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Changes of T-helper type 1/2 cell balance by anticholinergic treatment in allergic mice

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ARTICLE INFO

Article history:

Received for publication August 13, 2013.

Received in revised form November 16, 2013.

Accepted for publication December 8, 2013.

ABSTRACT

Background: Anticholinergic drugs or vidian neurectomy can alleviate the symptoms of allergic rhinitis.

Objective: To show that inhibition of the cholinergic nerve influences the balance of T-helper type 1 and 2 cells in allergic rhinitis mice.

Methods: Twenty-four mice were randomly allocated to 1 of 4 groups: control, model, model with ipratropium bromide treatment, and model with 6-hydroxydopamine treatment. Allergic model-treated mice were sensitized with ovalbumin. Evaluation of allergic symptoms was recorded according to a symptom score. Ovalbumin serum IgE was measured by enzyme-linked immunosorbent assay. Expression of interleukin-4, interferon- γ , forkhead box P3, substance P, and vasoactive intestinal peptides was detected by immunohistochemistry and imaging analysis.

Results: Symptoms in allergic mice were significantly alleviated by ipratropium bromide. Ovalbumin serum IgE and eosinophils of nasal mucosa were significantly decreased. Interleukin-4 expression level was significantly higher in the allergic model group than in the control group and significantly decreased by ipratropium bromide ($P < .05$). In contrast, the expression of forkhead box P3 was lower in the allergic model group than in the control group and increased with treatment by ipratropium bromide ($P < .05$). Conversely, interferon- γ expression was not changed by anticholinergic treatment in the nasal mucosa of allergic mice. Expression of substance P and vasoactive intestinal peptide was significantly increased in allergic mice and decreased by ipratropium bromide. Sympathetic denervation did not change the expression of interleukin-4, interferon- γ , forkhead box P3, substance P, and vasoactive intestinal peptide.

Conclusion: inhibition of the cholinergic nerve not only alleviated symptoms of allergic rhinitis by inhibiting the impulse of the parasympathetic nerve but also modulated the T-helper type 2–predominant immune reaction, expression of neuropeptides, and related inflammation factors.

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Introduction

Allergic rhinitis (AR) is an inflammatory disorder of the nasal mucosa characterized by sneezing, rhinorrhea, nasal obstruction, nasal itching, and non-nasal symptoms.¹ These symptoms arise from the interaction between mediators and neural, vascular, and glandular structures within the nose.² Nasal biopsy examinations have shown an accumulation of mast cells, eosinophils, and basophils³ and exhibited a T-helper type 2 (T_H2) cell predominance in AR.⁴ Those accumulated cells are in an activated state, and it is the mediators and cytokines released by those cells that lead to the generation of symptoms through the stimulation of specific receptors on the sensory and autonomous nerves and blood vessels within the nasal mucosa.⁵

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Disclosures: Authors have nothing to disclose.

Funding: This study was supported by Funds of Hunan Scientific Plan in China (grant S2013F1023).

Although AR is a T_H2-predominant disease, some symptoms are induced exclusively by the nervous system. Exaggeration of normal defensive functions owing to exposure to allergens, environmental stimuli, or pathologic processes leads to the release of mediators and cytokines, some of which act by stimulating the nervous system, so there is a great possibility that neural functions interact with the immune system, which influences the inflammatory process by up- or downregulation. This physiologic aspect needs more exploration, at least in the nose, but several pieces of evidence have indicated its importance in rhinitis.⁶

T-cell cholinergic receptors have been found to play an important role in the differentiation of naive CD4 T cells toward the T_H1, T_H2, and T_H17 lineages.⁷ Inhibition of the cholinergic nerve by tiotropium bromide has been shown to decrease the accumulation of T_H2 cells in lower airway in patients with asthma.⁸ Posterior nasal neurectomy (cholinergic nerve) has been shown to lead to a decrease of interleukin (IL)-5 and mast cells in severe AR.⁹ Bilateral vidian neurectomy has been found to significantly alleviate symptoms in moderate to severe persistent AR.¹⁰ Therefore, the relation

Table 1Changes in symptom score and OVA sIgE level in allergic mice treated with ipratropium bromide or 6-OHDA (mean $\pm$ SD)

Group (n) ^a	Symptom score			OVA sIgE (ng/mL)		
	Baseline	After treatment	<i>P</i> value ^c	Baseline	After treatment	<i>P</i> value ^c
1 (6)	0.8 $\pm$ 0.75 ^b	0.7 $\pm$ 0.81 ^b	.664	22.1 $\pm$ 4.33 ^b	26.1 $\pm$ 4.13 ^b	.137
2 (5)	6.4 $\pm$ 1.14	6.2 $\pm$ 1.09	.784	112.5 $\pm$ 16.3	120.3 $\pm$ 16.1	.422
3 (6)	6.8 $\pm$ 1.17	3.8 $\pm$ 0.98	<.001	123.5 $\pm$ 16.6	74.4 $\pm$ 9.87	<.001
4 (6)	6.3 $\pm$ 1.21	7.0 $\pm$ 1.09	.341	124.4 $\pm$ 17.1	130.7 $\pm$ 16.1	.547

Abbreviations: OH, ovalbumin; 6-OHDA, 6-hydroxydopamine hydrobromide.

^aGroup 1, normal controls treated with normal saline; group 2, allergic mice treated with normal saline; group 3, allergic mice treated with ipratropium bromide for 14 days; group 4, allergic mice treated with 6-OHDA.^b*P* < .001 compared with groups 2, 3, and 4, respectively.^c*P* value compared with baseline.

between neural functions and the immune system deserves to be studied in many aspects.

This study investigated the role of sympathetic and parasympathetic denervation in an AR mouse model and the correlation between the sensory neuropeptide and AR.

Methods

Experimental Animals

Six-week-old healthy female BALB/c mice (20–30 g), free of murine-specific pathogens, were used in this study. Twenty-four of these mice were purchased. The mice were housed in a controlled environment with a 12-hour light-and-dark cycle with free access to

food and water in the Experimental Animal Center of Third Xiangya Hospital, Central South University (Changsha, Hunan, China; experimental animal license number SCXK-[Hunan] 20020003, environmental Facilities Certificate of Conformity SYXK-(Hunan) 200,200.20). All experimental animals used in this study were handled according to a protocol approved by the institutional animal care and use committee of Central South University.

Reagents

Chicken ovalbumin (OVA), Al(OH)₃, and 6-hydroxydopamine hydrobromide (6-OHDA) were purchased from Sigma-Aldrich (St Louis, Missouri). Ipratropium bromide (no 100522-200601) was provided by China Pharmaceutical and Biological Products (Beijing,

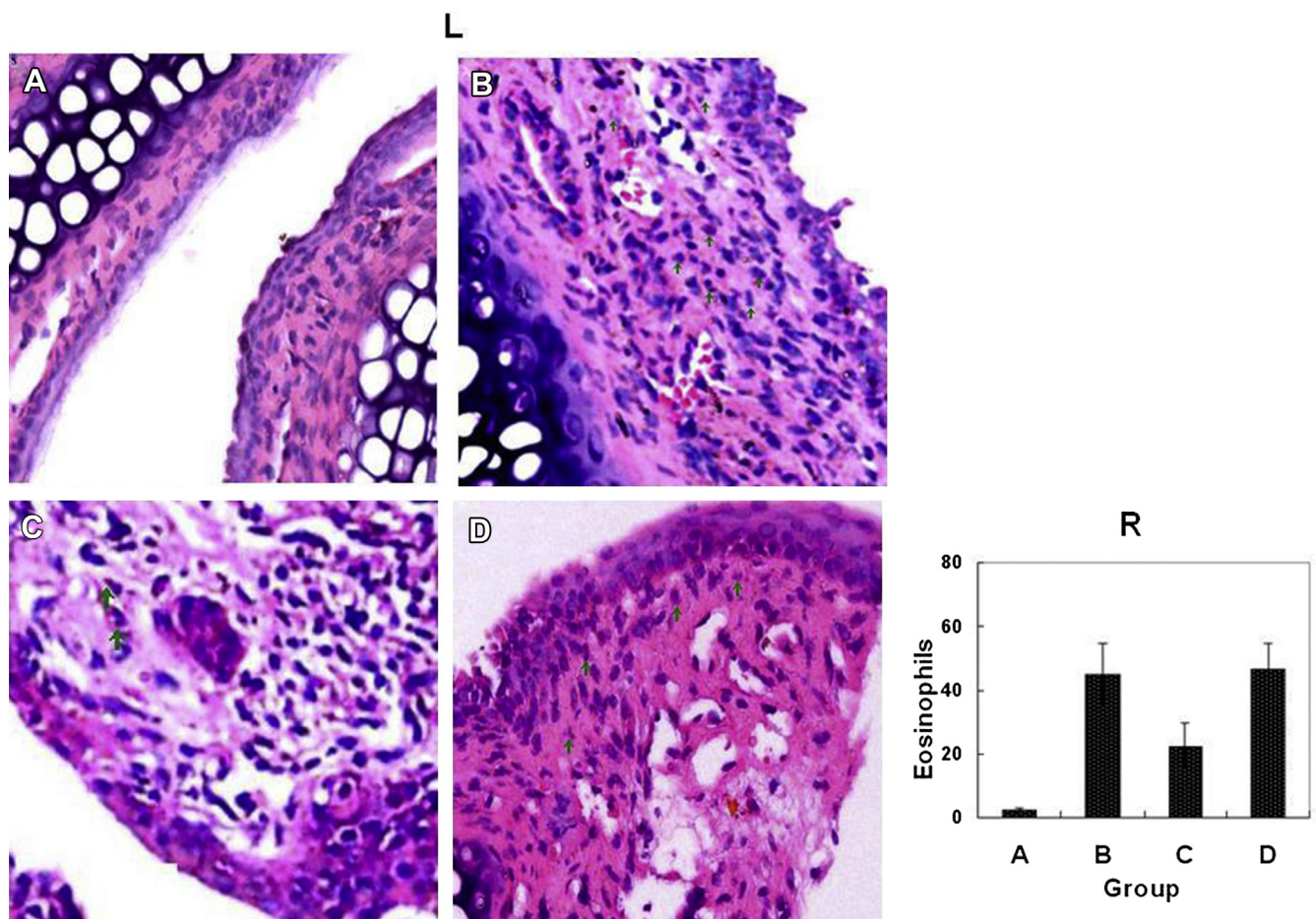


Figure 1. Infiltration of eosinophils (arrows) in nasal mucosa ($\times 100$). (L) Representative histochemical photographs. (R) Statistical histogram. (A) Normal control mice. (B) Allergic mice. (C) Allergic mice treated with ipratropium bromide. (D) Allergic mice treated with chemical sympathectomy.

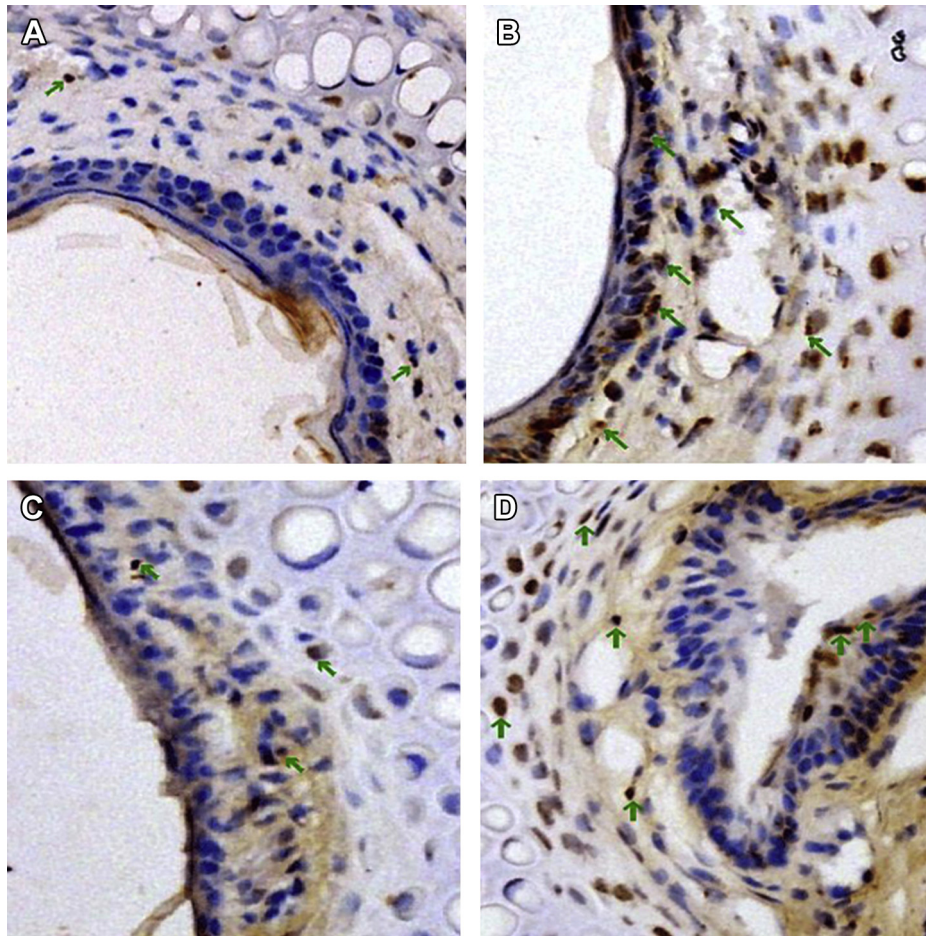


Figure 2. Representative immunohistochemical images of interleukin-4 expression (brown-staining cells, arrows; $\times 100$). (A) Normal control mice. (B) Allergic mice. (C) Allergic mice treated with ipratropium bromide. (D) Allergic mice treated with chemical sympathectomy.

China). An OVA serum IgE (sIgE) enzyme-linked immunosorbent assay kit was purchased from Huamei Biotech (Wuhan, China). Antibodies against IL-4, interferon- γ (IFN- γ), forkhead box P3 (Foxp3), vasoactive intestinal peptide (VIP), substance P (SP) and immunohistochemistry kits were purchased from Sigma-Aldrich.

Production of AR Model and Treatment Protocol

The allergic mouse model was produced as described by Saito et al.¹¹ Briefly, under pathogen-free conditions, mice were sensitized using OVA antigen as follows. A total of 0.5 mg of OVA diluted to 1 mL by sterile normal saline with 5 mg of aluminum hydroxide gel was administered to nonanesthetized animals 4 times on days 1, 5, 10, and 14 by intraperitoneal injection. After this intranasal challenge to the nasal mucosa, OVA diluted with sterile normal saline (20 μ L of OVA 25 mg/mL) was injected into the nasal mucosa once a day from day 15 to day 28. The allergic mice model was judged by symptom scores at day 28.¹²

Twenty-four mice were randomly allocated to 1 of 4 groups: control group (group A), model group (group B), model group with ipratropium bromide treatment (group C), and model group with 6-OHDA treatment (group D). In group A ($n = 6$), mice were not sensitized and were challenged with only saline. In group B ($n = 6$), allergic mice received an intranasal OVA challenge every 2 days (20 μ L of OVA 25 mg/mL) from day 29 to day 42. In group C ($n = 6$), allergic mice received the intranasal OVA challenge every 2 days and a nasal administration of 3.0 mg/mL of ipratropium bromide once a day from day 29 to day 42. The last intranasal OVA challenge was administered 30 minutes after the last drop of ipratropium

bromide. In group D ($n = 6$), chemical sympathectomy was performed in allergic mice by intraperitoneally injection of 6-OHDA at 200 mg/kg of body weight at days 29 and 35¹² and received intranasal OVA every 2 days from day 29 to day 42.

All mice were sacrificed within 15 minutes of the last intranasal OVA challenge on day 42. Evaluation of symptom scores and blood draws were performed before sacrifice.

Evaluation of Induced Allergic Symptoms

Sneezing and nasal rubbing were observed and counted for 10 minutes after the nasal challenge. Symptom scores were calculated as described by Wang et al.¹³ The allergic mice model was deemed successful when the symptom scores were higher than 5.

Measurement of OVA sIgE

Peripheral blood was obtained from the venae angularis before sacrifice. Serum was stored in a refrigerator. The enzyme-linked immunosorbent assay was carried out according to the manufacturer's instructions. Briefly, the antigen solution (mouse serum) was prepared at an appropriate concentration in a coating buffer, pipetted 100 μ L above the solution to each well of the microtiter plate, incubated at room temperature for 30 minutes, and then washed, blocked, labeled, and stained at room temperature. The optical density was measured using spectrophotometry at a λ value equal to 450 nm. Results were compared with the standard curve of OVA sIgE (10, 100, 500, and 1,000 ng/mL) and the concentration of OVA sIgE was expressed as nanograms per milliliter of serum.

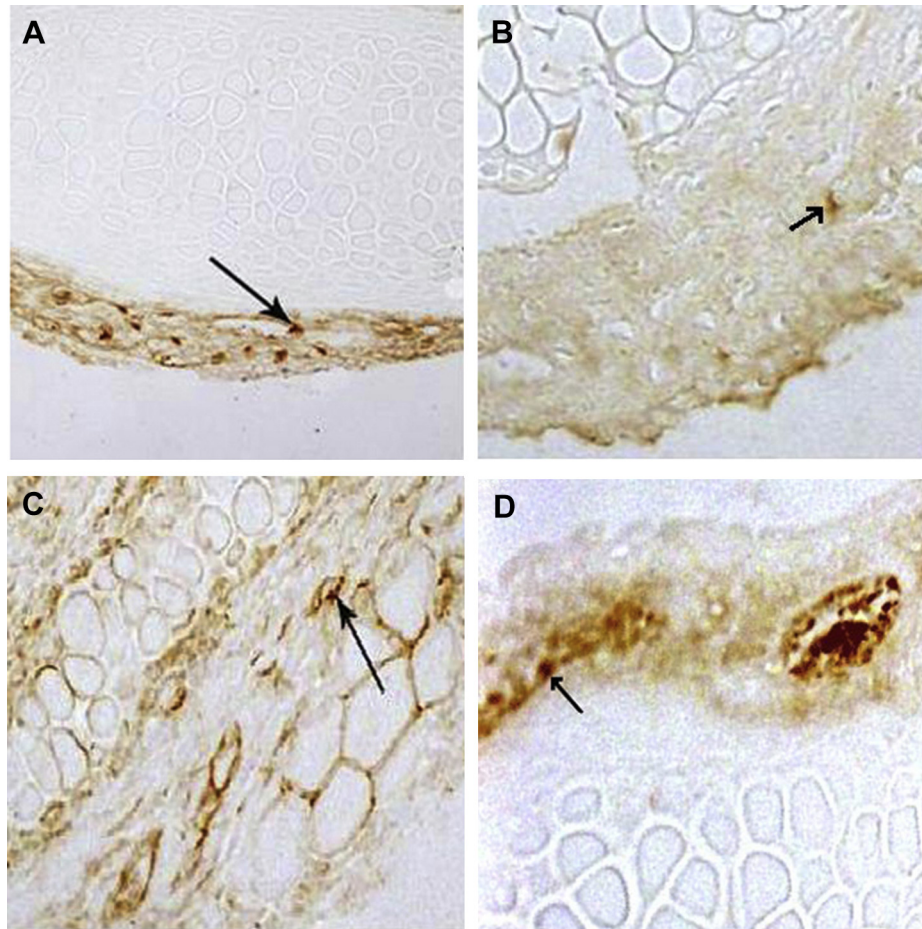


Figure 3. Representative immunohistochemical images of forkhead box P3 expression (brown staining cells, arrows; $\times 100$) in mice.

Histopathology and Immunohistochemistry

After the mice had been sacrificed in a carbon dioxide chamber, the nasal mucosa was fixed immediately in neutral buffered formalin and then embedded in paraffin. Sections were stained with hematoxylin and eosin. Immunohistochemistry was performed on tissues with antibodies to IL4, IFN- γ , Foxp3, SP, and VIP by the avidin-biotin complex method according to the

manufacturer's instructions. Slides were archived using digital imaging with a microscope and computer system. The percentage of positively stained cells was determined by automatically calculating the ratio of brown-stained cells to total cells in the field using Nikon NIS-Element software (Nikon, Tokyo, Japan). Brown-staining fibers also were calculated as positively stained cells for VIP and SP. Three independent slides for each sample were used for imaging analysis. All histologic and immunohistochemistry studies were performed and interpreted by pathologists who were blinded to the group of samples.

Statistical Analysis

The data are presented as arithmetic mean $\pm$ SD. Statistical comparisons were performed with the Student *t* test using SPSS 16.0 (SPSS, Inc, Chicago, Illinois). A *P* value less than .05 was considered statistically significant.

Results

Animal Model of AR Evaluated by Symptom Score and OVA sIgE

The numbers of sneezes and nasal rubbings were recorded for the symptom scores of the animal models. A successful animal model of AR was determined by symptom scores higher than 5.¹³ By day 28, 17 of the 18 OVA-sensitized mice had symptom scores meeting the criterion for successful induction of the AR model. The average score was significantly higher in the allergen-challenged than in the nonallergic groups (Table 1). Serum-specific OVA IgE

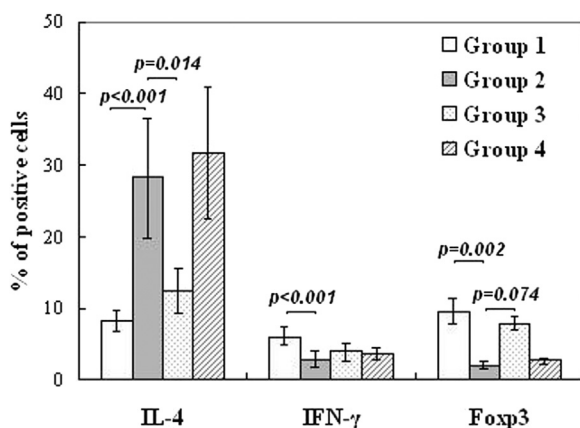


Figure 4. Percentages of cells positive for interleukin-4 (IL-4), interferon- γ (IFN- γ), and forkhead box P3 (Foxp3) in the nasal mucosa of allergic and control mice (mean $\pm$ SD).

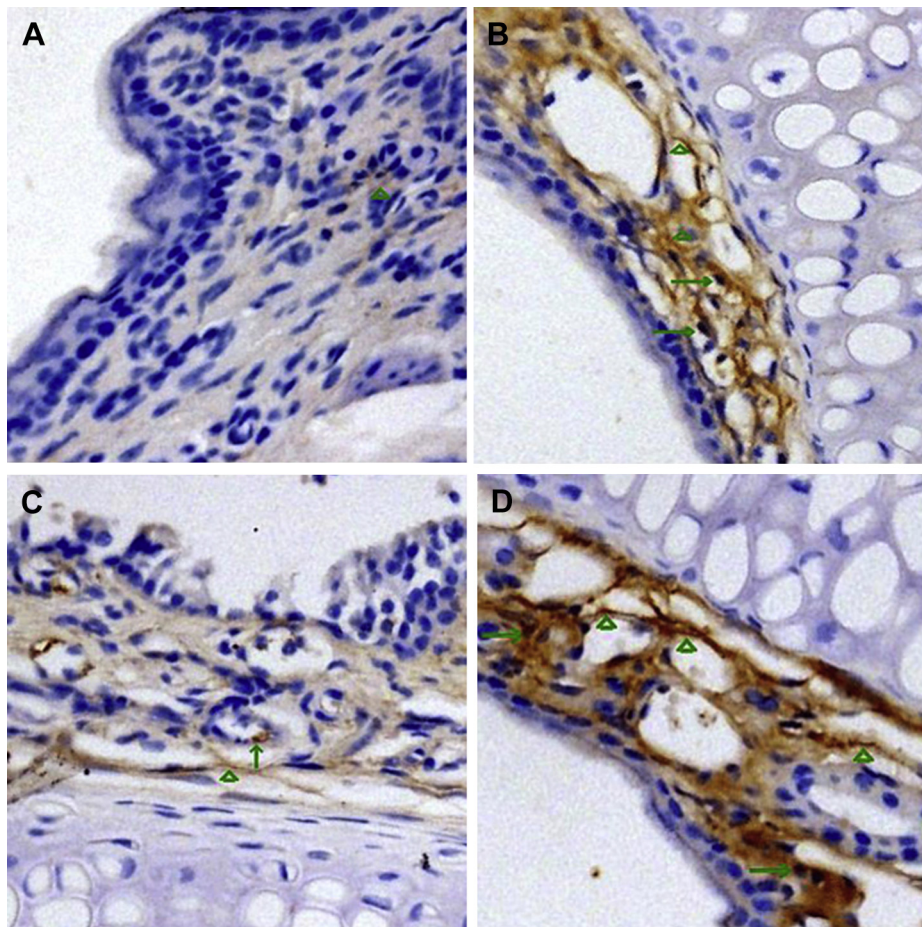


Figure 5. Representative immunohistochemical images of substance P expression in nasal mucosa (brown staining; arrows, positively stained cells; arrowheads, positively stained fibers; $\times 100$).

also was significantly elevated in the antigen-challenged groups compared with the nonallergic groups (Table 1).

Symptoms of AR Alleviated by Anticholinergic Drug

The symptom score decreased significantly to 3.9 ± 0.95 in the allergic mice after treatment with an ipratropium bromide nasal drop for 2 weeks compared with before treatment (6.8 ± 1.08) and did not change in allergic mice treated by normal saline. The specific OVA IgE level also decreased significantly to 74.4 ± 4.87 from 123.5 ± 6.68 ng/mL ($P < .01$; Table 1) after treatment with ipratropium bromide. However, inhibition of the sympathetic nerve by 6-OHDA did not change the symptom score or specific OVA IgE level in allergic mice ($P = .547$; Table 1).

Eosinophil Counts in Lamina Propria

Figure 1 shows the infiltration of eosinophils in the lamina propria of the nasal mucosa. Eosinophil counts per field ($\times 100$) were recorded as 2.5 ± 0.6 in the control group, 45.1 ± 9.6 in the allergic group, 22.5 ± 7.4 in the allergic group treated with ipratropium bromide, and 46.6 ± 7.9 in allergic group treated with 6-OHDA. The allergic groups showed a higher eosinophil count than the control group ($P < .05$). The eosinophil count in the allergic mice was significantly decreased after treatment with ipratropium bromide for 14 days ($P < .05$) and was not changed by 6-OHDA treatment ($P > .05$). These results suggest that the anticholinergic drug can inhibit eosinophil infiltration in nasal mucosa.

Protein Expression of IL-4, IFN- γ , and Foxp3

Interleukin-4 and IFN- γ are secreted mainly by T_H2 and T_H1 cells, respectively. Foxp3 is a hallmark of $CD4^+CD25^+$ Treg cells. In this study, the change of these T cells was detected using an analysis of immunohistochemistry by imaging software (Figs 2 and 3). Percentages of cells positive for IL-4, IFN- γ , and Foxp3 were 8.2 ± 1.45 , 6.1 ± 0.35 , and 9.5 ± 1.81 , respectively, in the control group and 28.2 ± 8.41 , 2.8 ± 0.63 , and 2.0 ± 0.52 , respectively, in the allergic groups. In the allergic group treated with 2.4 mg/kg of ipratropium bromide for 14 days, percentages of cells positive for IL-4, IFN- γ , and Foxp3 were 12.4 ± 3.12 , 3.9 ± 0.42 , and 7.8 ± 0.98 . The IL-4 expression level was significantly higher in the allergic group than in the control group ($P < .001$; Fig 4) and decreased significantly with ipratropium bromide treatment ($P = .014$; Fig 4). In contrast, the expression of Foxp3 was lower in the allergic groups than in the control group and was increased with ipratropium bromide treatment ($P < .001$; Fig 4). The percentage of cells positive for IFN- γ did not change significantly among allergic groups with treatments. The 6-OHDA treatments did not significantly change the expression of IL-4 and Foxp3 in the allergic groups ($P > .05$; Fig 4).

Expression of SP and VIP in Allergic Mice

Neuropeptides have been reported to be related to AR. Expressions of SP and VIP were detected by immunohistochemistry in allergic mice (Figs 4 and 5). Data from the imaging analysis software showed that a 4.6-fold increase in SP expression and a 3.4-fold

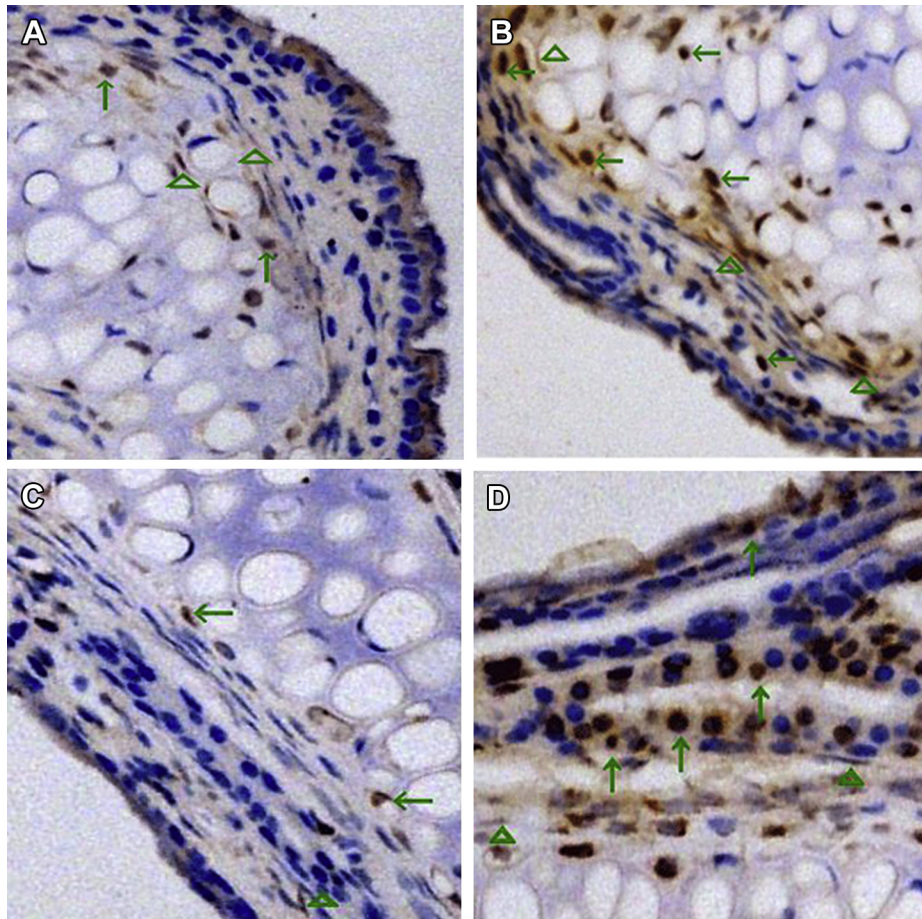


Figure 6. Representative images of vasoactive intestinal peptide expression in nasal mucosa depicted as brown-staining cells and fibers (arrows, positively stained cells; arrowheads, positively stained fibers; $\times 100$).

increase in VIP expression was observed in allergic mice compared with normal control mice ($P < .001$). The expression of SP and VIP was significantly inhibited by treatment with ipratropium bromide for 14 days ($P < .01$; Figs 6 and 7). However, antisympathetic treatment did not change the expression of SP and VIP in allergic mice ($P > .05$).

Discussion

Correlations between the immune system and the nervous system in AR needed to be explored. Inhibition of parasympathetic receptors by ipratropium bromide could alleviate symptoms of AR, although blocking the sympathetic receptors did not relieve symptoms in allergic mice. This observation might relate to a parasympathetic advantage of AR.¹⁴ These results are in agreement with previous studies showing that ipratropium bromide significantly decreased sneezing, lacrimation, nasal secretion, and congestion in a guinea pig model of AR.¹⁵ Eosinophils have been observed to localize to cholinergic nerves in asthma and AR and can induce the expression of several genes involved in the synthesis, storage, and metabolism of acetylcholine.¹⁶ In contrast, an anticholinergic drug, acclidinium bromide, decreased eosinophils from bronchoalveolar lavage fluid in a murine model of asthma.¹⁷ The present data demonstrated that inhibition of cholinergic signaling could decrease the accumulation of eosinophils in the nasal mucosa of AR. Moreover, OVA sIgE was decreased in allergic mice after ipratropium bromide treatment, which suggests that inhibitors of cholinergic signaling can decrease the inflammatory reaction of AR.

Parasympathetic activity has been observed to have the capacity to activate dendritic cells to release OX40 ligand, which induces

CD4⁺ T cells to produce IL-4 in nasal mucosa.¹⁸ Inhibitors of muscarinic cholinergic receptors, such as tiotropium bromide, could alleviate symptoms and decrease T_H2 cells in the bronchial tract in asthma.⁸

In the present study, the IL-4 expression level was significantly higher in the allergic model group than in the control group and decreased significantly with ipratropium bromide treatment ($P < .05$). In contrast, the expression of Foxp3 was lower in the allergic model group than in the control group and increased with

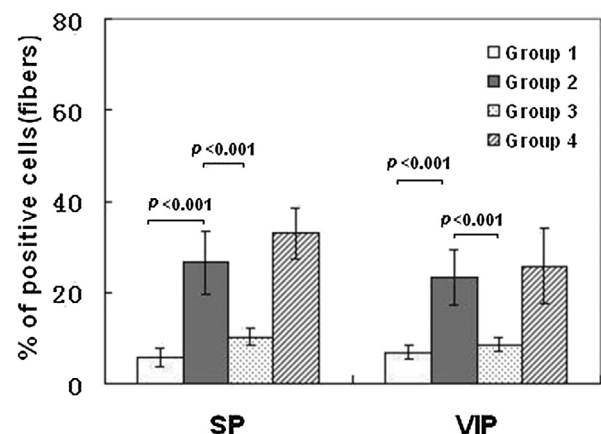


Figure 7. Percentages of cells and fibers positively stained for substance P (SP) and vasoactive intestinal peptide (VIP) in nasal mucosa of allergic and control mice (mean $\pm$ SD).

ipratropium bromide treatment ($P < .05$). This reciprocal relation between Foxp3 and IL-4 expression has been noted in other studies.¹⁹

Blocking of the sympathetic signaling did not change the expression of IL-4 and Foxp3 in allergic mice. These results show the correlation between the parasympathetic nervous and immune systems in AR.

The balance between T_H1 and T_H2 cells plays an important role in the pathogenesis of AR. High levels of T_H2 cells and low levels of T_H1 cells induced by allergens are known to be the main mechanism of allergic diseases. The authors measured the T_H2-produced cytokine IL-4 and T_H1-produced cytokine INF- γ by immunohistochemistry. The increased expression of IL-4 in allergic mice was significantly decreased by treatment with ipratropium bromide. However, the percentage of INF- γ -positive cells was not significantly changed by the inhibition of cholinergic signaling. This means that cholinergic activity can modulate the cytokine expression of T_H2 cells but not stimulate the T_H1 immune response in the nasal mucosa of AR. A detailed molecular process requires further investigation.

Substance P, a neuropeptide of the tachykinin family, is present in type C nociceptive sensory neurons of the human nasal mucosa. SP-containing nerve fibers are found around nasal arterial and venous vessels, gland acini, and in the epithelium.²⁰

There is growing evidence that SP participates in allergic reactions in the nasal mucosa. Patients with AR have higher tissue concentrations of SP than healthy individuals and this higher concentration of SP has been found to induce histamine release from nasal mucosa.²¹ VIPs have many functions in the nasal airways, including glandular discharge, vasodilatation, and sinusoidal engorgement,^{22–24} and higher expression levels of VIP and its receptor have been observed in the nasal mucosa of AR.²⁵ In the present study, the expression of SP and VIP in allergic mice was detected by immunohistochemistry. Data from the imaging analysis software showed that a 4.6-fold increase in SP expression and a 3.4-fold increase in VIP expression in the allergic model mice compared with normal control mice ($P < .001$). The increased expression of SP and VIP in allergic mice was significantly decreased by treatment with ipratropium bromide for 14 days ($P < .01$). Hence, ipratropium bromide treatment decreased SP and VIP expression, which in turn might be accompanied by the alleviation of symptoms of AR.

The present data showed that the inhibition of muscarinic cholinergic receptors not only alleviates the symptoms of AR by inhibiting parasympathetic signaling but also modulates the T_H2-predominant immune reaction, the expression of neuropeptides, and related inflammatory factors.

Acknowledgments

The authors thank Dr Zhou Jun, a pathologist at Third Xiangya Hospital, for her help with the immunohistochemistry and pathologic examination. They also are grateful to Dr Tarek Fouad, professor of otorhinolaryngology at Suez Canal University, for his help in editing this report.

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