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A Novel Rat Model to Determine Interaction between Reflux Esophagitis and Bronchial Asthma

Short title: GERD and Asthma

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Abbreviations used in this study: GERD, gastro-esophageal reflux disease; RE, reflux esophagitis; BA, bronchial asthma; BALF, bronchoalveolar lavage fluid; OVA, ovalbumin; PBS, phosphate-buffered saline

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Abstract

Background: Several studies have shown a strong association between reflux esophagitis (RE) and bronchial asthma (BA). The precise mechanisms of interaction between RE and BA are uncertain, possibly due to lack of animal models.

Aims: We established a novel rat model and examined pathogenic interaction of RE and BA.

Methods: RE and BA were induced in Brown-Norway rats by ligating the transitional region between the forestomach and the glandular portion and wrapping the duodenum near the pylorus, and by ovalbumin (OVA) sensitization and challenge with OVA aerosol. Rats were divided into four groups: control, RE, BA, and RE+BA. OVA-induced airway inflammation was assessed by number of infiltrating cells and cytokine levels in bronchoalveolar lavage fluid (BALF). Esophageal lesion index, histology, and cytokine mRNA expression as determined by real-time RT-PCR were also examined.

Results: Significant increases in number of cells, especially eosinophils, and IL-13 but not IFN- γ concentration in BALF were observed in the RE+BA group compared with the BA group. These enhancements of OVA-induced airway inflammation were prevented by treatment with rabeprazole. Although the esophagitis lesion index in the RE+BA group did not differ from that in the RE group, eosinophilic infiltration in the esophageal submucosa and levels of mRNA expression of cytokines such as IL-5, IL-10, IL-13, and RANTES were significantly increased.

Conclusion: We established a novel rat model of RE and BA, and found significant interactions of the two diseases. This model thus appears to be useful for examining the association between gastroesophageal reflux disease and bronchial asthma.

Introduction

Gastroesophageal reflux disease (GERD) is characterized by specific symptoms such as heartburn and acid regurgitation [1]. Epidemiological studies have shown that the prevalence of GERD ranges from 10% to 48% in Western countries [2] and 6.6% to 9.8 % in Japan [3, 4]. In the Montreal definition and classification recently published as a global evidence-based consensus [5], GERD is defined as a condition that develops when reflux of gastric content causes troublesome symptoms and/or complications. It was subclassified into esophageal and extra-esophageal syndromes. Among a variety of extra-esophageal manifestations, causal relationships between GERD and cough, laryngitis, asthma, and dental erosion were strongly suggested.

The prevalence of GERD among patients with asthma is high, and estimated to be between 33% and 89% [6], whereas asthma symptoms reportedly occur in 17–60% of patients with GERD [7, 8]. Using ambulatory esophageal pH monitoring studies, patients with asthma have been found to more frequently experience abnormal acid reflux than healthy controls. A large case-control study demonstrated that reflux esophagitis (RE) is significantly associated with asthma, with an odds ratio of 1.51 [9]. These clinical findings are evidence of a strong association between GERD and asthma.

Several studies have suggested possible mechanisms of interaction between GERD and bronchial asthma. Esophageal acid-induced bronchoconstriction can be provoked by vagal reflexes, microaspiration, heightened bronchial activity, and neurogenic inflammation, while autonomic dysregulation, an increased pressure gradient between the thorax and the abdominal cavity, high prevalence of hiatal hernia, altered crural diaphragm function, and use of bronchodilators are factors potentially predisposing to development of GERD in patients with asthma [10]. However, these mechanisms cannot fully explain the interactions between GERD and asthma. This might be due to the lack of animal models to examine in detail the associations between GERD and asthma. The aim of the present study was therefore to establish a novel rat model of GERD and asthma, and to examine the pathogenic interactions between these two diseases.

Materials and Methods

Animals

Eight-week-old male Brown Norway rats weighing approximately 200 g were purchased from Charles River Japan (Yokohama, Japan). They were housed at the animal research facility of Osaka City University Graduate School of Medicine in an air-filtered and specific pathogen-free environment during experiments. Rats were divided into four groups as follows: a control group (sham-operated, non-sensitized), RE group (subjected to operation and non-sensitized), BA group (sham-operated, sensitized), RE+BA group (subjected to operation and sensitized). Figure 1 shows the experimental protocol. Rats were killed 48 hr after exposure to 1% ovalbumin (OVA) aerosol. Experimental procedures were approved by the Animal Care Committee of Osaka City University Graduate School of Medicine.

Induction of esophagitis

Chronic acid reflux esophagitis was induced by the methods of Omura et al [11]. In brief, the duodenum near the pylorus was covered with a piece of 18Fr N  laton catheter (width, 2 mm; Sapheed  , Terumo Co., Tokyo, Japan) as a ring, and the transitional region between the forestomach and the glandular portion was ligated with 2-0 silk thread to enhance reflux of gastric acid into the esophagus (Figure 2). Solid food was withdrawn for two days after induction of esophagitis, but rats were allowed drinking water. The lesion index was determined as the sum of the product of length and width of erosions per esophagus. Tissues with esophagitis were excised and immediately frozen in liquid nitrogen and stored at -80   C until measurement of mRNA expression. For histological study, samples were gently rinsed with saline and fixed in 10% buffered formalin. Samples were embedded in paraffin and 4   m-thick sections were cut. Hematoxylin and eosin staining was performed for standard morphological analysis. To identify macrophages, sections were stained with mouse anti-ED1 antibody (Serotec Ltd., Oxford, UK). Immunohistochemical staining was performed with a streptavidin-biotin peroxidase method (LSAB2 kit, Dako Japan Co.). Numbers of eosinophils and ED-1-positive cells infiltrating six randomly selected high-power fields were counted on coded slides.

Induction of bronchial asthma (sensitization and challenge)

According to well established method previously reported [12,13], rats were sensitized by intraperitoneal injection of 1 mg OVA (Grade V; Sigma Chemical Co., St Louis, MO, USA) and 200 mg Al(OH)₃ (Sigma) in 1 ml saline. Fourteen days after sensitization, rats were placed in a Plexiglas chamber and challenged with an aerosol of 1% OVA in saline. The aerosol was delivered by an ultrasonic nebulizer (NE-U17, Omron Healthcare, Kyoto, Japan). For rats with the combination of RE and BA, sensitization by OVA was performed 7 days after induction of esophagitis.

Bronchoalveolar lavage (BAL)

Rats were anesthetized by intraperitoneal injection of pentobarbital and killed by aortic exsanguination. A cannula was inserted into the trachea and the lungs were lavaged with 5 ml phosphate-buffered saline (PBS). The fluid was retrieved by gentle aspiration, and this procedure was repeated 3 times. Recovery of fluid was over 90% in all animals. The lavage fluid was centrifuged at 500g for 10 min, and the cell pellet was resuspended in 1 ml PBS. Total cell count was determined using an automated cell counter (MEK6258, Nihon Kohden Co, Shinjuku, Tokyo, Japan). To determine differential cell population in BAL fluid (BALF), the cell suspension was poured into the chamber of a Cytospin centrifuge (Shandon Southern Instruments Inc., Sewckley, PA, USA) and centrifuged at 1000 rpm for 8 min. The resulting slides were stained with May-Giemsa. Numbers of cells identified as neutrophils, eosinophils, lymphocytes, and macrophages by standard morphological criteria were counted in four randomly selected high-powered fields on

coded slides.

Measurement of cytokines in BALF

The concentrations of IL-13 and IFN- γ in BALF were measured by commercially available rat-specific IL-13 (BioSource, Camarillo, CA, USA) and IFN- γ (R&D, Minneapolis, MN, USA) ELISA kits with limits of detection of < 1.5 pg/ml for IL-13 and < 10 pg/ml for IFN- γ according to the manufacturers' instructions. A microplate reader (MTP-500, CORONA electric, Ibaraki, Japan) was used to measure absorbance at 450 nm. Background absorbance of blank wells was subtracted from the standards and unknowns before determination of sample concentrations.

RNA isolation and real time RT-PCR

Total RNA was extracted and purified from frozen tissues of the entire esophagus using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The PCR primers and TaqMan probes for IL-1 β , IL-5, IL-10, IL-13, IFN- γ , RANTES, and eotaxin were designed using Primer Express, a software program (PE Applied Biosystems, Inc., Foster City, CA, USA). TaqMan probes were labeled with a reporter fluorescent dye, FAM (6-carboxyfluorescein), at the 5' end and a fluorescent dye quencher, TAMRA (6-carboxy-tetramethyl-rhodamine), at the 3' end. The PCR primers and TaqMan probes used in this study are shown in Table 1. Real-time quantitative RT-PCR analyses were performed using an ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Inc.). The reaction mixture was prepared according to the manufacturer's protocol using the PLATINUM quantitative RT-PCR THERMOSCRIPT One-Step System (Invitrogen, Carlsbad, CA, USA). The thermal cycling conditions included 50 °C for 30 min and 95 °C for 5 min, followed by 45 cycles of amplification at 95 °C for 15 sec and 60 °C for one min. Total RNA was subjected to real-time quantitative RT-PCR for measurement of target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard using TaqMan GAPDH control reagents (PE Applied Biosystems, Inc.). Each resulting gene amount was divided by the amount of GAPDH to obtain a normalized value. The mRNA levels are expressed as ratios to the mean value for normal esophageal tissue.

Effect of proton-pump inhibitor on OVA-induced airway inflammation

To determine the role of acid in worsening of OVA-induced airway inflammation, rats were intraperitoneally administered 30 mg/kg of sodium rabeprazole (Eisai Co., Ltd. Tokyo, Japan) once daily from three days before OVA challenge. Rabeprazole was dissolved in saline and injected in a volume of 5 ml/kg. BALF was collected 48 hr after OVA inhalation. Total and differential cell count and cytokine concentrations in BALF were measured.

Statistical analysis

Statistical analysis was performed with Statview software version 5.0 J (SAS Institute Japan, Tokyo, Japan). Differences between groups were examined for significance by one-way ANOVA followed by the Fisher PLSD test. p-values less than 0.05 were considered significant. All values are presented as means and SEM.

Results

Inflammatory cells in BALF

BALF was collected and analyzed 48hr after exposure to 1% OVA aerosol. The majority of cells identified in BALF were macrophages with smaller populations of eosinophils, neutrophils, and lymphocytes in both the control group and RE group, suggesting that acid reflux alone did not cause airway inflammation. There was a significant increase in number of total cells in BALF in the BA group compared with the control group ($p < 0.01$). Differential counts of cells in BALF showed that eosinophils, neutrophils, and lymphocytes but not macrophages in BALF were significantly increased in the BA group compared with the control group, with a particularly remarkable increase in eosinophils. There were significant

further increases in numbers of total cells and eosinophils in BALF in the RE+BA group compared with the BA group ($p<0.05$), while there were no differences in numbers of macrophages, neutrophils, or lymphocytes in BALF between these two groups (Figure 3).

Cytokine levels in BALF

IL-13 and IFN- γ concentrations in BALF was measured 48 hr after exposure to 1% OVA aerosol. There was no significant difference in IL-13 concentration in BALF between the control and RE groups. IL-13 concentration in BALF of rats with BA was significantly increased compared with that in the control group ($p<0.01$), and there was a significant further increase in IL-13 concentration in BALF of rats in the RE+BA group compared with the BA group ($p<0.01$) (Figure 4A). IFN- γ level in BALF was low, and there were no significant differences in IFN- γ level in BALF among the four groups (Figure 4B).

Macroscopic and histological findings of esophagitis

Figure 5 A-D shows the macroscopic appearance of the esophagus. There were no obvious esophageal lesions in the BA group (Figure 5B), and esophageal lesions were observed in the lower and middle parts of the esophagus in rats with RE (Figure 5C) or with RE+BA (Figure 5D). However, there was no difference in lesion index (7.62 ± 2.0 in RE group versus 7.84 ± 2.9 in RE+BA group, $p=0.951$) between the RE and RE+BA groups. Histologically, normal esophagus exhibited a thin epithelial layer with squamous cells, and few inflammatory cells were found in the submucosal layer (Figure 5E). No microscopic changes were observed in the BA group (Figure 5D). Marked mucosal thickening with elongation of the lamina propria papillae, basal cell hyperplasia, and inflammatory cell infiltration was found in both the RE group (Figure 5G) and RE+BA group (Figure 5H). Eosinophilic infiltration in the submucosa was predominant in the RE+BA group (Figure 5H,I) compared with the RE group (Figure 5G), while intraepithelial infiltration of eosinophils was rarely observed in rats only in the RE+BA group (Figure 5I). There were a few ED-1-positive cells in the esophageal mucosa in both the control (Figure 5J) and BA groups (Figure 5K), while several ED-1-positive cells was observed in the RE group (Figure 5L) and RE+BA group (Figure 5M). To quantify the severity of eosinophilic and macrophage infiltration, numbers of eosinophils and macrophages were counted. Number of infiltrating eosinophils was significantly increased in rats with RE ($p<0.05$), and a further significant increase in number of infiltrating eosinophils was found in rats with RE+BA ($p<0.01$) (Figure 6A). Although number of ED-1-positive cells was increased in rats with RE and RE+BA ($p<0.01$), there was no significant difference between the two groups (Figure 6B).

Determination of expression of cytokine mRNA by real-time RT-PCR

To determine the mechanisms of increase in eosinophil infiltration of the esophagus in rats with RE+BA, levels of mRNA expression of cytokines and chemokines such as IL-1 β , IFN- γ , IL-5, IL-10, IL-13, RANTES, and eotaxin in esophageal tissue were measured (Figure 7). IFN γ mRNA level did not vary among the four groups. IL-1 β and eotaxin mRNA expressions were significantly increased in rats with RE and RE+BA ($p<0.05$), but without difference between these two groups. There was no differences in IL-5, IL-10, or RANTES mRNA expression in rats with RE or BA compared with control, while significant increase in expression of these mRNAs was observed in rats with RE+BA compared with the control ($p<0.01$). IL-13 mRNA level was significantly increased in rats with RE and those with BA ($p<0.05$), and a further significant increase was found in rats with RE+BA ($p<0.05$).

Effects of PPI on OVA-induced airway inflammation in rats with BA and RE

To determine role of acid in worsening of OVA-induced airway inflammation in rats with RE+BA, rats were treated with rabeprazole from three days before OVA inhalation. Rabeprazole treatment did not affect the lesion index of RE in either rats with RE alone or those with RE+BA. Rabeprazole treatment did not affect either total cell number, cell

distribution, or IL-13 level in BALF of rats in the control group and RE group (data not shown). Although rabeprazole did not affect OVA-induced airway inflammation in rats with BA alone, it significantly reduced the acid-induced increase in total cell and eosinophil counts as well as IL-13 concentration in BALF of rats with RE+BA compared with the vehicle-treated group (Figure 8).

Discussion

The present study demonstrated that the combination of RE and BA enhanced OVA-induced airway inflammation, including increase in number of total cells, especially eosinophils, and IL-13 level in BALF compared with BA alone, and that it induced increase in eosinophil infiltration and cytokines, especially levels of Th2 cytokine mRNA expression, in esophageal tissue compared with RE alone. These findings are the first evidence of interaction between RE and BA obtained using a novel animal model.

Bronchial asthma is a complex chronic inflammatory disease of the airways characterized by reversible airway obstruction, inflammatory mediator production, and airway hyper-responsiveness, and is a Th2 disease with a specific cytokine profile including IL-4, IL-5, and IL-13. In this study, we used OVA-induced allergic inflammation of Brown Norway rats, which are genetically Th2-predisposed [12]; this model is well established and mimics human bronchial asthma [13]. Number of total cells, and especially that of eosinophils, and IL-13 but not IFN- γ level in BALF of rats with BA were significantly increased compared with control, suggesting that OVA-induced airway inflammation is Th2-predominant. Since the combination of RE and BA enhanced OVA-induced airway inflammation compared with BA alone, and since these enhancements of inflammatory response were completely prevented by treatment with a proton-pump inhibitor, acid plays a major role in the mechanisms of worsening of OVA-induced airway inflammation in rats with RE and BA.

The precise mechanisms by which esophageal acid reflux enhanced OVA-induced airway inflammation in this study are unknown. Two major mechanisms, vagal reflexes and microaspiration, have been proposed to explain how GER might cause asthma. These proposals are based on findings of animal and human studies: 1) Esophageal acid infusion increased respiratory resistance, which was prevented by bilateral vagotomy [14]. 2) Esophageal acid infusion reduced bronchial airflow, which was inhibited by treatment with atropine [15]. 3) Tracheal acid infusion increased total lung resistance [8]. 4) Direct evidence of microaspiration using ^{99m}Tc colloid scintigraphy [16] or pH monitoring [17]. If acid reflux alone affects respiratory tissue, significant change in airway inflammation should be observed in rats with RE alone through vagal reflexes or microaspiration. Since acid-induced airway inflammation was enhanced only in the presence of BA, vagal reflexes and microaspiration alone cannot explain the interaction between RE and BA in the present model. These findings suggest that acid reflux-induced worsening of OVA-induced airway inflammation is mediated through heightened bronchial reactivity. The finding by Herve et al [18] that heightened bronchial reactivity is associated with vagal nerve activity suggests that interaction between RE and BA might involve complex pathways. Further studies are needed.

Our previous study [19] showed that increases in various cytokines and chemokine expression play a significant role in the pathogenesis of rat RE, the same model as used in the present study, and also mimic the histology of human GERD [20]. These cytokines were mainly of Th1 type [19], and clinical studies have demonstrated that the squamous epithelium of the esophagus in patients with RE exhibited a Th1 pattern while the specialized intestinal metaplasia of Barrett's esophagus exhibited Th2 inflammatory responses [21]. In the present study, the combination of RE and BA increased esophageal eosinophil infiltration and levels of mRNA expression of Th2 cytokines such as IL-5, IL-10, and IL-13, while it did not affect expression of mRNA of Th1 cytokines, including IL-1 β and IFN- γ . These findings might be due to the species of rats used, since Brown Norway rats are known to be Th2-predisposed [12]. The present study demonstrated that the combination of RE and BA did not affect macroscopic lesions of esophagitis. This finding

might be related to the short duration between OVA challenge and sacrifice, and to the severity of BA, because we used mild BA model (only one OVA challenge) to observe worsening airway inflammation by combination of RE and BA. In the present study, rabeprazole did not affect erosive esophagitis since rats were treated with rabeprazole from three days before OVA inhalation to determine effects of PPI on OVA-induced airway inflammation in rats with RE+BA. In our previous study [19], rat reflux esophagitis was almost completely inhibited when rats were treated with rabeprazole from the day of induction of esophagitis. Timing and short duration of rabeprazole administration might be responsible for the absence of effect on severity of esophagitis in this study.

Several histological findings, such as mucosal thickening with elongation of papillae and basal hyperplasia and inflammatory cell infiltration, have been proposed as characteristics of GERD [22]. In addition to these histological characteristics, it is known that mild eosinophilic infiltration occurs in esophageal mucosa in patients with GERD [23], though the role of eosinophils and mechanisms of eosinophil infiltration in esophageal mucosa are quite unclear. Since the level of eotaxin mRNA expression was significantly increased in rats with RE alone compared with control and there was no significant difference in eotaxin mRNA level between rats with RE and those with RE+BA, eotaxin might play a role in eosinophilic infiltration in RE. However, levels of IL-5, IL-10, IL-13, and RANTES mRNA expression were significantly further increased in rats with RE + BA compared with RE, suggesting that these cytokines are related to enhancement of esophageal eosinophilia in rats with RE+BA. Since most of the cytokines with increase in level were of Th2 type, the elevation in cytokine expression might have been due to allergic response to OVA inhalation. The precise mechanisms of increase in esophageal IL-13 mRNA level despite the lack of esophageal eosinophil infiltration in BA group are unknown. Bronchial allergen or bronchial inflammation might affect esophageal tissue in BA group. Increase in esophageal IL-13 mRNA level with normal bronchial IL-13 level in RE group suggest that acid reflux alone affect esophageal tissue only, and might be related to the esophageal eosinophilic infiltration which is commonly found in reflux esophagitis.

Eosinophilic esophagitis is an allergic disease characterized by esophageal symptoms such as dysphagia and food impaction, as well as dense esophageal eosinophilia [24]. Clinical evidence suggest that eosinophilic esophagitis is an immune-mediated response to swallowed antigens. The precise pathogenic mechanisms of eosinophilic esophagitis are uncertain. Microarray analysis using human biopsy specimens of the esophagus demonstrated that expression of the gene encoding eotaxin-3 was significantly increased [25]. Mishra et al. [26] developed a murine model of eosinophilic esophagitis using the aeroallergen *Aspergillus fumigatus*, and found with use of IL-5 and eotaxin null mice that esophageal eosinophilia was IL-5-dependent and partially eotaxin-dependent. Another study of a murine model showed that direct delivery of IL-13 into the pulmonary tree caused esophageal eosinophilia, and that this response was blocked by IL-13 antibody [27]. Other studies proposed Th2 cytokines or other cytokines, chemokines, or arachidonic acid metabolites contained in eosinophils as candidates for involvement in the pathogenesis of eosinophilic esophagitis. There are several similarities between eosinophilic esophagitis and the present RE+BA model. Spechler et al. [28] reviewed the possibility of association between GERD and eosinophilic esophagitis, and showed that eosinophilic esophagitis might contribute to GERD via an eosinophil secretory product with cytotoxic and dysmotility-inducing effects or via a structural change, and that GERD might contribute to eosinophilic esophagitis since GERD-induced epithelial damage could expose the deep layers to antigens. Taken together, these findings suggest that the present model will be useful in clarifying the triangle of GERD, asthma, and eosinophilic esophagitis.

In conclusion, we established a novel rat model of reflux esophagitis and bronchial asthma, and found significant interactions of the two diseases. This animal model might be useful for clarifying the pathogenic association between GERD and bronchial asthma.

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Competing Interest: None declared.

Figure legends

Figure 1. Experimental protocol

OVA or saline was injected intraperitoneally 7 days after induction of esophagitis or sham operation. Two weeks later, rats were challenged with OVA aerosol. Rats were killed 48 hrs after OVA inhalation.

Figure 2. Induction of esophagitis

Chronic acid reflux esophagitis was induced by the methods of Omura et al. The duodenum near the pylorus was covered with a piece of 18Fr Nélaton catheter as a ring, and the transitional region between the forestomach and the glandular portion was ligated with 2-0 silk thread to enhance reflux of gastric acid into the esophagus.

Figure 3. Inflammatory cells in BALF

There were significant increases in numbers of total cells, eosinophils, neutrophils, and lymphocytes but not macrophages in BALF in the BA group compared with the control group. The increase in eosinophils was particularly remarkable. There were significant further increases in numbers of total cells and eosinophils in BALF in the RE+BA group compared with the BA group. * $p < 0.05$ vs control. ** $p < 0.01$ vs control. *** $p < 0.05$. Values are the mean and SEM of 5-10 experiments each.

Figure 4. Cytokine levels in BALF

A: IL-13 concentration in BALF of rats with BA was significantly increased compared with that in the control group, and there was a significant further increase in rats with RE+BA.

B: There were no significant differences in IFN- γ level in BALF among the four groups. * $p < 0.05$ vs control. ** $p < 0.01$. Values are the mean and SEM of 4-5 experiments each.

Figure 5. Macroscopic appearance and histological findings for the esophagus

A-D: There were no obvious esophageal lesions in the control (A) or BA group (B), while esophageal lesions were observed in the lower and middle parts of the esophagus in rats with RE (C) or RE+BA (D).

E: Normal esophagus exhibited a thin epithelial layer with squamous cells, and few inflammatory cells were found. **F:** There were no microscopic lesions in the BA group. **G:**

Marked mucosal thickening with elongation of the lamina propria papillae, basal cell hyperplasia, and inflammatory cell infiltration including eosinophils was found in the RE group. **H, I:** Similar histological findings were observed, but eosinophil infiltration was more predominant in the RE+BA group than the RE group and intraepithelial infiltration of eosinophils (arrow) was rarely observed. **J:** Few ED-1-positive cells were found in normal

esophagus. **K:** There were a few ED-1-positive cells in esophageal mucosa in the BA group. **L:** Several cells were ED-1-positive in the RE group. **M:** Several cells were ED-1-positive in the RE+BA group. Original magnifications G,H: $\times 200$, E,F,I-M: $\times 400$.

Figure 6. Numbers of eosinophils and macrophages in esophagus

A: Number of infiltrating eosinophils was significantly increased in rats with RE, and a further significant increase in infiltrating eosinophils was found in rats with RE+BA. **B:** Although number of ED-1-positive cells was increased in rats with RE and RE+BA, there was no significant difference between these two groups. * $p < 0.05$ vs control. ** $p < 0.05$. Values are the mean and SEM of 5 experiments each.

Figure 7. Determination of expression of cytokine mRNA by real-time RT-PCR

Levels of IL-1 β , IFN- γ , IL-5, IL-10, IL-13, RANTES, and eotaxin mRNA expression in esophageal tissue were measured. # $p < 0.05$ vs control. ## $p < 0.01$ vs control. * $p < 0.05$. ** $p < 0.01$. Values are the mean and SEM of 8-11 experiments each.

Figure 8. Effect of PPI on OVA-induced airway inflammation in rats with BA and RE

A: Total cell count in BALF. **B:** Eosinophil count in BALF. **C:** IL-13 concentration in BALF. Rabeprazole treatment significantly reduced the acid-induced increase in total cell and eosinophil counts and IL-13 concentration in BALF of rats with RE+BA compared with the vehicle-treated group. * $p < 0.01$. □ vehicle-treated rats, ■ rabeprazole-treated rats. Values are the mean and SEM of 8 experiments each.

Table 1. PCR primers and TaqMan probes

Gene	Primer and Probe	
IL-1 β	Primer (forward)	5'-CACCTCTCAAGCAGAGCACAG-3'
	Primer (reverse)	5'-GGGTTCATGGTGAAGTCAAC-3'
	Probe	5'-FAM-TGTCCCGACCATTGCTGTTTCCTAGG-TAMRA-3'
IL-5	Primer (forward)	5'-ACGGACAAAAGAGAAGTGTGG-3'
	Primer (reverse)	5'-AGGAACTCTTGCAAGTAATCCAG-3'
	Probe	5'-FAM-AAGAGAGGCGGAAGACCAGGCATTT-TAMRA-3'
IL-10	Primer (forward)	5'-CCAGGTTGCTCCTTCCATGAT-3'
	Primer (reverse)	5'-AGTGGTTGCAGAGTGATGGCA-3'
	Probe	5'-FAM-ATCCTTGCAACAGCTCAGCGCATCTC-TAMRA-3'
IL-13	Primer (forward)	5'-CCTGGAATCCCTGACCAACAT-3'
	Primer (reverse)	5'-CAGAGGCCATTCAATATCCTCTG-3'
	Probe	5'-FAM-TCCAGTTGCAATGCCATCCACAGGAC-TAMRA-3'
IFN- γ	Primer (forward)	5'-CAGCTGTCAACGAACTAGCC-3'
	Primer (reverse)	5'-ATTGGCACACTCTCTACCCAG-3'
	Probe	5'-FAM-AAGCGGAAAAGGAGTCGGTGCTGAT-TAMRA-3'
RANTES	Primer (forward)	5'-CCTTGCACTCGTCTTTGCACT-3'
	Primer (reverse)	5'-CTTGAACCCACTTCTTCTCTGG-3'
	Probe	5'-FAM-AAGGAACCGCCAAGTGTGTGCCAA-TAMRA-3'
eotaxin	Primer (forward)	5'-AGCCATAGTCTTCAAGACCAAGC-3'
	Primer (reverse)	5'-GCATCCTGGACCCACTTTTC-3'
	Probe	5'-FAM-TGGCAAAGAGATCTGTGCTGACCCCA-TAMRA-3'

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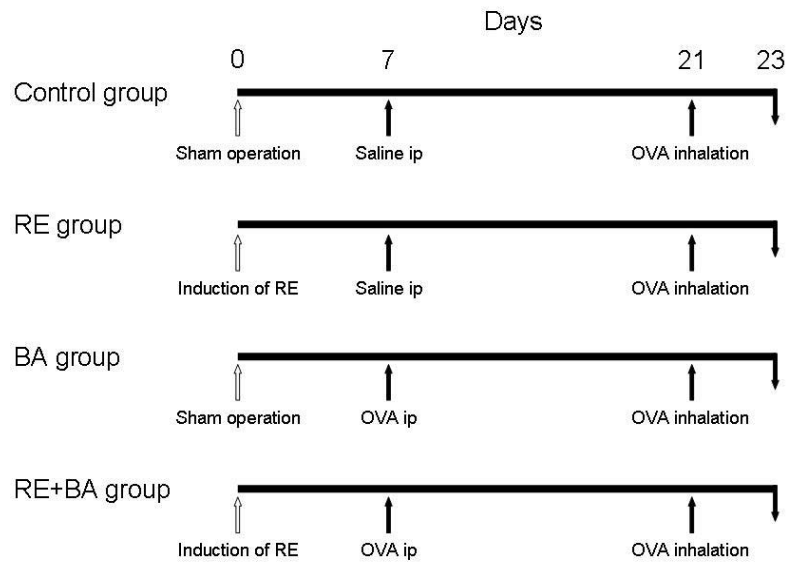


Figure 1

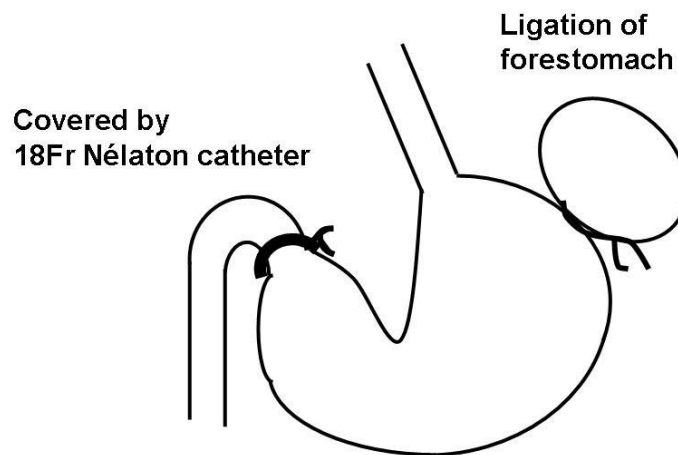


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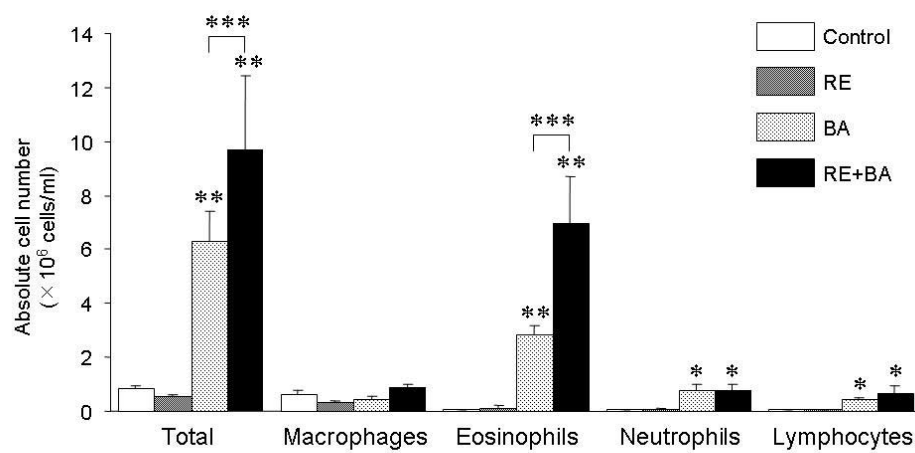


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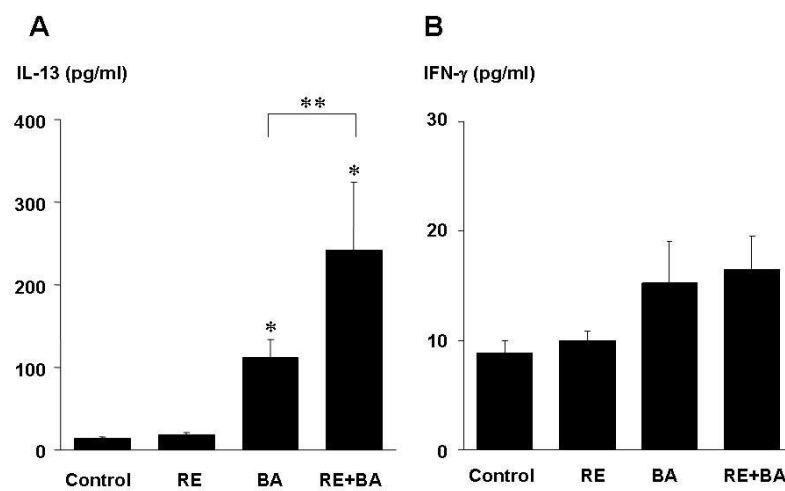


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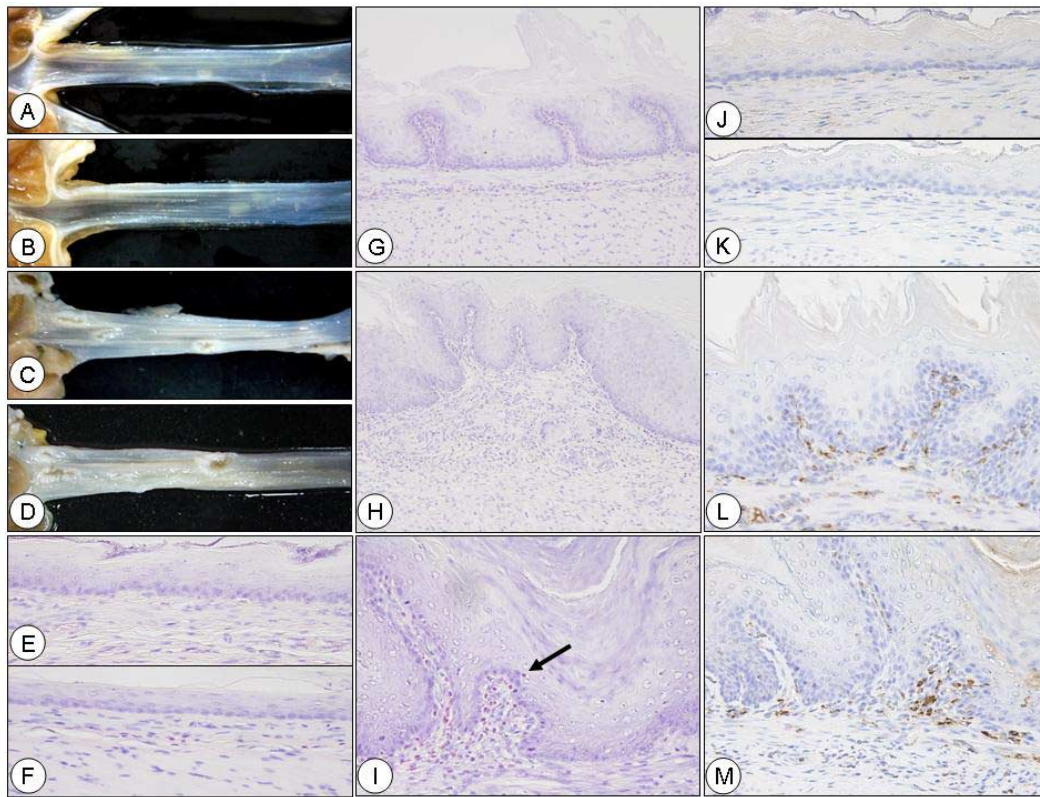


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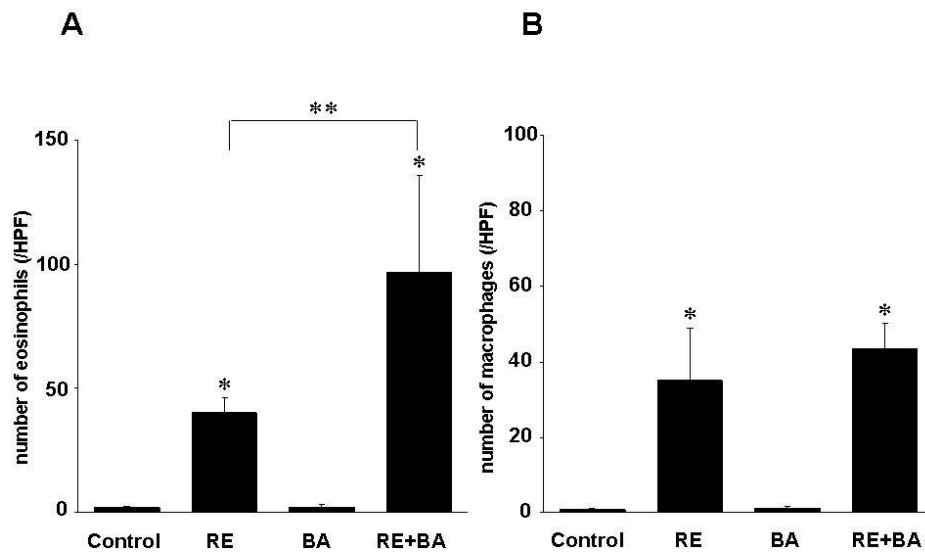


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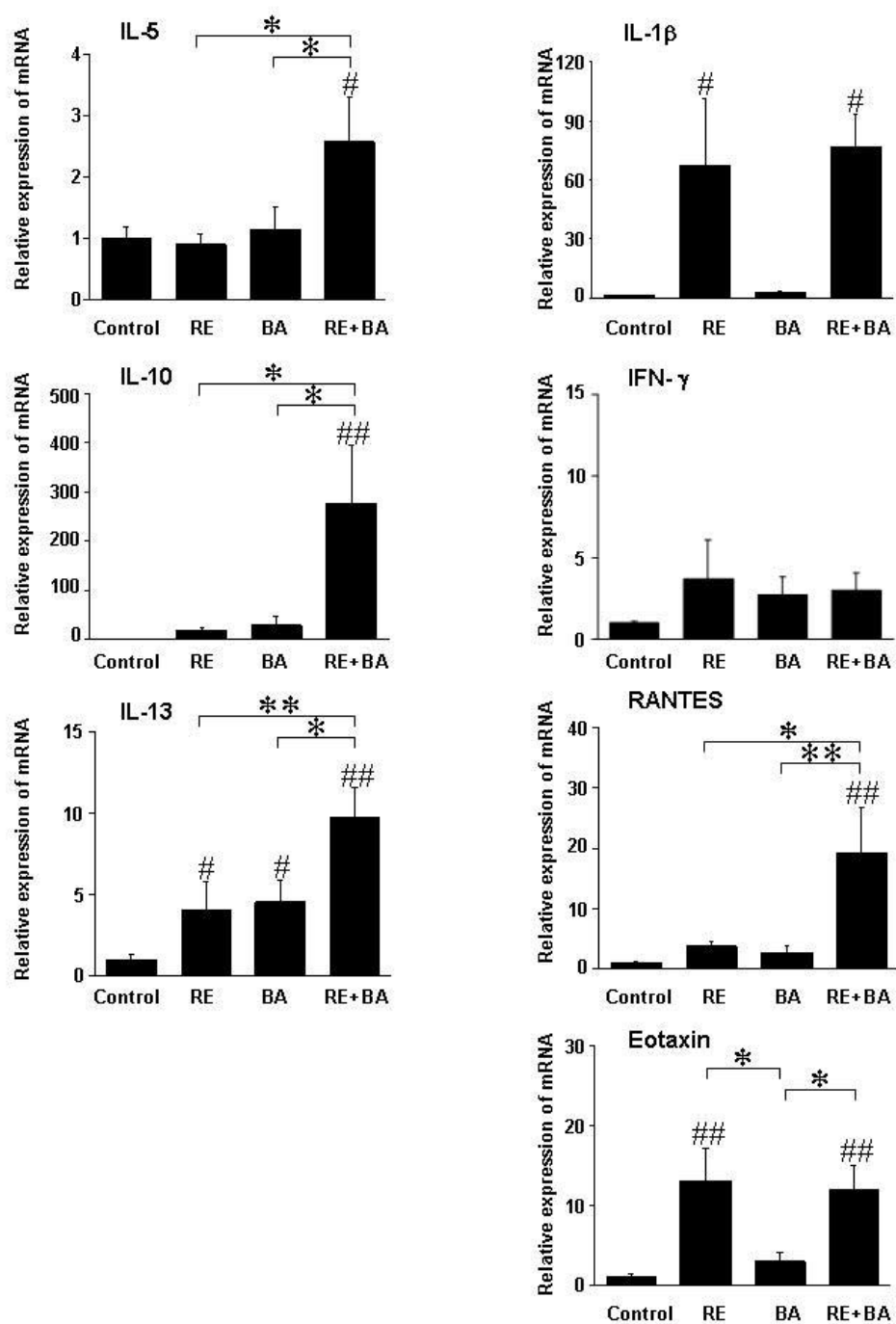


Figure 7

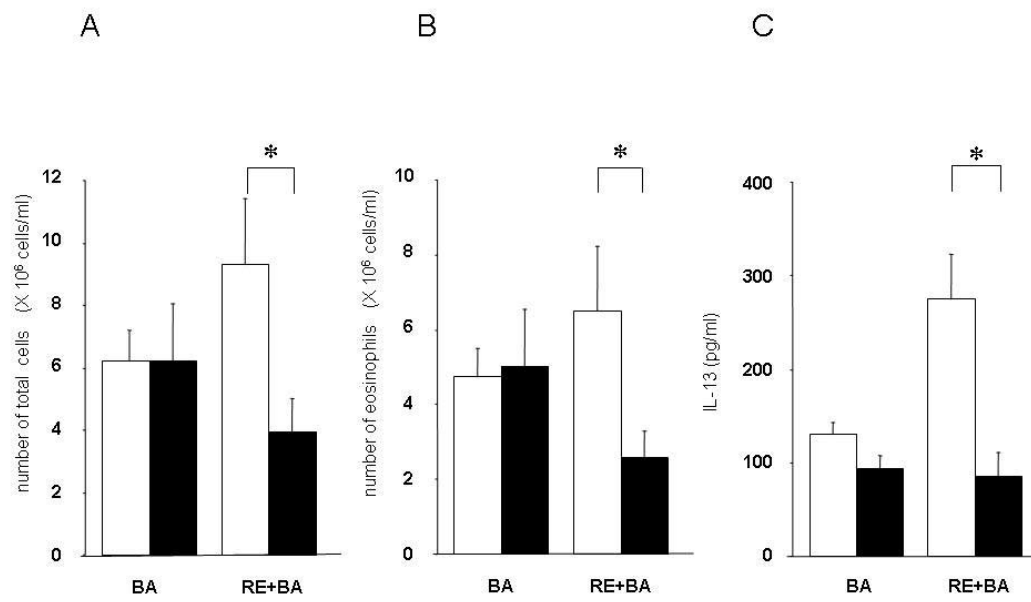


Figure 8