

Eosinophilic Venulitis in the Small Intestines in a Mouse Model of Late Asthma

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Abstract—The allergen-unchallenged enteric lesions in late allergic asthma are largely unknown. To clarify this point, BALB/c mice were sensitized by ovalbumin (OVA)/aluminum adjuvant intraperitoneally two times (on days 0 and 10) and then challenged with OVA intranasally on day 14 (asthma group). Four days after the challenge, small intestinal lesions were examined. By this treatment, diarrhea was not observed in the asthma group. Compared to the controls with or without OVA sensitization and/or OVA challenge, the asthma group developed eosinophilic venulitis without an increase in mucosal mast cells in small intestines, whereas intestinal epithelial cells were relatively intact. A few numbers of interleukin (IL)-4⁺ and IL-5⁺ lymphoid cells were recognized in intestines in the asthma group, but not in the controls. Expression of vascular cell adhesion molecule-1 on venular endothelium and eotaxin-2⁺ eosinophils, but not epithelial cells, in intestines were detected in the asthma group, but not in the controls. Total IgE, OVA-specific IgE and eotaxin, and IL-5, but not interferon- γ , were produced systemically in the asthma group compared to the controls. The present study suggests that eosinophilic venulitis without mast cells in the intestine may be induced by the systemic, but not by local, helper T 2-type responses. In addition, eosinophilic venulitis in small intestines may be subclinical enteric lesions.

KEY WORDS: mouse; allergic late asthma; Th2 cell; eotaxin-2; eosinophil; venulitis; small intestine.

INTRODUCTION

Allergic asthma is characterized by immediate type, which is mediated by IgE and mast cell, and/or by late inflammation, which is mediated by eosinophils and helper T 2 (Th2) cells in the bronchi-bronchiole [1–5]. These are believed to be driven by an immune response with critical role of Th2 cytokines such as interleukin (IL)-4, IL-5, and IL-13 in immediate and delayed types [6–9]. Moreover, Eotaxin together with IL-5 was important for regulating eosinophil recruitment at the site of inflammation [10]. Eotaxin production is

increased in diseased tissues that are characterized by eosinophilic inflammation, including lesions in patients with asthma [11, 12]. It is also increased in response to respiratory allergen challenge in animals [13, 14].

Allergic respiratory inflammation might even spread systemically to involve nonrespiratory organs. Eosinophilic enteritis and eosinophilic esophagitis are reported during pollen seasons in patients with seasonal allergic rhinitis in human [15]. Also, eosinophil-associated gastrointestinal disorders in allergic diseases including allergic asthma and rhinitis have been reported [16]. On the other hand, the macroscopically normal duodenal mucosa of patients with allergic asthma and allergic rhinitis has been reported, despite the presence of major cellular components of allergic inflammation in small intestines [17]. Although the systemic inflammatory events in local allergic diseases may lead to pathologic response in other parts of body [15], the underlying pathogenesis of subclinical enteric lesions, which are allergen-unexposed sites, in allergic airway disorders is largely unknown.

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The present study examined the enteric lesions in a mouse model of late asthma without the requirement for an early IgE/mast cell-dependent responses [1, 2].

MATERIALS AND METHODS

Animals

Specific pathogen-free, 8-week-old female BALB/c mice (Japan Charles River Co., Kanagawa, Japan) were used (total number of mice in experimental groups, $n=29$). They were kept at $25\pm2^\circ\text{C}$ room temperature, $55\pm10\%$ humidity, and at 12-h light–dark cycle (lightening time, 08:00–20:00 hours) in metal cages sterilized by dry heating (180°C , 30 min), and animals were given autoclaved (120°C , 30 min) pellets (CA-1, Clea Japan INC., Tokyo, Japan) and water freely. The number of mice used in each experimental group is given in parenthesis of experimental protocol unless otherwise stated. The animal experiments were approved by the Research Ethics Board of the Faculty of the Agriculture, Yamaguchi University.

Experimental Protocols and Sampling

The sensitization and challenge procedures were performed by the method described previously [18, 19]. As shown in Fig. 1, mice (group 1) were sensitized intraperitoneally (i.p.) by the injection of $10\ \mu\text{g}$ of ovalbumin (OVA, grade V; Sigma-Aldrich, St. Louis, MO, USA) in $1.2\ \text{mg}$ of aluminum hydroxide gel (Alum; SERVA, Heidelberg, Germany) adjuvant (OVA/Alum), which was suspended in phosphate-buffered saline (PBS, pH 7.4), on day 0 and boosted on day 10 i.p.. Four days after the second sensitization, the mice were

challenged intranasally (i.n.) with $200\ \mu\text{g}$ of OVA in $50\ \mu\text{l}$ of PBS (both right and left nasal cavities by a micropipette alternately) under anesthesia by injection with $0.5\ \text{ml}$ mixture of ketamine and xylazine (group 1, $n=12$) i.p.. The control mice were divided into the following three groups: group 2 ($n=6$), mice were sensitized with OVA/Alum two times and challenged with PBS; group 3 ($n=5$), mice were sensitized with PBS two times and challenged with OVA; and group 4 ($n=6$), mice were sensitized with PBS two times and challenged with PBS. After the challenge, clinical signs especially diarrhea were examined every day (two times a day). Four days after the challenge, blood, left lung, small intestines (duodenum, jejunum, and ileum), and spleen were obtained from all groups.

Plasma

Plasma was obtained from angular vein of eye on days 0 and 18 by a heparinized micropipette (Terumo, Tokyo, Japan) to count the number of blood leukocytes. Also, IgE values in the plasma were measured.

Leukocyte Number in Blood

The absolute number of leukocytes per microliter was counted on day 18. Percentage of eosinophils, neutrophils, lymphocytes, and monocytes was calculated after blood smear stained with Giemsa. Then, the total number of each leukocyte was determined.

Histologic Evaluation of Lung and Small Intestine

Samples (groups 1–4) from intestines (duodenum, jejunum, and ileum) and left lung were fixed in 10% of neutral buffered formalin (pH 7.0) and embedded in paraffin wax. Sections of $4\ \mu\text{m}$ in thickness were stained with hematoxylin and eosin (HE), alcian blue for goblet cells, methylene blue for mucosal mast cells [20], congo red for eosinophils, and methyl green-pyronin for plasma cells. The severity of lesions in each transversely sectioned bronchi–bronchiole was evaluated by light microscopy and scored as described previously [18] as follows: no lesions, 0; mucus or desquamated epithelial cells in the lumen, 1; a few goblet cells in the epithelial layer, 1; many such cells, 2; thickened basement membrane, 1; mild infiltration of eosinophils and lymphocytes in mucosa, 1; severe infiltration of those cells in the mucosa, 2; and hypertrophy of smooth muscle cells in mucosa, 1. In each mouse the score index (S.I.) was calculated by

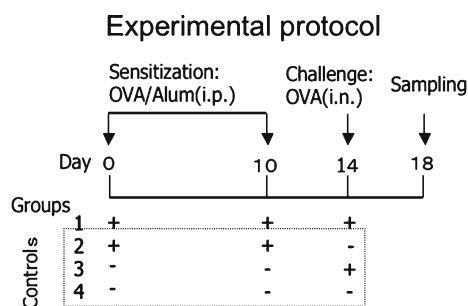


Fig. 1. Experimental protocol in the induction of late asthma. Positive sign OVA exposure, negative sign placebo exposure. See details in “Materials and methods.”

dividing the total score by the number of bronchi-bronchiole examined. The S.I. average was then determined for each group. The number of eosinophils, lymphocytes, mast cells, and goblet cells in intestines was determined by ten well-orientated villous-crypt units (VCU) [21] at random ($\times 400$ magnification). The number of plasma cells in the intestine was determined on three VCU. The mean number of other inflammatory cells and goblet cells was expressed per ten VCU. S.I. and the number of leukocytes and goblet cells were examined blindly by two different observers. In addition, each leukocyte count in and around Peyer's patches was excluded.

Immunohistochemistry

Deparaffinized sections of lung and jejunum from all groups ($n=3$) were treated with microwave oven (700 W for 3 min) in 10 mM citrate buffer (pH=6.0). Sections were treated with 5% bovine serum albumin (Sigma-Aldrich Co., St Louis, MO, USA) in PBS for 5 min at room temperature to block non-specific binding of antibody. Sections were then incubated with 3% hydrogen peroxide in methanol for 10 min to eliminate endogenous peroxidase activity. The sections were treated with avidin/biotin blocking kit (Vector Laboratories, Inc., Burlingame, USA). Then, sections were reacted with rat anti-mouse VCAM-1 monoclonal antibody (mAb) (Antigenix America Inc., Huntington Station, NY, USA), rat anti-mouse IL-4 mAb (Pharmingen, San Diego, CA, USA), rat anti-mouse IL-5 mAb (Southern Biotechnology Associates, Birmingham, AL, USA), or goat anti-eotaxin-2 polyclonal antibody (Santa Cruz Biotech., <http://www.scbt.com>), which is reported to stain eotaxin-2 of murine eosinophil [22]. Thereafter, sections were treated with biotin-labeled rabbit anti-rat antibody (Vector Laboratories, Burlingame, CA, USA) for IL-4, IL-5, and VCAM-1 or biotin-labeled rabbit anti-goat polyclonal antibody (DakoCytomation, Carpinteria, CA, USA) for eotaxin-2. Then, sections were treated with peroxidase-labeled streptavidin (Invitrogen Corporation, Camarillo, CA, USA). After that, sections were incubated with 3,3-diaminobenzidine substrate (Roch Diagnostics, GmbH, Penzberg, Germany) for 10 min. The reaction without primary antibody served as negative control. As positive controls, lung sections of group 1 were used. All sections were counterstained with methyl green for 1 min. Incubation was

done at room temperature for 30 min, unless otherwise stated.

Isolation of Splenocytes

Spleens were aseptically removed and placed in phosphate-buffered balanced salt solution (pH 7.4). Splenic cells were obtained by the method described previously [19]. In brief, single-cell suspensions were collected in sterile conical tubes (Assist Trading Co., Tokyo, Japan) after red blood cells were hemolyzed by Tris-buffered NH_4Cl solution and washed in PBBS containing 0.1% heat-inactivated fetal calf serum (FCS; Gibco, Grand Island, NY, USA) followed by centrifugation at $800\times g$ for 10 min at 4°C . Total number of cells was counted using a hemocytometer and diluted in minimum essential medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan) with kanamycin (100 $\mu\text{g}/\text{ml}$) to a density of 1×10^6 cells per milliliter. Viability of cells was more than 81% by trypan blue dye exclusion test. Splenocytes ($2\times 10^5/200\ \mu\text{l}$ MEM with 5% FCS) were cultured for 48 h at 37°C in 5% CO_2 in the presence of 5.5 $\mu\text{g}/\text{ml}$ of concanavalin A (Con A; ICN Biomedical Inc., Aurora, OH, USA) in 96-well plates (Coster, Cambridge, MA, USA) and supernatants were obtained.

Values of IFN- γ and IL-5 in Supernatants of Cultured Cells and Values of Total and OVA-specific IgE and Eotaxin in Blood

Concentrations of interferon (IFN)- γ (eBioscience, San Diego, CA, USA) and IL-5 (R&D System, Inc., Minneapolis, MN, USA) in supernatants from cultured splenocytes and values for total IgE (Morinaga, Yokohama, Kanagawa, Japan), OVA-specific IgE (DS Pharmacia Biomedical Co., Ltd, Suita, Osaka, Japan), and mouse CCL11/eotaxin (R&D System, Inc., Minneapolis, MN, USA) were measured by mouse ELISA kits according to the manufacturer's instructions. Detectable amounts of IFN- γ , IL-5, total IgE, OVA-specific IgE, and eotaxin are 6, 5, 500, 50, and 3 pg/ml, respectively. In case cytokines could not be detected, their concentration was estimated as 0.

Statistical Analysis

Data were analyzed using SPSS 12.0 for windows (SPSS Inc., Chicago, IL, USA). One-way ANOVA test was employed for the statistical analyses, and then a multi-group comparison test with $P<0.05$ were consid-

ered to be significant. The data are expressed as the mean of sample examined $\pm$ standard error (SE).

RESULTS

Number of Leukocytes in Blood

The number of each leukocyte is shown in Fig. 2. In the asthma group (group 1), there was a significant increase in numbers of eosinophils ($P < 0.01$) and lymphocytes ($P < 0.001$, compared to all of the controls). The number of neutrophils in group 1 was slightly increased compared to all of the controls ($P < 0.01$). There was no difference in numbers of monocytes among the four groups.

Pulmonary and Small Intestinal Lesions

Macroscopically, slight congestive changes of lungs were observed in the asthma group (group 1), whereas there are no macroscopic changes in all the controls. As shown in Fig. 3a in group 1, there were prominent goblet cell metaplasia and hyperplasia of the hypertrophic bronchial–bronchiolar epithelial cells with mucous secretion in bronchi–bronchial lumens in general. Edema and hyperemia, hypertrophic smooth muscle cells, and thickening of basement membranes were observed in bronchi–bronchiole walls. Severe infiltration of lymphocytes and eosinophils with some neutrophils was seen in bronchi–bronchiole walls (Fig. 3b) and alveolar walls. In addition, hyperemia and eosinophil infiltration were seen in and around capillaries and venules with destruction of them in pulmonary interstitium. Moreover, there was infiltration of eosinophils and lymphocytes in and around small arteries

in pulmonary interstitial tissues. On the other hand, those changes were not observed in all the controls (Fig. 3c, d). There were no diarrhea and prominent macroscopic changes in intestines in all of the four groups. In group 1, intestinal epithelial cells (duodenum, jejunum, or ileum) are relatively intact in general. Basically, infiltration of eosinophils was restricted in and around capillaries and

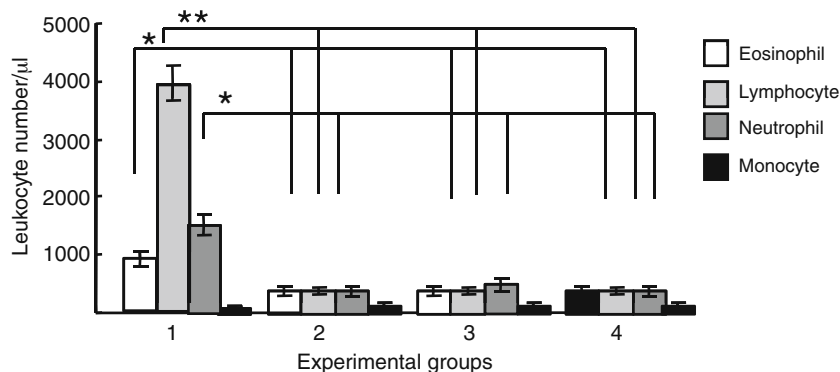
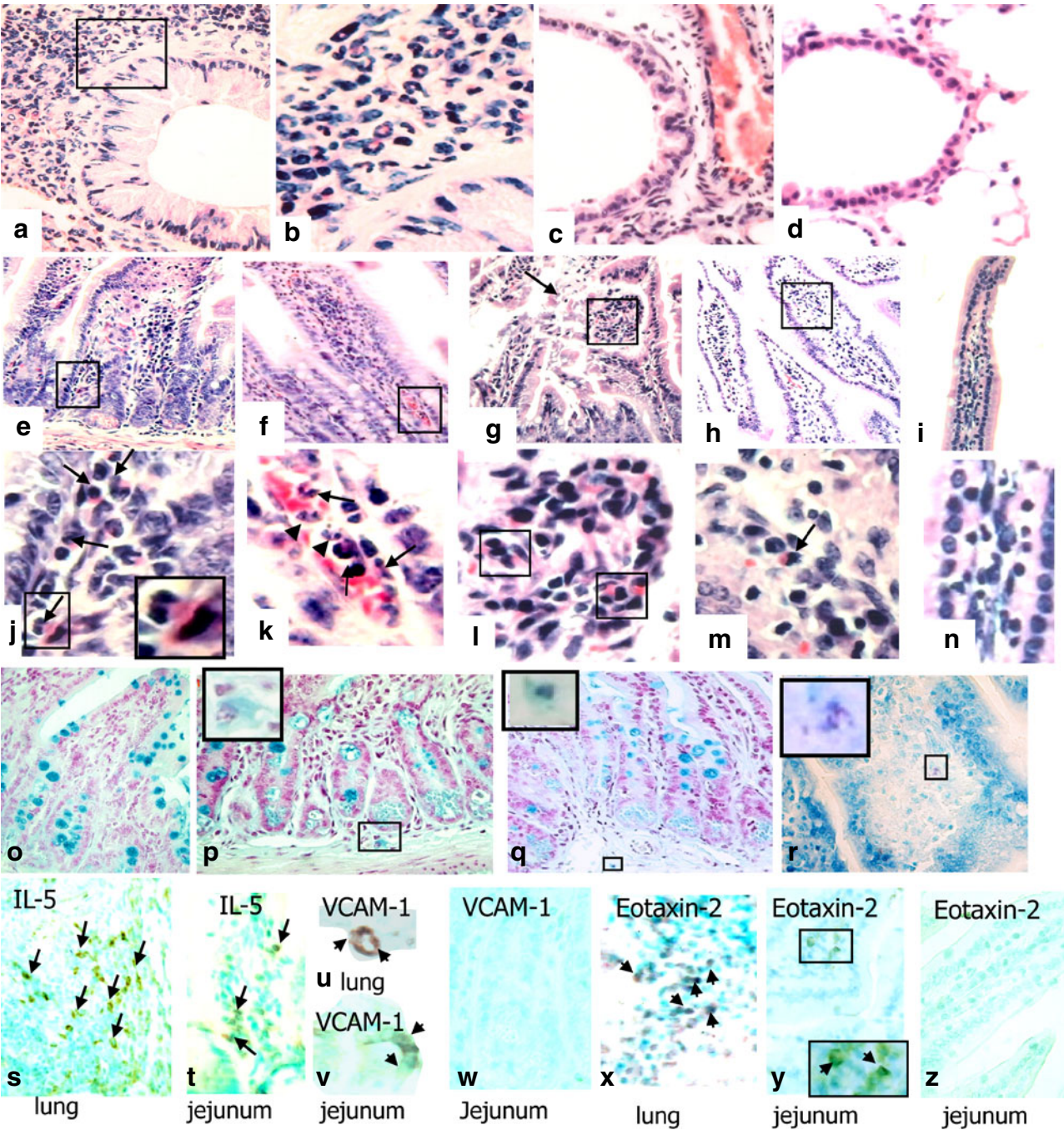


Fig. 2. Number of each leukocyte in blood in each group. Each value represents the mean $\pm$ SE. * $P < 0.01$; ** $P < 0.001$.



venules (eosinophilic venulitis) (Fig. 3e-g, j-l) accompanied with edema and hyperemia in lamina propria of intestines. In affected vessels, there were swelling, degeneration, and desquamation of endothelial cells. At these sites, nuclear fragmentation of eosinophils with destructed endothelium and vascular walls (leukocyte clastic vasculitis) was observed (Fig. 3f, k). Some such vessels were accompanied with hemorrhage. Also leukocyte-occlusive vasculopathy by eosinophils was observed (Fig. 3g, l). In group 1, hyperplasia of epithelial cells (Fig. 3e) was sometimes observed, and degeneration and necrosis with sloughing of intestinal epithelial cells were rarely observed

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(Fig. 3g). On the other hand, a few eosinophils were observed in lamina propria of small intestines in group 2 (Fig. 3h, m), but there were no destructive changes of venules. There was no increase in eosinophil infiltration in the groups 3 and 4. (Fig. 3i, n). There were a few eosinophils at the base of villi in all four groups.

In general, there was no increase or decrease in number of goblet cells in group 1 (Fig. 3o) compared to the controls (Fig. 3q). Decrease in number of goblet cells was observed at the sites with infiltration of relatively large numbers of eosinophil (Fig. 3p) in group 1. In addition, there was no increase in connective tissue (Fig. 3p) and mucosal (Fig. 3r) mast cells in group 1 compared to those in all of the control groups (Fig. 3q).

Immunohistochemistry of Bronchi–Bronchiole and Jejunum

In group 1, but not in all of the controls, a number of IL-4⁺ and IL-5⁺ cells (Fig. 3s) were observed not only in bronchi–bronchiole mucosa but also in alveolar septa. On the other hand, in jejunum, a small number of IL-4⁺ and IL-5⁺ (Fig. 3t) cells were found in group 1, whereas none of these cells were found in all the controls. An intense VCAM-1 expression on venular endothelial cells in lungs (Fig. 3u) was recognized, whereas its expression on those cells was weak in jejunum (Fig. 3v) in group 1. There was no VCAM-1 expression on vascular endothelium of lungs and jejunum (Fig. 3w) in all the controls. Some bronchial mucosal epithelium and alveolar epithelium and many polymorphonuclear leukocyte probably eosinophils (morphologically) expressed eotaxin-2 in bronchi–bronchiole walls (Fig. 3x), whereas ecotaxin-2⁺ eosinophils, but not intestinal epithelial cells, were observed in jejunum (Fig. 3y). No detectable amounts of eotaxin-2 expression on infiltrated leukocytes and epithelium in lungs and jejunum were observed in all the controls (Fig. 3z).

Score of Bronchial Lesions and Inflammatory Cell Reaction in Small Intestine

The development of bronchi–bronchiole lesions in group 1 was observed (S.I.; 4.8 ± 0.4), whereas there were no bronchi–bronchiole lesions in all the controls (Fig. 4a). The number of eosinophils (Fig. 4b) and plasma cells (Fig. 4c) increased significantly in duodenum, jejunum, or ileum in group 1 compared to all the controls ($P < 0.001$). There were no significant difference and increase in the number of lymphocytes (Fig. 4d) and

mast cells (Fig. 4e) including neutrophils (data not shown) in group 1 compared to all of the controls ($P < 0.001$). Although the number of goblet cells varied in each individual, there was no difference of those cell numbers among four groups (Fig. 4f) in various parts of the intestinal tract.

Total IgE, OVA-Specific IgE, and Eotaxin Values in Blood and Production of IL-5 and IFN- γ in Supernatants from Cultured Splenocytes

Total IgE values increased on day 18 compared to the values on day 0 ($P < 0.001$) in group 1, but not in the control groups (Fig. 5a). In addition, compared to all the control groups, there was a significant increase in total IgE values on day 18 ($P < 0.01$) (Fig. 5a). Moreover, compared to group 2, there was an increase in OVA-specific IgE values in group 1 ($P < 0.01$) (Fig. 5b). OVA-specific IgE was not produced in the groups 3 and 4 (Fig. 5b). A significant increase in eotaxin values (Fig. 5c) in group 1 compared to all the control groups statistically ($P < 0.001$) was observed. IL-5 values (Fig. 5d) in group 1 increased compared to those in the control groups ($P < 0.01$). IFN- γ values increased slightly in group 1, but the difference was not significant among the four groups (Fig. 5e).

DISCUSSION

Late allergic bronchial lesions characterized by severe infiltration of lymphocytes and eosinophils with goblet cell hyperplasia developed [23], whereas there was eosinophil dominant reaction in venules of the intestine without goblet cell hyperplasia in the asthma group. Also, a number of IL-4⁺ and IL-5⁺ lymphoid cells in bronchi–bronchiole were recognized, whereas those cells were few in the intestine. The interaction of allergen (OVA)-sensitized CD4⁺ Th2 cells reacted with OVA may lead to severe inflammation in the bronchial mucosa, whereas those events may be minor in the intestine. A slight to mild eosinophilic enteritis with destruction of epithelial cells at a certain degree developed, but it may be secondary events following venulitis, since in general, infiltration of eosinophils was restricted in venules. In addition, accumulated eosinophils in and around venules may lead to the damage to the venular wall by the release of granule proteins such as eosinophilic cationic protein, eosinophil-derived neurotoxin, and myelin basic protein [24]. It seems likely, since degranulation from eosinophils was observed. Although

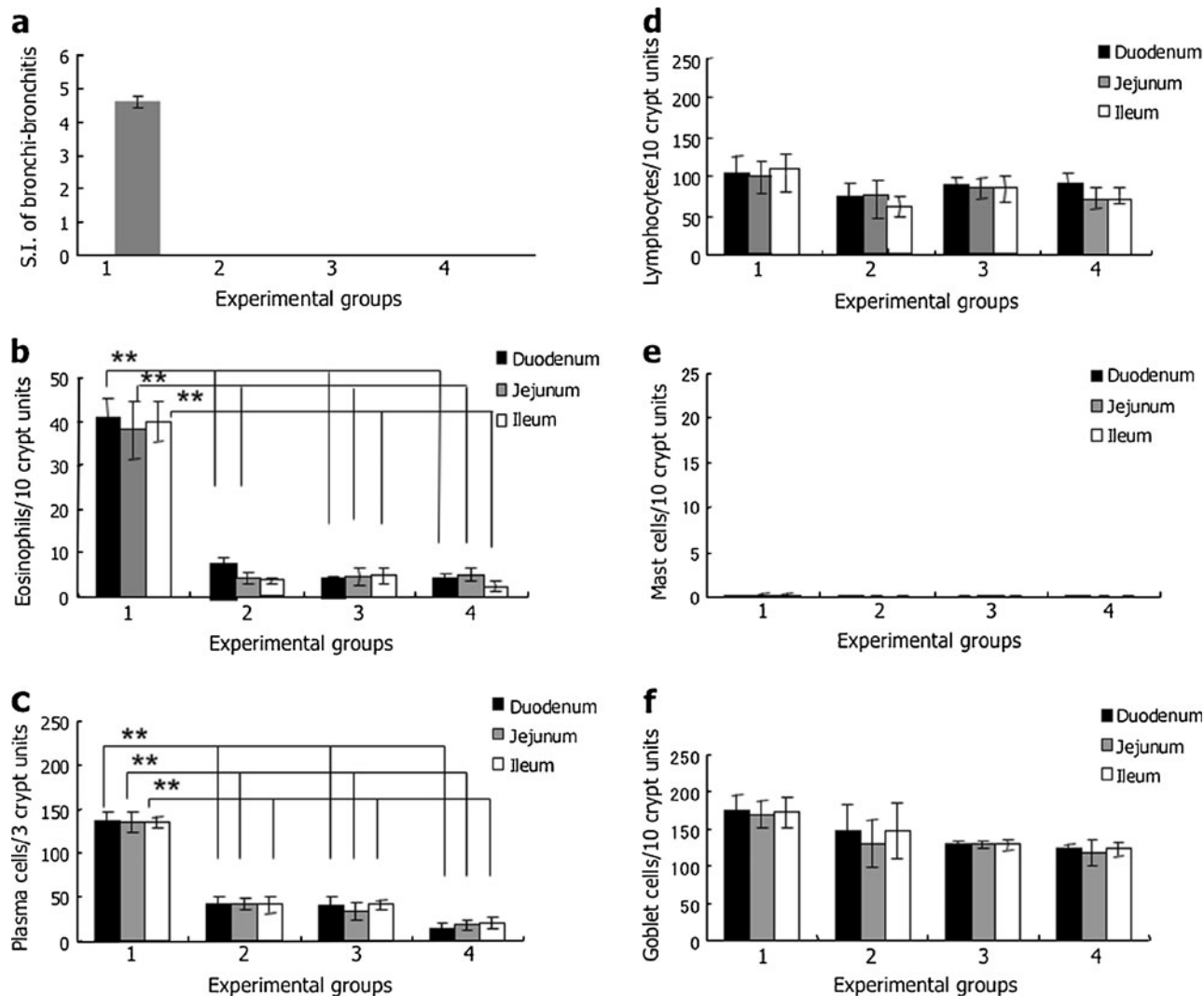


Fig. 4. S.I. (severity of bronchi-bronchiole lesions) (a) and number of eosinophils (b), plasma cells (c), lymphocytes (d), mast cells (e), and goblet cells (f) in duodenum, jejunum, or ileum, respectively, are shown. Group 1 ($n=12$), group 2 ($n=6$), group 3 ($n=5$), and group 4 ($n=6$). Each bar represents the mean $\pm$ SE. ** $P<0.001$.

eosinophil clastic vasculitis was observed (Fig. 3k), the mechanisms are unknown. Further study is needed to clarify this point. Alternatively, it has been reported that mice challenged with repeated intranasal inoculations of *Aspergillus fumigatus* antigens induced a significant increase in the level of eosinophils in the peripheral blood and broncho-alveolar lavage fluid, but no change in the level of gastrointestinal eosinophils was observed [25]. Taken together, different types of gastroenteric lesions may develop in airway allergic diseases in each individual depending on the sorts of allergens and exposure doses including allergic types such immediate type and/or late type.

IgE and OVA in the circulation might reach systemic circulation not only at the site of allergen exposure but also at the site of allergen unexposure [26]. We have reported previously in late allergic rhinitis model that IgE and OVA leaked from damaged venules in dermis might interact IgE receptor (Fc ϵ RI) on mast cells and eosinophils [27] in dermis [19], resulting in the development of immediate reaction in dermis which is allergen-unexposed sites [19]. If that is the case, the leak of IgE and OVA may occur in the intestine. However, the lack of immediate reaction in intestinal lamina propria may be due to the fact that there was no significant increase in mucosal and connective mast

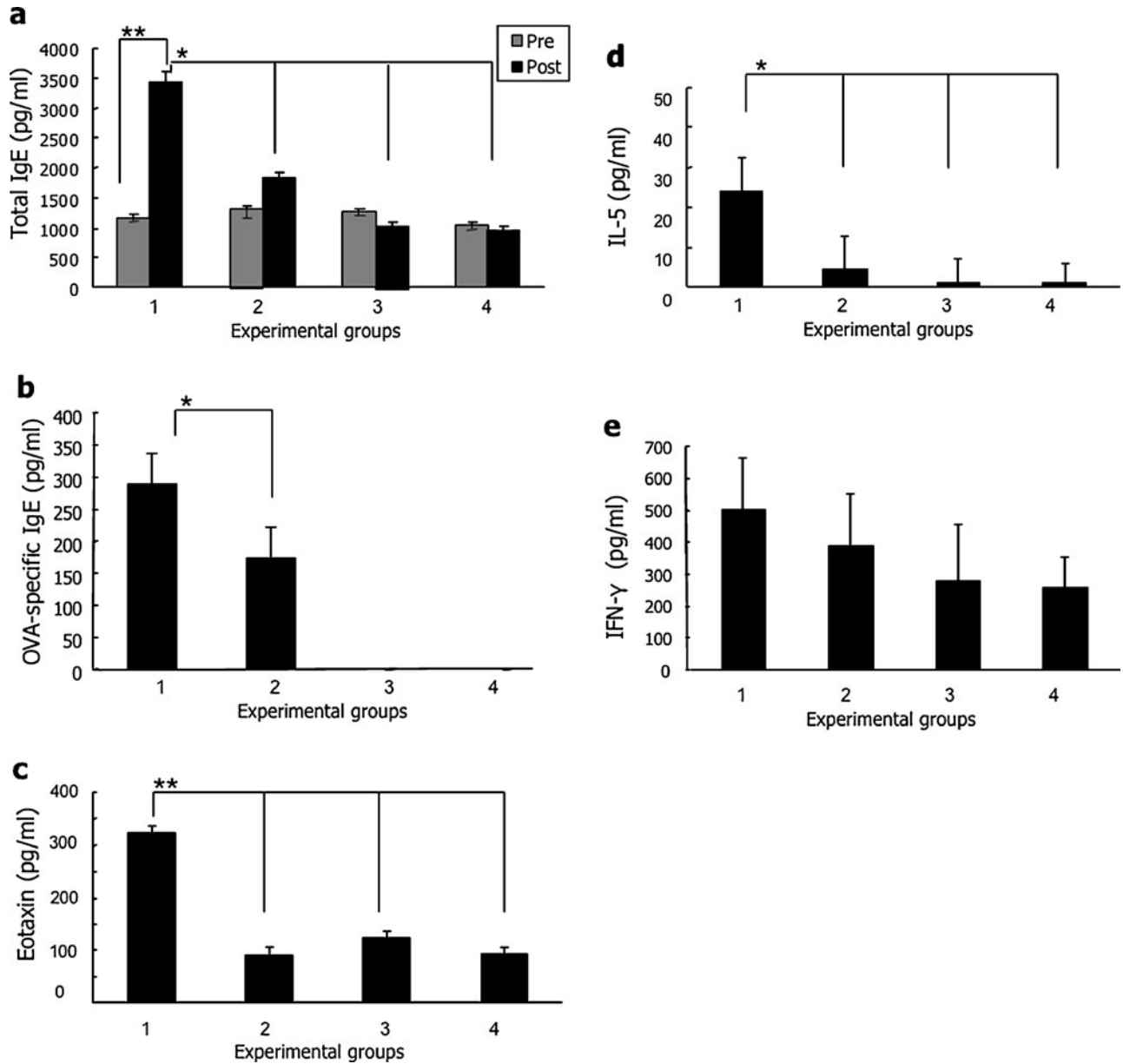


Fig. 5. Total blood IgE (a), OVA-specific IgE (b), and eotaxin (c) values, and productions of IL-5 (d) and IFN- γ (e) in each group are shown. Total IgE values were compared not only on days 0 and 18 in the same group but also between group 1 and all of the control groups. Other cytokine values on day 18 were compared between group 1 and the control groups. Groups 1–4 ($n=4$, respectively). Each bar represents the mean $\pm$ SE. * $P<0.01$; ** $P<0.001$.

cells. Increased values of IgE, which is induced by Th2 cytokines in this model [18], may not play a role in the development of eosinophilic venulitis in intestines. Taken together, different types of reaction may occur in allergen-unexposed sites in sensitized individuals.

We previously reported that very late activation antigen-4 (VLA-4), a ligand for VCAM-1, on eosinophils and vascular cell adhesion molecule-1 (VCAM-1) on

capillary endothelium as demonstrated here may be induced by circulating Th2 cytokines (*e.g.*, IL-4), but not Th1 cytokines (*e.g.*, IFN- γ), at the allergen-unexposed dermis in a delayed allergic rhinitis model [19]. Then, firm attachment of eosinophils to venular endothelium via those adhesion molecules, which also causes activation of eosinophils, may occur. However, VCAM-1 expression on vascular endothelium in intestines was weak compared

to that in lungs. Thus, weak VCAM-1 expression cannot accumulate efficiently eosinophils and lymphocytes, both of which have VLA-4, in blood vessels of intestines. In addition, eosinophil reaction was restricted at blood vessels. No detectable amounts of eotaxin-2 expression on intestinal epithelial cells may be due to a few IL-4⁺ T cells [28] in the intestine. It has been reported that Th2 cytokines such as IL-4 and IL-13 stimulate intestinal epithelial cells and alveolar epithelium, resulting in the expression of eotaxin-2, which acts as chemoattractant for eosinophils [22]. Although increased eosinophils in the circulation were observed, a few number of IL-5⁺ lymphoid cells in the intestines could not promote local eosinophil accumulation [29]. Moreover, eosinophils cannot migrate from venular lumen to lamina propria by lacking of eotaxin-2 expression by epithelial cells together with less in number of IL-4⁺ and IL-5⁺ lymphoid cells in lamina propria in the intestines. In contrast, eosinophil infiltration in bronchial and septal walls may be induced by infiltrated eotaxin-2⁺ eosinophils being responsible for the further accumulation of eosinophils at the site of bronchial and alveolar lesions other than eotaxin released from bronchial and alveolar epithelium [30]. Furthermore, cooperation of IL-5 produced from OVA-sensitized Th2 cells interacting OVA together with eotaxin-2 [30, 31] may stimulate the release of eosinophils from the bone marrow into the peripheral circulation [31], leading to high eosinophilia in blood. It seems likely, since those may contribute to bone marrow eosinopoiesis and migration of eosinophils from the bone marrow to the sites of inflammation [10] and may stimulate the effector capacity of mature eosinophils [11]. Taken together, lack of enough kind of these mediators in the intestine may attribute to restriction of eosinophil accumulation at venules.

Increased plasma cell infiltration was observed in the intestine, but not in the lung, of the asthma group. At present, the reasons are unclear. Intestinal microenvironmental changes in terms of venular lesions may permit the several bacterial components (e.g., lipopolysaccharide) from intestinal lumens, since impairment of the mucosal barrier system increases gut-derived endotoxin in the portal blood [32]. Thus, venular changes may be responsible for increased plasma cell infiltration in intestines.

One might suppose that there may be a possibility of gastrointestinal absorption of OVA protein after intranasal application and that antigen administration in the lung is gradually removed from the lung by the muco-ciliary escalator and passes through the intestine. However, that possibility may be minor or negligible, since the main lesions were limited at the site of venules.

In addition, a large number of OVA localized in bronchi-bronchiole walls were observed, whereas it was not detected in intestinal lamina propria by immunohistochemistry (our unpublished data).

The present study demonstrated the development of eosinophilic venulitis in intestines, which are allergen-unexposed sites in late allergic asthma model. There may be, at least, two separate steps such as, primarily, microvessel-oriented inflammation as discussed above and, secondary, the development of intestinal inflammation with changes of mucosal epithelium at certain degree, although the later changes were minor. The absence of clinical symptoms such as diarrhea suggests subclinical enteric lesions. There supposed to be heterogeneity in the development of enteric lesions in allergen-unexposed site in allergen-sensitized individuals, for example, allergic status (e.g., severity) and duration and exposure of allergen doses may determine the direction of the development of enteritis in allergen-unexposed sites.

In conclusion, intestinal venular lesions may be induced by systemic Th2 reaction rather than local Th2 reaction. The present experimental system may be useful to understand the pathogenesis of subclinical enteric lesions in patients with late allergic asthma.

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Conflict of Interest. We declare that we have no conflict of interest.

REFERENCES

1. Haselden, B.M., A.B. Kay, and M. Larché. 1999. IgE-independent MHC-restricted T cell peptide epitope-induced late asthmatic reactions. *The Journal of Experimental Medicine* 189: 1885–1894.
2. Ali, F.R., W.L. Oldfield, N. Higashi, M. Larché, and A.B. Kay. 2004. Late asthmatic reactions induced by inhalation of allergen-derived T cell peptides. *American Journal of Respiratory and Critical Care Medicine* 169: 20–26.
3. Verstraelen, S., K. Bloemen, I. Nelissen, H. Witters, G. Schoeters, and R. Van Den Heuvel. 2008. Cell types involved in allergic asthma and their use in *in vitro* models to assess respiratory sensitization. *Toxicology In Vitro* 22: 1419–1431.

4. Meyts, I., J.A. Vanoirbeek, G. Hens, B.M. Vanaudenaerde, B. Verbinnen, D.M. Bullens, L. Overbergh, C. Mathieu, J.L. Ceuppens, and P.W. Hellings. 2008. T-cell mediated late increase in bronchial tone after allergen provocation in a murine asthma model. *Clinical Immunology* 128: 248–258.
5. Hatzivlassiou, M., C. Grainge, V. Kehagia, L. Lau, and P.H. Howarth. 2010. The allergen specificity of the late asthmatic reaction. *Allergy* 65: 355–358.
6. O'byrne, P.M., J. Dolovich, and F.E. Hargreave. 1987. Late asthmatic responses. *The American Review of Respiratory Disease* 136: 749–751.
7. Coyle, A.J., G.L. Gros, C. Bertrand, S. Tsuyuki, C.H. Heusser, M. Kopf, and G.P. Anderson. 1995. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *American Journal of Respiratory Cell and Molecular Biology* 13: 54–59.
8. Foster, P.S., S.P. Hogan, A.J. Ramsay, K.I. Matthaei, and I.G. Young. 1996. Interleukin-5 deficiency abolishes eosinophilia, airways hyperactivity, and lung damage in a mouse asthma model. *The Journal of Experimental Medicine* 183: 195–201.
9. Mehlhop, P.D., M. van de Rijn, A.B. Goldberg, J.P. Brewer, V.P. Kurup, T.R. Martin, and H.C. Oettgen. 1997. Allergen-induced bronchial hyperactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proceedings of the National Academy of Sciences of the United States of America* 94: 1344–1349.
10. Foster, P.S., A.W. Mould, M. Yang, J. Mackenzie, S.P. Hogan, S. Mahalingam, A.N. McKenzie, M.E. Rothenberg, I.G. Young, K.I. Matthaei, and D.C. Webb. 2001. Elemental signals regulating eosinophil accumulation in the lung. *Immunological Reviews* 179: 173–181.
11. Lamkhioed, B., P.M. Renzi, S. Abi-Younes, E.A. Garcia-Zepeda, Z. Allakhverdi, O. Ghaffar, M.D. Rothenberg, A.D. Luster, and Q. Hamid. 1997. Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *Journal of Immunology* 159: 4593–4601.
12. Brown, J.R., J. Kleimberg, M. Marini, G. Sun, A. Bellini, and S. Mattoli. 1998. Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. *Clinical and Experimental Immunology* 114: 137–146.
13. Jose, P.J., D.A. Griffiths-Johnson, P.D. Collins, D.T. Walsh, R. Moqbel, N.F. Totty, O. Truong, J.J. Hsuan, and T.J. Williams. 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *The Journal of Experimental Medicine* 179: 881–887.
14. Pope, S.M., N. Zimmermann, K.F. Stringer, M.L. Karow, and M.E. Rothenberg. 2005. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. *Journal of Immunology* 175: 5341–5350.
15. Togias, A. 2004. Systemic effect of local allergic disease. *The Journal of Allergy and Clinical Immunology* 112: S8–S14.
16. Shirai, T., A. Komiyama, H. Hayakawa, D. Hashimoto, T. Suda, and K. Chida. 2009. Eosinophil-associated gastrointestinal disorders with asthma: immunohistochemical analyses. *Internal Medicine* 48: 1315–1321.
17. Pires, G.V., H.S. Souza, C.C. Elia, C. Zaitman, A.T. Carvalho, C.J. Tortori, H.C. Garrofé, and J.R. Lapa e Silva. 2004. Small bowel of patients with asthma and allergic rhinitis: absence of inflammation despite the presence of major cellular components of allergic inflammation. *Allergy and Asthma Proceedings* 25: 253–259.
18. Hayashi, T., Y. Adachi, K. Hasegawa, and M. Morimoto. 2003. Less sensitivity for late airway inflammation in male than females in BABL/c mice. *Scandinavian Journal of Immunology* 57: 562–567.
19. Hayashi, T., and T. Fujii. 2008. Acute urticaria-like lesions in allergen-unexposed cutaneous tissues in a mouse model of late allergic rhinitis. *International Journal of Experimental Pathology* 453: 177–188.
20. Friend, D.S., N. Ghildyal, K.F. Austen, M.F. Gurish, R. Matsumoto, and R.L. Stevens. 1996. Mast cells that reside at different locations in the jejunum of mice infected with *Trichinella spiralis* exhibit sequential changes in their granule ultrastructure and chymase phenotype. *The Journal of Cell Biology* 135: 279–290.
21. Allen, A., D.A. Hutton, A.J. Leonard, J.P. Pearson, and L.A. Sellers. 1986. The role of mucus in the protection of the gastroduodenal mucosa. *Scandinavian Journal of Gastroenterology* 1125: 71–78.
22. Zimmermann, N., S.P. Hogan, A. Mishra, E.B. Brandt, T.R. Bodette, S.M. Pope, F.D. Finkelman, and M.E. Rothenberg. 2000. Murine eotaxin-2: a constitutive eosinophil chemokine induced by allergen challenge and IL-4 overexpression. *Journal of Immunology* 165: 5839–5846.
23. Hayashi, T., A. Ishii, S. Nakai, and K. Hasegawa. 2004. Ultra-structure of goblet cell metaplasia from clara cell in the allergic asthmatic airway inflammation in a mouse model of asthma. *Virchows Archiv* 444: 66–73.
24. Venge, P., and J. Byström. 1998. Molecules in focus eosinophil cationic protein (ECP). *The International Journal of Biochemistry & Cell Biology* 30: 433–437.
25. Mishra, A., S.P. Hogan, E.B. Brandt, and M.E. Rothenberg. 2000. Peyer's patch eosinophils: identification, characterization, and regulation by mucosal allergen exposure, interleukin-5, and eotaxin. *Blood* 96: 1538–1544.
26. Hens, G., S. Bobic, K. Reekmans, J.L. Ceuppens, and P.W. Hellings. 2007. Rapid systemic uptake of allergens through the respiratory mucosa. *The Journal of Allergy and Clinical Immunology* 120: 472–474.
27. Ying, S., L.T. Barata, Q. Meng, J.A. Grant, J. Barkans, S.R. Durham, and A.B. Kay. 1998. High-affinity immunoglobulin E receptor (Fcε RI)-bearing eosinophils, mast cells, macrophages and Langerhans' cell in allergen-induced late-phase cutaneous reactions in atopic subjects. *Immunology* 93: 281–288.
28. Conroy, D.M., and T.J. Williams. 2001. Eotaxin and the attraction of eosinophils to the asthmatic lung. *Respiratory Research* 2: 150–156.
29. Lee, J.J., M.P. McGarry, S.C. Farmer, K.L. Denzler, K.A. Larson, P.E. Carrigan, I.E. Brenneise, M.A. Horton, A. Haczku, E.W. Gelfand, G.D. Leikauf, and N.A. Lee. 1997. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *The Journal of Experimental Medicine* 185: 2143–2156.
30. Rothenberg, M.E., R. Ownbey, P.D. Mehlhop, P.M. Loiselle, M. van de Rijn, J.V. Bonventre, H.C. Oettgen, P. Leder, and A.D. Luster. 1996. Eotaxin triggers eosinophil-selective chemotaxis and calcium flux via a distinct receptor and induces pulmonary eosinophilia in the presence of interleukin 5 in mice. *Molecular Medicine* 2: 334–338.
31. Collins, P.D., S. Marleau, D.A. Griffiths-Johnson, P.J. Jose, and T. J. Williams. 1995. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation *in vivo*. *The Journal of Experimental Medicine* 182: 1169–1174.
32. Fillon, S., Z.D. Robinson, S.P. Colgan, and G.T. Furuta. 2009. Epithelial function in eosinophilic gastrointestinal diseases. *Immunology and Allergy Clinics of North America* 29: 171–178.