

The contribution of mast cells to the late-phase of allergic asthma in rats

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Abstract. *Introduction:* Mast cells are thought to be the main cause of an immediate asthmatic response, but their contribution to the late-phase of asthma is unknown.

Objective: To prove the contribution of preactivated mast cells to the late phase of allergic asthma by advanced activation.

Methods: Mast cell function in the late-phase of asthma was studied. Rats (wild, +/+ and mast cell deficient, *Ws/Ws*) were challenged with OVA to investigate the relationship between the contraction of airways and the population of inflammatory cells in the trachea.

Result: During the entire asthmatic period, the contraction of the airway after OVA challenge in +/+ rats was enhanced significantly compared to *Ws/Ws* rats, especially in the late phase. The bronchoalveolar lavage fluid histamine in +/+, but not *Ws/Ws*, rats increased 5.3-fold in 30 min and 3.4-fold in 8 h after challenge, significantly. The number of mucosal mast cells in the tracheal epithelial layer in +/+ rats increased significantly 2.2-fold over controls at 8 h after challenge, as demonstrated by in situ hybridization.

Conclusions: Mast cells may contribute to the late phase of asthmatic response by continuous mast cell activation and the mucosal mast cell number increased in the late phase of asthmatic response.

Key words: Asthma – Mast cell deficient rat – Histamine – PenH – RMCP-II

Introduction

About 250 millions asthmatic patients in the world represent a major health problem. In response to the rising incidence and the world-wide health impact of asthma, the understanding of asthma has increased substantially over the past 30 years.

The cellular and molecular mechanisms of atopic asthma have been extensively investigated. Inhalation challenge of atopic asthmatics with a specific allergen evokes biphasic responses

that are separated by several hours [1]. There is a discrete early-phase airway response (EAR) that is followed by late-phase airway responses (LAR). The evolution of the allergic asthma response begins when a processed allergen is presented to naive Th-precursor cells by antigen presenting cells within a cytokine milieu that favors the selective expansion of Th₂-polarized memory cells. Eventually B cells, driven by IL-4, produce specific IgE [2]. Re-exposure to allergen elicits the EAR that is triggered by crosslinking of antibody-loaded, high-affinity IgE receptors (FcεRI) on mast cells or basophils, and histamine, cytokines and chemokines are secreted. After several hours the LAR occurs, during which inflammatory mediators, including IL-3, IL-5, and GM-CSF are produced by both Th₂ cells [3] and eosinophils. Mast cells, basophils, macrophages, neutrophils, and platelets may also make additional contribution to the generation of inflammatory mediators [1].

Mast cells are localized at the interface of the internal and external environment within the airway where they may respond to stimulation by allergens. Mast cells are thought to cause the acute symptoms of the acute allergic inflammation by release of histamine and other lipid mediators [4]. Our previous research also discussed the role of mast cells in the EAR [5]. Mast cells are now known to contribute to recruitment and activation of secondary effector cells, in particular eosinophils, which are hallmarks of the chronic asthmatic response and airway remodeling [6]. However the involvement of mast cells in the progression to the LAR is still unclear. To study the roles of mast cell function in the LAR, we used unrestrained whole body plethysmography, which is regarded as a credible technique for examining late-phase bronchoconstrictive events in animal models of asthma [7–9] to compare the respiratory changes in mast cell deficient (*Ws/Ws*) and wild type (+/+) rats. *Ws/Ws* rats are completely devoid of mast cells due to the deletion of 12-nucleotide bases in the tyrosine kinase domain of the *c-kit* receptor gene. The histamine levels in tracheal tissue from *Ws/Ws* rats are approximately 0.83% of that in tissue from +/+ rats [5]. The results of our research demonstrate the direct relationship between mast cells and the LAR.

Materials and methods

Chemicals

Ovalbumin, naphthol AS-D chloroacetate, pararosaniline, and 3, 3-diaminobenzidine tetrahydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). ISOGEN was purchased from Wako Pure Chemical Industries (Osaka, Japan). Tissue-Tek OCT compound was purchased from Sakura Finetechnical Company (Tokyo, Japan). DIG Easy Hyb was purchased from Roche Molecular Biochemicals. All other chemicals were the highest grade commercially available.

Animals

Male *Ws/Ws* and *+/+* rats were obtained using the mating procedure described by Niwa et al. [10]. The male rats weighing between 250–300 g and 3–4 months old were used for experiments. The animals were housed at a constant temperature of $22 \pm 2^\circ\text{C}$ with a humidity of $55 \pm 10\%$ on an automatically controlled 12:12 h light-dark cycle, with light on at 7:00 A.M. Rats were given food and water *ad lib*. The experimental protocols were in accordance with the guidelines of the Animal Care Committee of Ehime University and approved by the University Committee for Animal Research.

Sensitization protocol

Rats were sensitized by subcutaneous injection of 1.0 ml of 1.0 mg/ml ovalbumin mixed with 10% aluminum hydroxide suspension in physiologic saline. At the same time, an intraperitoneal injection of Bordetella pertussis antigen solution containing 1.0×10^9 organisms/ml was given as an adjuvant. A booster injection of 1.0 ml of 1.0 mg/ml ovalbumin suspended in 10% aluminum hydroxide and physiologic saline was injected after 7 days. The rats selected for the experiment were used 14 days after the first injection, in which IgE titer had been shown a peak of the time-dependent increase [5].

Airway antigen challenge and measurement of asthmatic response

Airway responsiveness was analyzed in sensitized rats (twenty *+/+* rats and twenty *Ws/Ws* rats) placed in a whole-body plethysmographic chamber (Buxco Electronics, Sharon, CT). After a 10-min stabilization period, ten rats in each group were challenged by inhalation of 2% ovalbumin or saline for 10 min. Enhanced pause, or PenH (= pause $\times$ PEP/PIP) described the bronchoconstriction, using the peak expiratory pressure (PEP) and the peak inspiratory pressure (PIP). The pause was defined as $(T_c - T_r)/T_c$, where T_c is the time of expiration and T_r is the relaxation time. The T_r was the time of pressure decay to 36% of the total expiratory pressure signal. The constriction of the airway is shown as mutative PenH. PenH was monitored for 24 h after inhalation challenge and the maximal PenH readings were taken over 10-min windows every 30 min in the first hour and every 1 h thereafter within 24 h.

Released histamine measurement in bronchoalveolar lavage fluid (BALF)

After challenge with ovalbumin or saline, rats were anesthetized by nembutal at 30 min, 8 h and 24 h. BALF was collected via tracheal cannulation using 8 ml of phosphate buffered saline solution and collections were done 3 times on each time point. Routinely, more than 90% of the lavage fluid was recovered from lungs. Supernatants obtained by centrifugation of the BALF at $250 \times g$ for 10 min were assayed for histamine.

The concentrations of released histamine in BALF were determined by HPLC-fluorometry [11]. Briefly, the BALF was diluted with half volume of perchloric acid (7.5%), centrifuged at $10,000 \times g$ for 10 min, and the supernatant was transferred to a fresh tube. A 10% volume of 2M KOH/1M KH_2PO_4 was added, followed by centrifugation at $10,000 \times g$ for 10 min, and the resulting supernatant was injected into a column packed with TSK gel SP-2SW cation exchanger (150 mm $\times$ 6 mm i.d., Tosoh, Tokyo, Japan). The histamine was post-labeled with o-phthalaldehyde under alkaline conditions and detected fluorometrically by an FL Detector L-7480 (Hitachi, Tokyo, Japan).

Histochemical analysis

For histological analysis, control group were non-sensitized rats and sensitized rats that were challenged with saline. Histological sections of trachea obtained at times after challenge with ovalbumin were examined for inflammatory cells, including eosinophils and mast cells. Rats were euthanized by inhalation of diethyl ether. The tracheas were quickly removed and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 12 h at 4°C . After washing with 0.1 M phosphate buffer, specimens were immersed in 20% sucrose/0.1 M phosphate buffer overnight at 4°C . Fixed tracheas were embedded in OCT compound and stored at -80°C . The frozen tracheas in OCT compound were cut into 4–5 μm sections with a cryotome.

The cyanide-resistant eosinophil peroxidase activity was used for histochemical visualization of eosinophils [12]. In brief, cryosections were incubated for 15 min at room temperature in phosphate buffered saline (pH 7.4) supplemented with 3, 3-diaminobenzidine tetrahydrochloride (0.06%), H_2O_2 (0.1%), KCN (0.12%). Slides were rinsed in water and mounted with permount.

For mast cell identification by enzyme histochemistry, the chloroacetate esterase procedure was used [13]. Briefly, the sections were incubated for 1 h at 30°C with chloroacetate substrate solution consisting of 0.5% naphthol AS-D chloroacetate and 0.02% hexazotized pararosaniline, counterstained with hematoxylin, and mounted on slides with permount.

The eosinophils, as well as both connective tissue mast cells and mucosal mast cells were scored individually in each section and the average counts of 4 sections per trachea was used for semi-quantitative analysis.

Northern blot analysis

An 835-base pair fragment of rat mast cell protease II (RMCP II) mRNA, which is primarily in rat mucosal mast cells [14], was used for the digoxigenin-labeled mRNA antisense probe. A 614-base pair fragment of the β -actin mRNA was used to produce the positive control probe. Briefly, RNA was extracted from the trachea tissue of *+/+* and *Ws/Ws* rats with ISOGEN. After separation on an agarose gel, RNA was transferred overnight to a positively charged nylon membrane. The probes were hybridized overnight at 68°C to the blotted RNA in DIG Easy Hyb. After stringency washes, the bound probe was detected by chemiluminescence.

In situ hybridization analysis

Rat trachea fixation for in situ hybridization was the same as for histochemical analysis except for the addition of diethylpyrocarbonate to the fixing solution to inhibit RNase. Dried frozen sections were rinsed with phosphate buffered saline supplemented with 0.3% Tween-20 (PBT) for 3 times at room temperature and fixed with 4% paraformaldehyde for 20 min. Then sections were rinsed 3–5 times with PBT. The digoxigenin-labeled mRNA antisense probe of RMCP-II was hybridized with section overnight in a humidified chamber at 60°C . As posthybridization washes the sections were incubated in $5 \times$ SSC with 50% formamide and 1% SDS for 30 min and 45 min twice in

2 × SSC with 50% formamide at 60°C. After rinsing 2–3 times with 0.1 M Tris buffered saline solution containing 0.15 M NaCl, 0.03 M KCl and 1% Tween 20 at room temperature, sections were placed in 0.5% blocking reagent in rinse solution for 1 h at room temperature and incubated with digoxigenin antibody overnight at 4°C. The sections were washed 3 times at room temperature with 2 mM levamisole in 0.1 M Tris buffered saline solution containing 0.15 M NaCl, 0.03 M KCl and 1% Tween 20 for 20 min and developed overnight with 4-nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate color-reagent in a dark humid chamber after infiltration for 5 min with equilibration solution containing 0.1 M NaCl, 0.5 M MgCl₂, 0.1 M Tris, 0.1% Tween 20 and 2 mM levamisole. The sections were washed gently using 0.1 M Tris in 1 mM EDTA and mounted with 50% glycerol. The positive cells, detected as blue points, were counted microscopically.

Statistical analysis

All experiments were designed as completely randomized multifactorials with 3–10 rats per group. The results are expressed as the means ± SEM. The difference of the airway response to antigen challenge and histamine content in BALF among the groups was analyzed using repeated measure ANOVA. The difference between every time point in airway response and histamine content in BALF was analyzed using Tukey test. The two-sample t-test assuming unequal variance was done to evaluate statistical significance of the inflammatory cell number in trachea. *P* values < 0.05 indicated significant differences.

Results

Asthmatic response induced with antigen in the +/+ and *Ws/Ws* rats

Throughout the entire asthmatic period, the contraction of the airway to ovalbumin challenge in +/+ rats was obviously more sensitive than that of other groups of rats (*P* < 0.05), especially in the LAR. The significant increase of PenH value in +/+ rats challenged with OVA was observed in 6 h after challenge and sustained at this level until 16 h (Fig. 1). The *Ws/Ws* rats showed weaker responses, as measured by PenH values, than +/+ rats (*p* < 0.05, *n* = 10). No significance was found among the +/+ rats challenged with saline, the *Ws/Ws* rats challenged with saline and the *Ws/Ws* rats challenged with ovalbumin. These data show a close relationship between airway responsiveness and mast cell activation in an antigen induced asthmatic response.

Histamine released in BALF

Histamine was rarely detected in BALF of *Ws/Ws* rat and no significant difference was shown between before and after ovalbumin challenge (Fig. 2). The histamine levels in BALF of +/+ rats were increased during the asthmatic response by 5.3-fold at 30 min, 3.4-fold at 8 h, and 3.2-fold at 24 h after challenge. The histamine concentrations increased significantly in both the EAR at 30 min and the LAR at 8 h. However the difference between responses at the EAR and the LAR was not significant.

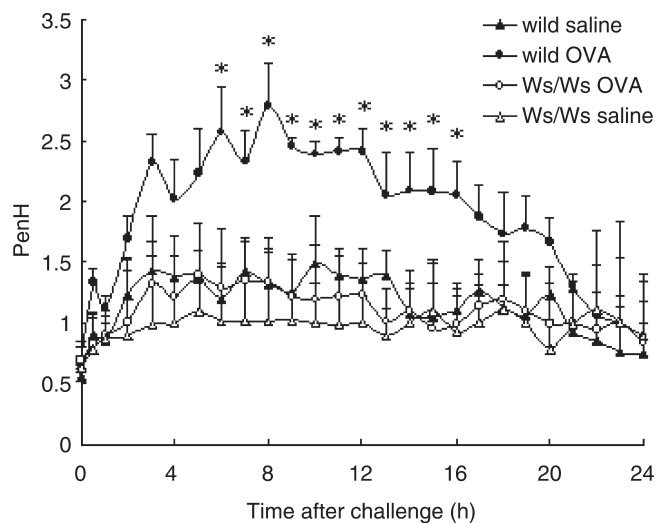


Fig. 1. Whole body plethysmograph measurement of airway hyperresponsiveness in *Ws/Ws* rats and +/+ rats induced by ovalbumin. After sensitization, rats were challenged by inhalation of 2% ovalbumin or saline for 10 min. Airway hyperresponsiveness was characterized by PenH, which was recorded using a whole body plethysmograph measurement system from 10 min before until 24 h after challenge. For each rat, the maximum PenH reading was taken over a 10-min time window at every 30 min point in the first hour and every 60 min point thereafter until 24 h. *, *P* < 0.05, Tukey test for different time point in every group compared with control group (*n* = 10 rats/group).

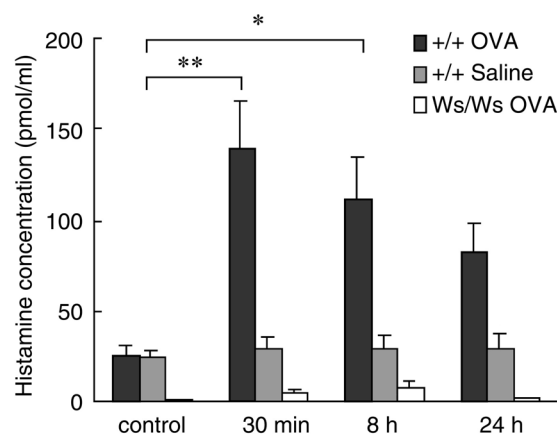


Fig. 2. Histamine levels in BALF after challenge with ovalbumin. Rat BALF was taken from *Ws/Ws* rats challenged with ovalbumin (open columns), +/+ rats challenged with ovalbumin (solid columns) and +/+ rats challenged with saline (gray columns) at 30 min, 8 h and 24 h after challenge by 2% ovalbumin inhalation. Both kinds of rats challenged with saline were used as control groups. *, *P* < 0.05; **, *P* < 0.01, Tukey test for different time point in every group (*n* = 5 rats/group).

Eosinophil recruitment and mast cells degranulation in the LAR

Histochemically, the numbers of either eosinophils or mast cells in non-sensitized rats were comparable to those in sensitized rats challenged with saline. In $+/+$ rats, the number of eosinophils in 24 h was nearly 6-fold higher than that of control group. No such changes were detected in the Ws/Ws rats (Table 1).

Mast cells were stained red (Figs. 4a and 4b). Connective tissue mast cells were found in the submucosal layer around smooth muscle cells and mucosal mast cells were abundant in the epithelial layer. On the other hand, mast cells were not detected in Ws/Ws rat trachea samples (Figs. 4c and 4d). The degranulation phenomenon was observed in the connective tissue mast cells in $+/+$ rats after challenge with inhaled ovalbumin. The degranulation was indicated a 40% decrease in the number of connective tissue mast cells near the region of smooth muscle cells. The decrease in connective tissue mast cells persisted for 24 h.

The 9% decrease in the number of mucosal mast cells observed 30 min after challenge was not significant. In the LAR, in which the PenH value increased significantly, the number of mucosal mast cells also increased in $+/+$ rats challenged with ovalbumin. When analyzed by histochemically, a 1.6-fold increase in the number of esterase-positive cells in the epithelial layer was observed at 8 h, but by 24 h esterase-positive cells had returned to the basal level (Table 1).

Increase in numbers of mucosal mast cells associated with RMCP-II in the LAR

Histochemical analysis showed an increase in the number of mucosal mast cells during the LAR. The cell population that increased in number was confirmed as being mucosal mast cells by specific in situ hybridization using digoxigenin-labeled RMCP II mRNA antisense probe.

The specificity of the probe was verified by Northern blot analysis of the mRNA extracted from the trachea of $+/+$ and Ws/Ws rats. The hybridization with the DIG-labeled RMCP II mRNA antisense probe revealed a single band with trachea mRNAs in $+/+$ rats, but no labeling with the Ws/Ws rat trachea mRNAs (Fig. 3). The mRNAs of tracheas in both types

of rats gave positive hybridization results with the control probe for β -actin.

Upon microscopic examination after in situ hybridization, all of the dark blue staining cell were located in the epithelial layer and positive cells were not detected in the submucosa (Figs. 4e and 4f). No positive cells were found in the trachea sections of Ws/Ws rats (Figs. 4g and 4h).

After challenge, the population of positive cells in the epithelial layer of the trachea that were labeled with RMCP II mRNA probe was increased 2.2-fold at 8 h and 2.3-fold at 24 h when compared to the group challenged with saline, while the number of cells that were positive by enzyme histochemistry returned to control levels in 24 h (Fig. 5 and Table 1).

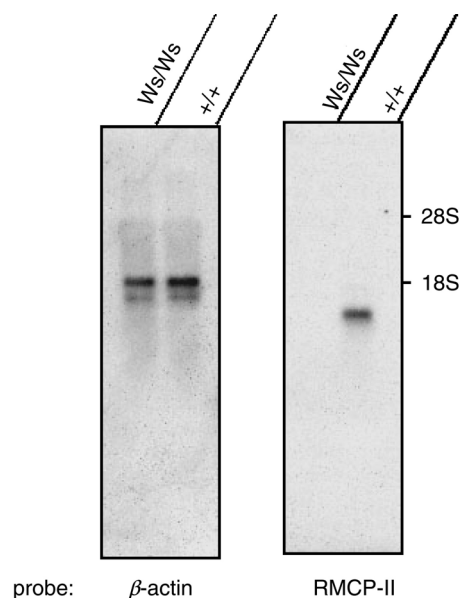


Fig. 3. Northern blot analysis of RMCP-II and β -actin mRNA prepared from trachea of $+/+$ rats and Ws/Ws rats. Extracted RNA samples (10 μ g per lane) were denatured, electrophoresed through an agarose gel, transferred to the membrane and hybridized using following probes: digoxigenin-labeled antisense RMCP II mRNA probe and digoxigenin-labeled antisense β -actin mRNA probe. The hybridized probe was detected by chemiluminescence.

Table 1. Histochemical quantification of inflammatory cells in rat trachea after challenge with ovalbumin.

Sensitization/challenge	Eosinophil		Mast cell			
	Wild type	Ws/Ws	Wild type CTMC	MMC	Ws/Ws CTMC	MMC
Unsensitization	4 $\pm$ 2	4 $\pm$ 1	155 $\pm$ 10	130 $\pm$ 17	ND	ND
Saline challenged	3 $\pm$ 1	1 $\pm$ 0	105 $\pm$ 27	126 $\pm$ 43	ND	ND
OV A challenged (30 min)	2 $\pm$ 0	2 $\pm$ 1	63 $\pm$ 11 *	115 $\pm$ 7	ND	ND
OV A challenged (8 h)	7 $\pm$ 2	1 $\pm$ 1	59 $\pm$ 16 *	198 $\pm$ 47 *	ND	ND
OV A challenged (24 h)	18 $\pm$ 3 *	4 $\pm$ 1	63 $\pm$ 7 *	108 $\pm$ 24	ND	ND

Data are shown as means $\pm$ S.E.M/section (n= 3–5 rats/group). ND, not detected.

* P < 0.05, compared with the control group challenged with saline.

CTMC: connective tissue mast cells; MMC: mucosal mast cells; OVA: ovalbumin.

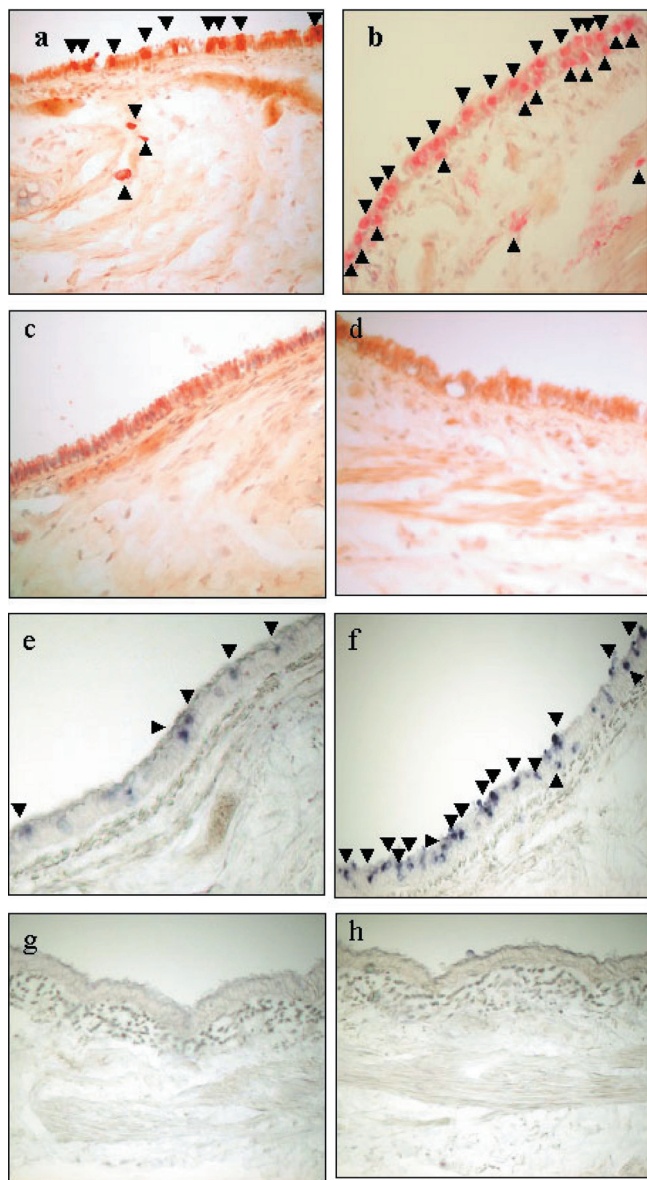


Fig. 4. Histological analysis of mast cells in asthmatic rat trachea. Light micrographs ($\times 100$) of serial sections of the membranous part of trachea for mast cell detection. Mast cells (arrow) were expressed in **a** (+/+, control), **b** (+/+, 8 h after ovalbumin challenge), **c** (*Ws/Ws*, control), **d** (*Ws/Ws*, 8 h after ovalbumin challenge) as detected by chloroacetate esterase activity, and **e** (+/+, control), **f** (+/+, 8 h after challenge), **g** (*Ws/Ws*, control), **h** (*Ws/Ws*, 8 h after challenge) by in situ hybridization. Rat trachea was harvested as quickly as possible at the indicated times after challenge by ovalbumin and fixed overnight in RNase free 4% paraformaldehyde. After cutting with a cryotome, the frozen sections were incubated with digoxigenin-labeled RMCP II mRNA probe.

Discussion

Mast cells are well positioned to respond to an antigenic stimulus by allergen because they are present on and within the airway surface. After antigen challenge of the airway, mast cells respond within minutes with release of preformed mediators and the response becomes maximum after 15–30 min. Some asthmatics develop the LAR that begins 3–6 h

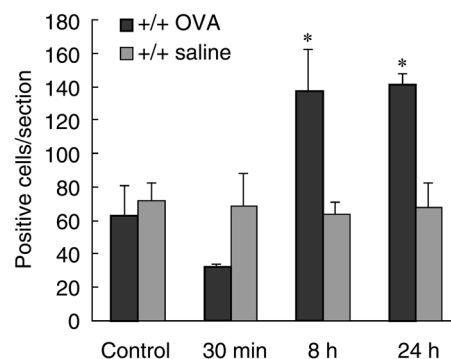


Fig. 5. Analysis by in situ hybridization of mucosal mast cells number in +/+ rats. The trachea samples were taken from +/+ rats challenged with ovalbumin (solid columns) and +/+ rats challenged with saline (gray columns) at 30 min, 8 h and 24 h after challenge by 2% ovalbumin inhalation. The mast cells on the frozen sections were labeled by digoxigenin-labeled RMCP II mRNA probe. The number of RMCP II positive mucosal mast cells was expressed as cell number/section. *, $P < 0.05$, compared to the group challenged with saline ($n = 3$ rats/group).

after allergen challenge. The EAR after allergen challenge has been thought to be mediated by mast cells, whereas eosinophils has been responsible for the LAR and airway remodeling due to their release of cys-leukotrienes. In this study, we demonstrate the role of mast cells in the asthmatic response, especially the LAR, based on several line of evidence in vivo using mast cell deficient rats.

The importance of the mast cell in evolution of the LAR is supported by several findings in our study. First, the *Ws/Ws* rats that were deficient in mast cells were significantly less responsive to ovalbumin challenge after sensitization than +/+ rats, as assessed by PenH recording. These data provide evidence that mast cells, either directly or indirectly, do participate in the LAR in rats. Our data are consistent with reports that mast cells play essential roles in asthmatic responses in mast cell deficient mice [15–17] and extend the concept to a mast cell contribution to the LAR in a mast cell deficient rat model.

Second, the significant increase was observed in the histamine in BALF in 30 min and 8 h after ovalbumin challenge in +/+ rats, along with the lack of histamine release in *Ws/Ws* rats. This increase suggests that mast cells are activated during both the EAR and the LAR, even though the EAR described by PenH value was unremarkable. Connective tissue mast cells were considered responsible to the EAR as the degranulation phenomenon was detected in histochemical staining. The combination of connective tissue mast cells and mucosal mast cells affected the histamine level in the LAR as both the degranulation of connective tissue mast cells and the increase in number of mucosal mast cells were found during this period. Histamine, along with other inflammatory mediators, may contribute to the LAR by increasing vasodilation and vascular permeability, mucus secretion and bronchoconstriction [18]. Subsequent increased expression of endothelial cell adhesion molecules, such as intracellular adhesion molecule-1, vascular cell adhesion molecule-1 and P-selectin, and the release of proinflammatory cytokines and chemokines, result in both intravascular trapping of leukocytes and their migration from blood into the inflamed tissue

site [19]. The cooperation of the numerous polypeptides, leukotrienes, cytokines and cytosolic enzymes of eosinophils culminates in the characteristic symptoms associated with the LAR. The cellular sources of released histamine in the airway response are not only mast cells but also basophils. However previous report indicated that the peak of basophils infiltration to BALF was in 24 h after antigen challenge [20]. The combination of the data demonstrating increased histamine levels in BALF and the histological observation of ves-tiges of mast cell degranulation provides the direct proof that mast cells are activated during the LAR.

How mast cells that are activated in the EAR can degranulate again in the LAR is an interesting question. The major basic protein, previously thought to be one of the representative basic proteins contained in eosinophils, stimulates antigen-activated mast cells to release histamine [21] and may also cause airway hypersensitivity and hyperreactivity of respiratory smooth muscle [21–23]. These were also reasons that eosinophils were thought to be important in the LAR. However, levels of major basic protein gene expression have been demonstrated in mast cells [24]. In our study, the number of mucosal mast cells, but not eosinophils, significantly increased within 8 h after allergen challenge. Although the relationship between the changes of the mast cell number in the LAR and major basic protein produced in mast cells is still unknown, this question will be a focus in our future studies.

Third, an increase in numbers of mucosal mast cells associated with RMCP-II in the LAR was observed in the epithelial layer of trachea in +/+ rats. This increase may be the result of either the migration and differentiation of immature mast cell-precursor cells or the proliferation of mucosal mast cells already localized at the epithelial layer. Cytokines, such as IL-4, IL-3, IL-5 and GM-CSF also play roles in promoting proliferation, differentiation and phenotypic switching of mast cells [6, 25]. While the source of those cells and the relationship between mucosal mast cells and the LAR are unclear, we hypothesize that the increased number of mucosal mast cells should promote the amplification of airway inflammation.

RMCP II, a kind of chymase, can cleave both basement membrane proteins and neuropeptides, as well as stimulate mucus secretion [19, 26]. As an activator of endothelin, a mitogenic molecule for airway smooth muscle cells and fibroblasts, and a stimulant for collagen synthesis in fibroblasts [27], the increase in expression of chymase in mucosal mast cells in the epithelial layer in the trachea will show us another aspect of the contribution of mast cells in the LAR and potentially effect on chronic airway remodeling.

At last, in addition to the direct activation, mast cells might also regulate the LAR and the following chronic inflammation by affecting the recruitment of eosinophils. Studies using mast cell deficient mice show that the recruitment of eosinophils during the LAR depends on mast cells [28]. The steps required for eosinophils trafficking from the peripheral circulation to the site of antigen challenge were closely related to mast cell activation [25]. Our histological results confirmed that the activation of mast cells induced the increase in the number of eosinophils in the trachea. The release of histamine and the decrease in respiratory function preceded the marked recruitment of eosinophils to the tra-

chea that was observed at 24 h after antigen challenge. In this study cys-leukotriens were not evaluated during the asthmatic response, but we could conclude that the role of eosinophils in the LAR was limited. During the LAR eosinophils may not be the major source of inflammatory mediators. This conclusion was also supported by the detection of cells and mediators in a computer model of chronic asthma [29].

We propose a sequence of events that results in the contribution of mast cells to the LAR. After the EAR, there is another release of histamine and other inflammatory mediators by mast cells. Subsequently, the increase in number of mucosal mast cells is accompanied by an increase in chymase gene expression. Re-activated mast cells also enhance the recruitment of eosinophils from peripheral blood to the airway. In cooperation with other inflammatory cells, the LAR that is characterized by perivascular edema in the airway, mucous plugging and activated Th2 cells, ensues.

It is of interest that the number of mucosal mast cells increased significantly as described by histochemical analysis. Prior to this study, the data from Giemsa and toluidine blue stained sections provided evidence for an increase in the number of mast cells in the dog trachea after allergen challenge [30] and in human bronchial mucosa after the LAR [31]. The activity of chymotrypsin-like serine protease could be detected not only in mucosal mast cells but also in globule leukocytes and basophils [32]. RMCP II is a typical major protease expressed in mucosal mast cells [33, 34] and using the digoxigenin-labeled antisense RMCP II mRNA probe as a specific marker for mucosal mast cells, the increase in the number of mucosal mast cells in the LAR was proven.

In addition, the degranulation of mast cells has been described as a decrease in cell number and/or a degranulation process [5, 35] using histochemical analysis. However the localization of mast cells in situ using histochemical techniques is not always accurate. The high specificity and sensitivity of in situ hybridization in the trachea tissue section simplified the investigation of gene expression of mucosal mast cells at the cellular level. The discrepancy between the number of mucosal mast cells described as histochemical analysis and as in situ hybridization at 24 h after challenge (shown in Table 1 and Fig. 5) can be explained by differences in sensitivities between a method dependent upon staining of granule enzymes and a method which detects cytosolic mRNA signals. Compared to other types of histological analysis, the in situ hybridization method is a powerful technique for detection of mast cells in pathological samples.

There were some points in our experiment that were difficult to interpret. The EAR with only small increments of PenH was observed in +/+ rats, as has been observed in other strains of Sprague-Dawley and the Flinders resistant line rats [36]. Eosinophil numbers were also less than those reported by other groups [15, 36, 37]. It may be because that the different sensitization way and the route of challenge cause the different severity of the asthmatic response [15, 17]. We have already reported that in vitro the contraction of trachea smooth muscle from the +/+ rats occurred in response to 5-hydroxytryptamine, but not histamine released from mast cells after allergen challenge. The poor EAR may be due to the absence of histamine H₁ receptors on the smooth muscle of the rat airway [11].

The reconstitution of tissue mast cells in *Ws/Ws* rats with in vitro derived mast cells of congenic *+/+* rats, as has been done in *W/W^v* mice with successful engraftment of bone marrow derived mast cells [38] would be helpful for understanding the role of mast cell function in the LAR. We could not reconstitute mast cells in *Ws/Ws* rats because *Ws/Ws* and *+/+* rats are not inbred strains.

In conclusion, the comparison of the late-phase of classical IgE-dependent airway response in mast cell-deficient and wild type rats has demonstrated an important role of mast cells that is initiated by release of immunomediators in advance activation. Through synthesis and secretion of proinflammatory cytokines, mast cells regulate the development of eosinophils in inflammatory response in the LAR. The mucosal mast cells increase in number and also produce several neutral proteases, such as chymase, which may potentially damage and activate the airway epithelium, thereby contributing to the LAR and remodeling of airway wall. Reevaluation of the role of mast cells in the LAR should promote our better understanding of the pathogenetic sequence and discovery of new therapeutic targets.

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