

Botulinum Toxin Therapy in the Ovalbumin-Sensitized Rat

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Key Words

Allergic rhinitis • Botulinum toxin type A • Ovalbumin • Substance P • Vasoactive intestinal peptide

Abstract

Objective: The aim of this study was to determine whether intranasal administration of botulinum toxin type A (BTX-A) could relieve the typical symptoms of allergic rhinitis (AR) and alter substance P (SP)- and vasoactive intestinal peptide (VIP)-immunoreactive (IR) expression in nasal mucosa of AR animals sensitized with ovalbumin (OVA). **Methods:** AR was induced by intraperitoneal injection of OVA followed by its repeated intranasal instillation in female Wistar rats. Some AR animals were intranasally treated with a cotton strip containing BTX-A (10 U per nostril) for 1 h. After BTX-A treatment, OVA was repeatedly instilled in AR and AR + BTX-A groups every 2 days for 10 days. Subsequently, nasal symptoms were evaluated, and nasal secretions collected. Finally, the nasal mucosae of all animals were prepared for histological and immunohistochemical assessment. **Results:** BTX-A administration alleviated typical AR symptoms including rhinorrhea, nasal itching and sneezing, and subsequent intranasal repeated challenge with OVA did not trigger AR symptoms. After BTX-A treatment, inflammatory histological characteristics within the nasal mucosa of AR animals were absent, but atrophy of serous glands was observed.

BTX-A decreased dense SP-IR and VIP-IR cells and fibers within and beneath the epithelium, around blood vessels and close to serous glands in AR animals. **Conclusion:** Local BTX-A treatment is an effective method to reduce AR symptoms. BTX-A decreased the excessive SP-IR and VIP-IR expression induced by OVA. Therefore, BTX-A may affect the nasal mucosa via the suppression of neuropeptides, playing a major role in autonomous mucosal innervation in the pathophysiology of AR.

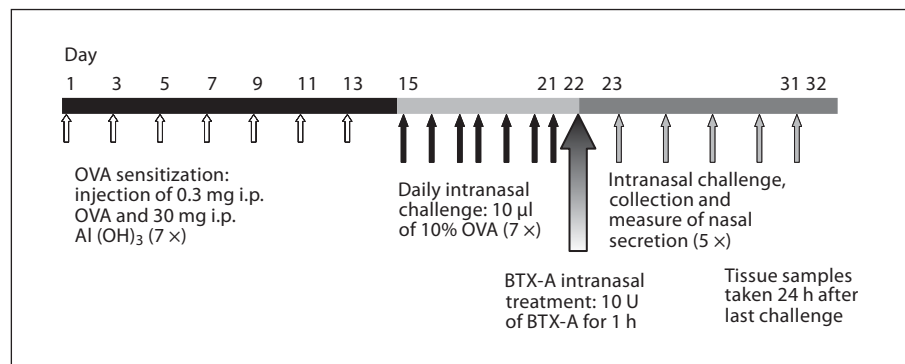
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Introduction

Allergic rhinitis (AR) is characterized by the recruitment and activation of eosinophils [1, 2] and nerves, indicated by symptoms such as itching and sneezing, pathophysiologic features such as (reflex-mediated) secretion, and the release of neurotransmitters and neuropeptides [3, 4]. It has been suggested that nerve activities may interact with inflammatory/immune cells and that these interactions are proinflammatory [5–7]. For example, it has been demonstrated that topical application of substance P (SP) and vasoactive intestinal peptide (VIP) may increase the nasal output of eosinophils in AR [5, 7].

In AR, sneezing and watery rhinorrhea can be suppressed by antihistamines, but nasal blockage is less af-

Fig. 1. The protocol of sensitization, challenge, BTX-A treatment and repeated challenge, and simultaneous collection and measure of nasal secretion is shown. A schematic representation of a rat experimental model of AR induced by OVA and treated with BTX-A. After BTX-A treatment, rats were repeatedly challenged, and nasal secretions were collected and measured. Animals were killed on day 32, and nasal tissue samples were taken for histological and immunohistochemical analyses.



fects by these drugs [8, 9]. On the other hand, decongestants [10] and glucocorticoids have been shown to relieve nasal blockage [11], but AR patients are less affected.

Botulinum toxin type A (BTX-A) is a potent neurotoxin produced by the bacterium *Clostridium botulinum*. A recent test of BTX-A in AR patients has demonstrated that BTX-A could relieve rhinorrhea, nasal obstruction and sneezing [12]. BTX-A is well known to inhibit acetylcholine (ACh) release from cholinergic nerves in skeletal muscles [13]. BTX-A acts by binding to a membrane receptor in cholinergic nerve endings, enters a fiber and selectively cleaves the synaptosomal-associated protein (SNAP)-25, leading to the inability of neurotransmitter-containing synaptic vesicles to undergo exocytosis and release neurotransmitters [14]. Studies on BTX-A-treated AR suggest that BTX-A is not inhibiting ACh release in voluntary muscles, but modulates the release of neuropeptides, which are involved in the pathophysiological processes inducing AR.

The aim of the current study was to investigate whether local BTX-A treatment of nasal mucosa could be used to treat ovalbumin (OVA)-sensitized AR animals [7, 15, 16], and whether it could relieve the typical AR symptoms including rhinorrhea, nasal obstruction and sneezing, and alter immunoreactive (IR) expression of SP and VIP, which play important neuroimmunomodulating roles in the pathogenesis of AR in the nasal mucosa.

Materials and Methods

Animals

Pathogen-free adult female Wistar rats (Experimental Animal Center, Lanzhou University, China) weighing 200–250 g were housed individually in cages, with food and water being available ad libitum, and kept on a 12-hour light-dark cycle (lights on 07:00–19:00 h) for 7 days before experiments; room temperature

was $21 \pm 1^\circ\text{C}$ and relative humidity 50%. Experimental procedures were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by the Institutional Animal Care and Use Committees of the Gansu Province Medical Animal Center and Lanzhou University. The study was designed to minimize animal suffering, and only the number of animals necessary to produce reliable scientific data was studied.

Model of AR Induced by OVA Sensitization

After acclimatization to laboratory conditions, the first phase of our experiments involving the immunization of animals was performed. All animals were sensitized with OVA (0.3 mg i.p., Sigma, St. Louis, Mo., USA) as antigen, administered together with aluminum hydroxide (30 mg) in saline (1 ml i.p.) every 2 days for 14 days. In the second phase of our experiments, sensitized experimental animals ($n = 20$) were used to develop a model of AR by repeated intranasal instillation of $10 \mu\text{l}$ of 10% OVA with a thin catheter daily for 7 consecutive days (fig. 1). Control animals ($n = 12$) were repeatedly sensitized and challenged with saline at the same dose as the AR animals. The immediate reaction of animals after nasal challenge was examined according to AR symptoms.

Evaluation of Symptoms

AR was evaluated from the occurrence of typical clinical symptoms with respect to nose and eye irritation, sneezing, conjunctival and nasal secretion, and nasal acoustic phenomenon reflected the degree of nose obstruction. Symptoms scores were graded on a four-point scale. Each grade was assigned a numerical score (0–3); nasal symptoms scores were graded in points as follows: (1) nasal itching motion: 0 – none; 1 – rubbing nose 2/min; 2 – rubbing nose 4–6/min; 3 – rubbing nose $>6/\text{min}$; (2) sneeze: 0 – none; 1 – 1–3/10 min; 2 – 4–9/10 min; 3 – $\geq 10/10$ min, and (3) nasal mucus: 0 – none; 1 – in a nostril; 2 – outside a nostril; 3 – overflowing. Total scores >5 might be successful AR mode [17, 18].

BTX-A Treatment Protocol

BTX-A was purchased from the Lanzhou Institute of Products (GMP Nos. C0868, S10970037, 2002501, Lanzhou, China). The concentration of 100 U was prepared freshly with a 0.9% NaCl vehicle in 1 ml of solution kept at -4°C and used within 4 h. On

the 2nd day, some animals with AR were treated with BTX-A (AR + BTX-A group, $n = 10$). A thin cotton strip soaked with 0.1 ml of solution containing 10 U BTX-A was inserted into each nostril for 1 h under chloral hydrate (3 g/kg, i.p.) anesthesia. However, animals in control and AR groups ($n = 10$, no BTX-A treatment) were treated with same-size cotton strips soaked with 0.1 ml saline and under identical anesthetic conditions. The day after BTX-A treatment, antigen challenges with 10 μ l of 10% OVA were repeatedly performed in both AR groups (AR and AR + BTX-A) every 2 days for 10 days (fig. 1). The evaluation of nasal symptoms and collection and measure of nasal fluids were performed during these 10 days.

Collection and Measure of Nasal Secretions

After each challenge with OVA, nasal fluid was intranasally collected by the filter paper method [17] for 30 min/day, every 2nd day for 10 days in the three groups (fig. 1). The quantity of nasal secretions was calculated by weighing and subtracting of the pre-collection from the post-collection weights. The results were statistically analyzed.

Histological and Immunohistochemical Preparation

Following nasal secretion collections, all animals were sacrificed with an overdose of chloral hydrate (4 g/kg, i.p.) and perfused with 0.9% NaCl (100 ml, -4°C), followed by 350 ml of 0.1 mol/l phosphate buffer (pH 7.4) containing 4% paraformaldehyde via the ascending aorta. After perfusion, nasal mucosa was moved carefully from the skull and post-fixed overnight in the same solution and then rinsed for 24 h with phosphate buffer containing 30% sucrose (4°C). Some nasal mucosa tissues were dehydrated in graded alcohol series and embedded in paraffin. A transverse section was cut (4 μ m) and stained with hematoxylin and eosin. Histological assessment was performed by light microscopy.

Other nasal mucosa tissues were cut into 30- μ m-thick sections using a freezing microtome, and 5 adjacent series were collected at 150- μ m intervals on the slides. Immunostaining for SP and VIP in nasal mucosa was performed with Strept Avidin Biotin Complex methods. The procedure of immunohistochemistry was performed as described as previously [19, 20]. The sections were incubated overnight at 4°C with rabbit anti-SP (1:200; Chemicon, Temecula, Calif., USA) and rabbit anti-VIP (1:200; Chemicon), followed by SABC kits (Boster, Wuhan, China) to be visualized under a microscope.

Statistical Analysis

Data were expressed as means $\pm$ SEM and statistically analyzed using one-way analysis of variance by SPSS 13.0 for Windows. Differences between means were considered significant at $p < 0.05$.

Results

Evaluation of the AR Rat Model

Following injection and initial intranasal challenge with OVA, the typical AR symptoms, such as frequent sneezing, nose rubbing and watery rhinorrhea, were observed, which then gradually disappeared within approx-

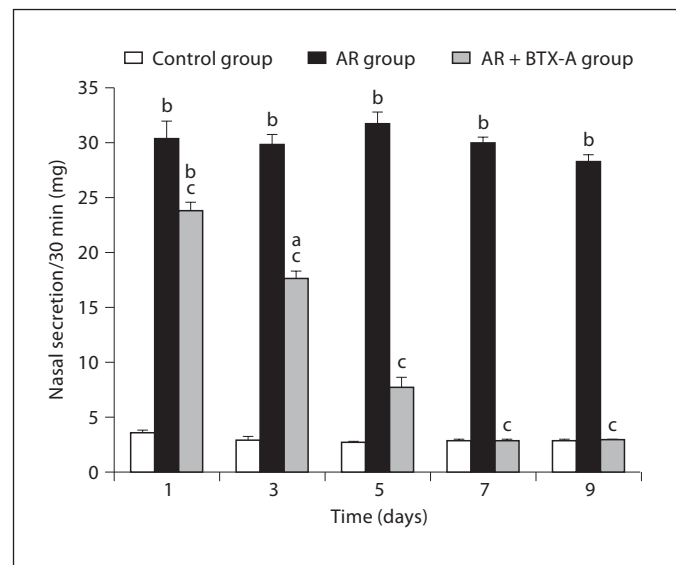


Fig. 2. Nasal secretions were collected and measured with the filter paper method for 30 min/day every 2nd day during a 10-day period in the three groups. In the AR group ($n = 10$), nasal secretions were significantly increased compared with the control group ($n = 12$, $p < 0.001$). However, in the AR + BTX-A group ($n = 10$), nasal secretions were reduced and comparable to controls on day 5. Data are expressed as means $\pm$ SEM. ^a $p < 0.05$, ^b $p < 0.001$, vs. control; ^c $p < 0.001$, vs. AR group.

imately 1.5 h. Subsequent intranasal challenges with OVA triggered more severe symptoms that persisted longer. In animals treated with BTX-A, symptoms were reduced and disappeared on day 2. Furthermore, subsequent intranasal challenges with OVA did not trigger AR symptoms.

Determination of Nasal Secretions

Each OVA challenge aroused nasal secretions in rats with experimental AR. In the AR group, nasal secretion (per 30 min measured by the filter paper method) increased significantly ($p < 0.001$, fig. 2) after intranasal challenge with OVA. In the AR + BTX-A group, nasal secretions significantly decreased following BTX-A treatment. On the 5th day and subsequent days, the quantity of nasal secretion was comparable to the control group, without enhancement following intranasal challenge with OVA (fig. 1, 2).

Histological and Immunohistochemical Assessment

Histological examination of the nasal mucosa revealed excessive vascular dilatation, congestion and edema in

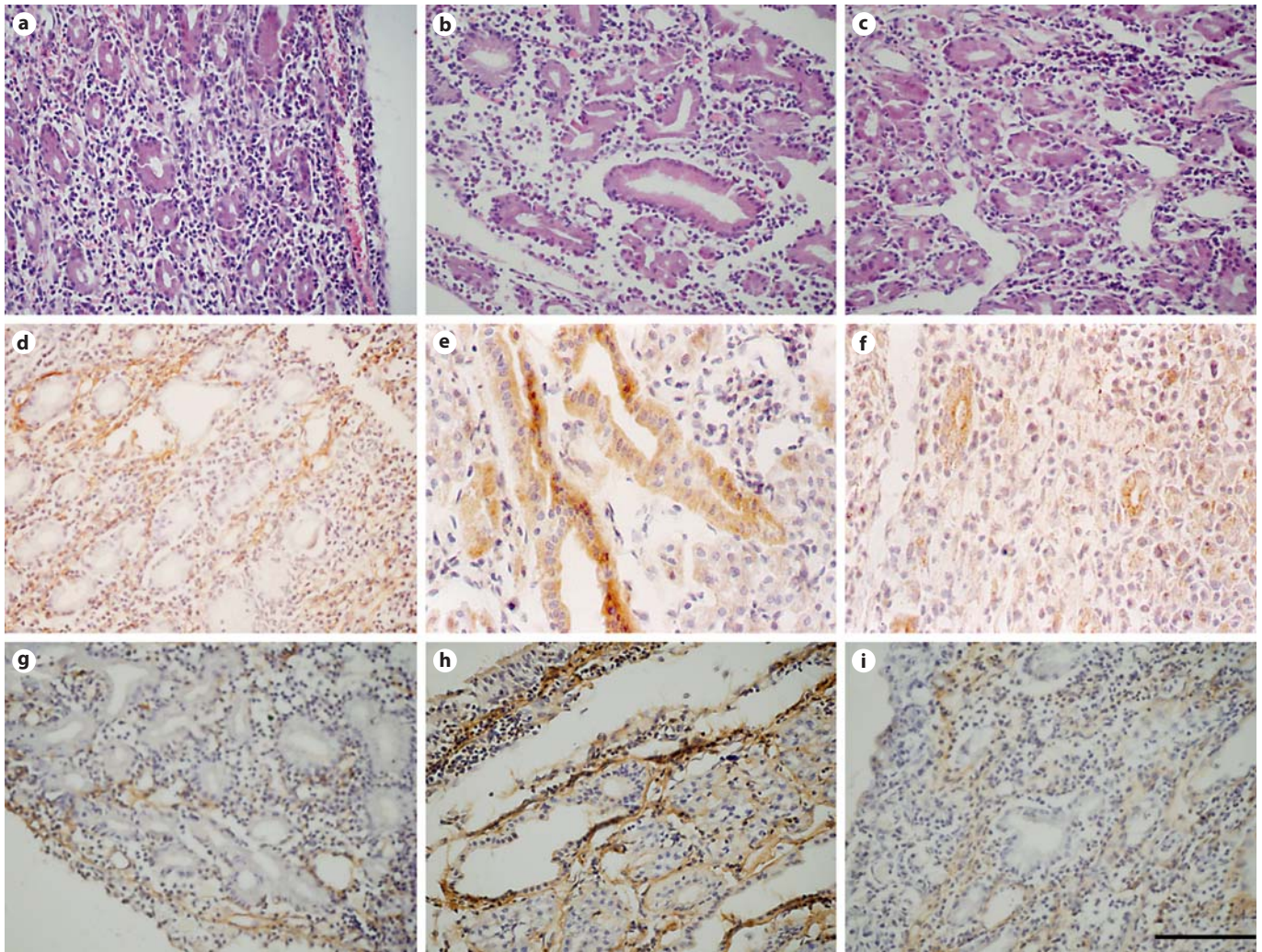


Fig. 3. Microscopic photographs of histologically and immunohistochemically stained sections of the nasal mucosa of the control (**a**, **d**, **g**), AR (**b**, **e**, **h**) and AR + BTX-A (**c**, **f**, **i**) groups. In AR animals, histological staining of mucosa with HE revealed excessive vascular dilatation, congestion, edema, eosinophils and hyperplasia of serous glands (**b**) compared with controls (**a**). In the BTX-A group, inflammatory characteristics were absent in the nasal mucosa, but atrophy of serous glands was observed (**c**).

More SP-IR cells and fibers were shown within and beneath the epithelium, around blood vessels and close to serous glands with hyperplasia in AR (**e**) than in control rats (**d**). Following BTX-A treatment, SP-IR was decreased and was only found in atrophic serous glands (**f**). VIP-IR staining was frequently found around glands and blood vessels in the AR group (**h**). In the BTX-A treated (**g**) and control animals (**i**), VIP-IR was generally weak. Bar = 10 μ m.

animals with AR. Within the mucosa, diffuse infiltration by an increased number of eosinophils was present, often in the presence of lymphocytes. In addition, diffuse hyperplasia of serous glands and hyperplasia of epithelium were noted (fig. 3b). After BTX-A treatment, inflammatory characteristics disappeared within nasal mucosa, but atrophy of serous glands was observed (fig. 3c).

Dense SP-IR cells and fibers were regularly observed within and beneath the epithelium, around blood vessels

and close to serous glands, and only small amounts of SP-containing nerve fibers were seen in the preparations around subepithelial glands and blood vessels in animals with AR (fig. 3e). VIP-IR staining was frequently found in the epithelium, around glands and in blood vessels (fig. 3h) in the AR group. In the BTX-A-treated and control animals, weak staining for SP- and VIP-IR was noted (fig. 3d, f, g, i).

Discussion

In the present study, BTX-A was used to treat OVA-sensitized rats. The OVA antigen is widely used as an animal model to induce AR due to the similarity of symptoms in human AR [7, 15–18]. The results showed that typical AR symptoms, such as rhinorrhea, nasal itching and sneezing, were relieved following BTX-A treatment. Our study further demonstrated that the severity of rhinorrhea was significantly diminished in the AR + BTX-A group compared with the AR group, being in accordance with the recent exploratory clinical treatment in patients with AR [12].

Secretions from numerous seromucinous glands are thought to contribute to the humidification of inspired air, as do secretions from other sources, including the vast network of fenestrated capillaries found immediately underneath the respiratory epithelium [17]. Neuropeptides, including SP and VIP, are primarily responsible for the glandular secretory nasal response and inflammatory process [21–23]. Previous studies have demonstrated that when humans and animals display AR, SP- and VIP-IR expression increases in the nasal mucosa [7, 21–23]. In addition, our study revealed that in the nasal mucosa of AR animals the density of SP- and VIP-IR cells and fibers within and beneath the epithelium, around blood vessels and close to serous glands was higher compared with controls. After BTX-A treatment, SP- and VIP-IR demonstrated in general weak staining in the nasal mucosa compared with AR animals. These results suggest that BTX-A reduces AR symptoms via suppressing the release of neuropeptides, e.g. SP and VIP, which play a major role in autonomic mucosal innervation and in the pathophysiology of AR.

In striated muscles it has previously been shown that BTX inhibits ACh release from cholinergic nerves [13]. Neurotransmitter release, most notably ACh, involves fusion of the synaptic vesicles with the plasma membrane and passage of ACh out of the neuron into the synapse. SNAP-25 is a membrane-bound protein anchored to the cytosolic face of membranes in the middle of the molecule [24], possibly accounting for the specificity of membrane fusion and direct fusion by forming a tight complex (the SNARE or core complex) that brings the synaptic vesicle and plasma membrane together. BTX consists of a heavy protein chain linked by a disulfide bond to a lighter protein chain. The heavy chain can bind to synaptic membranes, and the entire molecule is then taken up into the synaptic terminal by receptor-mediated endocytosis. The BTX-A light chain specifically hydrolyzes SNAP-25.

When the light chain interferes and cleaves SNAP-25, the ACh-containing vesicles can no longer fuse with the plasma membrane, and exocytosis of ACh is inhibited [25, 26] leading to paralysis in clinically developed botulism. However, the effects of BTX-A on the inhibitory mechanism at other non-adrenergic, non-cholinergic nerve terminals are insufficiently known. Our results indicate that BTX-A also inhibits SP and VIP release from nasal mucosa during AR. Recent evidence suggests an inhibitory effect of BTX-A on neuropeptide release from the trigeminal nerve terminals of the rabbit iris sphincter muscle [27] and blood vessels [28]. These data indicate that BTX-A may not only inhibit ACh release in striated muscles but also SP and VIP release from the trigeminal and parasympathetic nerves in nasal mucosa of AR.

Conclusion

Local BTX-A treatment is an effective method to reduce AR symptoms induced by OVA, including rhinorrhea, nasal obstruction and sneezing. The decrease in SP- and VIP-IR expression after BTX-A treatment suggests that the effect of BTX-A on nasal mucosa is via the suppression of neuropeptide release, which plays a major role in autonomic mucosal innervation and in the pathophysiology of AR.

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