinal mucosal microvessels *in situ* in a chronic food allergy model. However, the exact mechanism, including interactions between chemokines and chemokine receptors with integrins, remains to be further investigated. The increased trafficking of lymphocytes to the intestine exposed to antigen together with abnormal cell-mediated immune responses and IgE-mediated mast cell stimulation *in situ* might play a key role in the development of intestinal mucosal injury in patients with chronic food allergies.

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