ABSTRACT — In guinea pigs actively sensitized with ovalbumin, we have observed that nasal hyperreactivity and hypersensitivity to methacholine causes nasal secretion accompanied by an increase in the density of muscarinic acetylcholine receptor (m-ACh•R) following the appearance of nasal symptoms induced by the ovalbumin challenge. Therefore in the present investigation, we studied the relationship between the density of m-ACh•R and nasal hypersecretion in actively sensitized guinea pigs. There was no relationship between the quantity of nasal secretion and serum Ig G1 or Ig E levels. On the other hand, the sensitivity to methacholine and nasal secretion induced by methacholine were significantly related to the density of m-ACh•R located on the nasal mucosa. The quantity of nasal secretion induced by allergen was also correlated significantly to the density of m-ACh•R. These results suggest that the nasal hypersecretion observed in the nasal allergic model may be closely associated with an increase in the density of m-ACh•R located on the nasal mucosa.

Keywords: Muscarinic receptor, Nasal hypersensitivity, Allergic rhinitis, Nasal provocation, Serum antibody level

In the present study, the relationship between the nasal hypersecretion and density of m-ACh•R was investigated by using guinea pigs actively sensitized with ovalbumin to clarify the influence of impaired function of the parasympathetic nervous system on the appearance of nasal mucosal hypersensitivity.

MATERIALS AND METHODS

Experimental animals
Male Hartley guinea pigs (Japan SLC) weighing 400 to 500 g were used. The animals were housed in a temperature-controlled room at 24°C with a 12-hr light cycle (lights on from 08:00 to 20:00), and food and water were given ad libitum.

Chemicals
Chemicals were purchased from the following companies: Fluorescein sodium (uranin, Kishida), methacholine chloride, atropine sulfate (Wako), phentolamine hydrochloride (Ciba-Geigy), dl-propranolol hydrochloride (Sigma) and ovalbumin (egg albumin, Seikagaku Kogyo).
Radioligands were purchased from the following companies: L-quinuclidinyl [phenyl-4-3H] benzilate ([3H]QNB, 1.70 TBq/mmol; Amersham), [7-methoxy-3H] prazosin ([3H]prazosin, 3.03 TBq/mmol; New England Nuclear) and L-[phenyl-2,3-3H] dihydroalprenolol ([3H]DHA, 2.22 TBq/mmol; Amersham).

Active sensitization of guinea pigs and their nasal provocation

According to the method of Terada et al. (12), guinea pigs were intraperitoneally immunized seven times every 2 weeks with 20 μg of ovalbumin and 10 mg of aluminum hydroxide in 1 ml of saline. After that, airway sensitization was performed for 5 consecutive days by ultranebulization with 2 ml of 0.25% ovalbumin in saline. A week thereafter, nasal challenge was carried out by application of 50 μl of 1% ovalbumin on the anterior nares of both sides, which caused typical nasal symptoms in most of the guinea pigs used.

Antiserum and their titer in passive cutaneous anaphylaxis

Sera used for passive sensitization were collected from the actively sensitized guinea pigs used in our experiments. According to the method of Ovary et al. (13) with slight modifications, the Ig G1 and Ig E levels in the serum of each guinea pig were estimated by 4-hr and 7-day homologous passive cutaneous anaphylaxis (PCA) titer, respectively. In brief, serial doubling dilutions of serum were made with saline from 250- to 16,000-fold. Guinea pigs were sensitized by intradermal injection of 0.1 ml of antiserum. After 4 hr or 7 days, the animals were challenged with an intravenous injection of 0.1 ml of 0.5% Evans blue dye solution containing 0.5 mg of ovalbumin. After 30 min, the animals were sacrificed, and the diameter of blue spots were measured from the inner surface of the skin. The PCA titer was expressed as the highest dilution still giving a positive reaction of more than 5 mm in diameter.

Passive sensitization of guinea pigs

Guinea pigs were passively sensitized with pooled antiserum. The potency of the sera was 8,000 or 1,000 as titrated by 4-hr or 7-day homologous PCA, respectively. A series of each antiserum (dilutions: 1/1-1/16) was administered intravenously into the auricular vein in a volume of 1 ml/kg body weight 2 days prior to nasal provocation.

Quantitative measurement of nasal secretion

Using the method of Namimatsu et al. (14) based on capillary action, nasal secretion was measured by using defatted cotton threads (No. 40/2; Yokota, Japan). The threads were dyed with 10% fluorescein sodium in saline at one end and were cut into 100-mm-long pieces, of which 10 mm from one end was colored. Nasal provocation by allergen was carried out by application of 50 μl of 1% ovalbumin on the unilateral anterior naris. Ten minutes after nasal provocation, nasal secretion under immobilization with handling was measured with dyed thread, one end of which was inserted into the unilateral anterior naris and kept there for 60 sec. The stretch of color on the thread due to nasal secretion was regarded to indicate the quantity of nasal secretion.

Measurement of reactivity and threshold of sensitivity in nasal mucosa

Reactivity and sensitivity of nasal mucosa were tested by applying 10 μl of methacholine into the unilateral anterior naris. Methacholine solutions of different concentrations (dissolved in saline), a dilution series, were consecutively applied at intervals of 10 min to determine the reactivity and sensitivity of individual animals. The minimum concentration of methacholine which induced a stretch of color beyond 5 mm due to nasal secretion was regarded to be the threshold of sensitivity (14).

Preparation of membrane protein samples

Actively sensitized guinea pigs were sacrificed 2 or 3 days after nasal challenge with ovalbumin, and then their nasal mucosae were isolated. The nasal mucosa was weighed and immediately stored at −80°C until use. The nasal mucosa was homogenized in a Polytron in 100 volumes of ice-cold 50 mM Tris-HCl (pH 7.4). The homogenate was filtered through nylon mesh to remove connective tissue debris and then centrifuged at 50,000 × g for 20 min at 4°C. The pellet was suspended in 10 ml of incubation buffer (50 mM Tris-HCl buffer, 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, pH 7.4), and chilled in an ice bath until use.

Receptor binding assay

Radioligand binding assays for mACh-R, α1-R and β-R were carried out according to the method of Ishibe et al. (5) with slight modifications.

The standard binding assay was carried out in 1 ml of incubation buffer containing approximately 1 mg membrane protein and radioligand in the presence or absence of an excess of 2 × 10-6 M atropine for [3H]QNB binding, 10-5 M phentolamine for [3H]prazosin binding or 10-6 M propranolol for [3H]DHA binding. Specific receptor binding is defined as the total binding of the radioligands subtracted by the nonspecific binding observed in the presence of the excess competitors. Specific binding was usually 50–70% of the total bind-
The binding reaction was initiated by the addition of the membrane protein; and the reaction mixture was incubated for 30 min at 25°C for \(^{3}H\)QNB binding, for 40 min at 25°C for \(^{3}H\)prazosin binding, and for 10 min at 37°C for \(^{3}H\)DHA binding, in a water bath with shaking. After incubation, the mixture of membrane protein and radioligand was rapidly filtered in vacuo through a Whatman GF/F glass filter, and the filter was washed 3 times with 3 ml of ice-cold 50 mM Tris-HCl buffer. The filter was transferred to a scintillation vial; and after drying at 60°C for 5–12 hr, 5 ml of toluene-based scintillator (AL-1, Dojin) was added, and radioactivity was counted in a liquid scintillation counter. The protein content of the membrane samples was determined by the method of Lowry et al. (15) with bovine serum albumin as a standard.

**Analyses of data**

The data in the figures are expressed as the mean ± S.E. Scatchard analyses (16) were used to obtain values for the maximum number of binding sites (B\(_{\text{max}}\)) and dissociation constants (K\(_{D}\)). Statistical significance of difference was determined by Student's t-test or one way analysis of variance with Dunnett's multiple range test. The straight lines are the least squares linear regression fit of these data.

**RESULTS**

Hyperreactivity and hypersensitivity in nasal mucosa of actively sensitized guinea pigs

As parameters for nasal mucosal hyperreactivity and hypersensitivity, we used reactivity and sensitivity to methacholine in the nasal mucosa of guinea pigs.

In actively sensitized guinea pigs that were challenged with allergen after airway sensitization, the reactivity to methacholine was significantly increased compared with normal ones, and the threshold of sensitivity to methacholine in causing nasal secretion was correspondingly lowered. On the other hand, no significant change was observed in sensitized guinea pigs not subjected to nasal challenge or airway sensitization (Fig. 1).

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**Fig. 1.** Reactivity and sensitivity to methacholine in the nasal mucosa of guinea pigs actively sensitized with ovalbumin. **, **: significantly different from the non-sensitized group at P < 0.05 and P < 0.01, respectively. Data represent the mean values ± S.E. of 8 guinea pigs. Nasal mucosal reactivity and sensitivity were measured by applying 10 μl of methacholine into the left anterior naris 1 day following a nasal challenge with ovalbumin, or groups without challenge. A dilution series of methacholine was used to determine the concentration just required to cause nasal secretion.
Changes in autonomic nerve receptors in nasal mucosa

As shown in Fig. 2, the maximum binding ($B_{\max}$) of $[^3H]QNB$ to m-ACh•R in actively sensitized guinea pigs after challenge increased significantly compared with that in the non-sensitized group, whereas the $B_{\max}$ of $[^3H]prazosin$ to $\alpha_1$-R and the $B_{\max}$ of $[^3H]DHA$ to $\beta$-R significantly decreased. Though not shown in the figure, each individual $B_{\max}$ of $[^3H]QNB$ to m-ACh•R was not necessarily associated with each individual $B_{\max}$ of $[^3H]prazosin$ to $\alpha_1$-R or of $[^3H]DHA$ to $\beta$-R. On the other hand, our studies showed no significant difference between the two groups with regards to the affinities of these three receptors.

Relation between nasal secretion and serum antibody level in passively or actively sensitized guinea pigs

To investigate the relationship between the nasal secretion and serum antibody level, guinea pigs passively sensitized with various dilutions of antiserum as well as actively sensitized guinea pigs were used. In the passively sensitized group, the quantity of nasal secretion was found to be proportional to the serum Ig G1 and Ig E levels (Fig. 3). In the actively sensitized group, however, there were no relationships between the quantity of nasal secretion and serum Ig G1 or Ig E levels (Fig. 4).

Relationship between nasal hypersecretion and density of m-ACh•R in actively sensitized guinea pigs

The threshold of sensitivity to methacholine and nasal secretion induced by methacholine were significantly related to the density of m-ACh•R located on the nasal mucosa (Fig. 5). The quantity of nasal secretion induced by allergen, therefore, was also related to the density of m-ACh•R (Fig. 6).
Fig. 4. Relationship between nasal secretion and serum antibody levels in guinea pigs actively sensitized with ovalbumin. Data represent the mean values ± S.E. Nasal provocation was carried out by application of 50 μl of 1% ovalbumin on the unilateral anterior naris. Antisera for measurement of serum antibody levels were collected from actively sensitized guinea pigs 2 days following measurement of nasal secretion. The Ig G_{1} and Ig E levels in the serum of individual guinea pigs were estimated by 4-hr and 7-day homologous PCA titer, respectively.

Fig. 5. Relationship between nasal hypersecretion induced by methacholine and the density of m-ACh•R in guinea pigs actively sensitized with ovalbumin. Nasal mucosal reactivity and sensitivity were measured by applying 10 μl of methacholine into the left anterior naris. A dilution series of methacholine was used to determine the concentration just required to cause nasal secretion. Actively sensitized guinea pigs for measurement of m-ACh•R were sacrificed 2 or 3 days following measurement of nasal mucosal reactivity and sensitivity.
DISCUSSION

Nasal allergy is considered to be a Type I allergic reaction in which Ig E plays an important role (1). On the other hand, it has been empirically known that the appearance of non-specific hypersensitivity and hyperreactivity in the nasal mucosa of patients with nasal allergy may be due to imbalance of autonomic nervous systems in the nasal mucosa. Ando et al. (17) found that pathogenesis of hyperreactive nasal symptoms in nasal allergic patients may be associated with changes in the density of autonomic nerve receptors in the nasal mucosa. In our former study on SART (intermittent exposure to cold)-stressed guinea pigs (18), we also found that an increase in density of m-ACh-R located on nasal mucosa could be related to the appearance of nasal mucosal hypersensitivity and the aggravation of nasal allergy. Thus, in addition to its immunological mechanism, the changes in densities of autonomic nerve receptors seem to have an important role in the appearance of hypersensitivity in the nasal mucosa. To validate this speculation, we carried out the following experiments.

First to study the process of the appearance of nasal mucosal hypersensitivity, we tested guinea pigs actively sensitized with ovalbumin for the reactivity and sensitivity of the nasal mucosa to methacholine before and after nasal challenge with ovalbumin. Following the appearance of nasal symptoms induced by ovalbumin challenge, nasal mucosal hypersensitivity was accompanied by an increase in density of m-ACh-R and a decrease in densities of α1-R and β-R, although no significant changes could be observed by ovalbumin-sensitization alone. In the nasal mucosa of guinea pigs sensitized with bacterial crystalline α-amylase, which showed typical asthmatic symptoms but without nasal symptoms, no change in the density of m-ACh-R was observed (19). Thus, changes in the density of receptors observed in actively sensitized guinea pigs are not induced by systemic sensitization itself. The nasal symptoms induced by the allergen-antibody reaction on the nasal cavities may be of importance for the induction of nasal mucosal hypersensitivity with changes in receptor density. In addition, this actively sensitized animal would be useful as model for nasal mucosal hypersensitivity in the study of nasal allergy.

Secondly, the relationship between nasal hypersecretion and serum antibody level was studied. The quantity of nasal secretion in the actively sensitized model was independent of serum Ig G1 or Ig E levels, although in the passively sensitized model, it was proportional to the antibody levels. The gap between the two sensitized models could not explained by this experiment, but the result in the actively sensitized model seemed to be similar to that observed in patients with nasal allergy (20). Thus, nasal hypersecretion may have little association with serum antibody levels within the range observed in the actively sensitized model in this experiment.

Konno and co-workers (6, 19, 21) stated from their results with guinea pigs that the increase in density of m-ACh-R may facilitate nasal secretion while the decrease in density of α1-R may facilitate swelling of the nasal mucosa. However, the relationship between nasal symptoms and changes in densities of receptors is not clear because nothing was mentioned about the nasal symptoms in their reports. Therefore, we finally focused our study on the relationship between nasal hypersecretion and the density of m-ACh-R located on the nasal mucosa by using actively sensitized guinea pigs that had been subjected to nasal challenge. It was found that the threshold of sensitivity to methacholine or the quantity of nasal secretion induced by methacholine was significantly related to the density of m-ACh-R. The quantity of nasal secretion induced by allergen was also related significantly to the density of m-ACh-R. Although the localizations of m-ACh-R and the other receptors on the nasal mucosa could not be identified by the receptor binding assay alone, it was confirmed by the immunohistochromical technique that the parasympathetic or sympathetic nervous system was
mainly innervating nasal glands or around the arterioles, respectively (22). In addition, it was reported by Watase and Okuda (4) that various adrenergic agonists or antagonists had little influence on nasal secretion. These results suggest that the nasal hypersecretion observed in the nasal allergic model may be closely related to the increase in density of m-ACh-R located on the nasal mucosa.

In another study of ours, we experimentally confirmed (23) that nasal secretion from nasal glands mediated via a nerve reflex may be elicited by histamine released by the allergen-antibody reaction on histamine H1 receptors (H1-R) at the sensory nerve endings. Therefore, measurement of H1-R density also seems to be necessary to explain the nasal hypersecretion. However, we failed to show the relationship between H1-R density and nasal hypersecretion (24). Ishibe et al. (25) also reported that there was no significant correlation between H1-R density and clinical findings such as Ig E and peripheral eosinophils in patients with nasal allergy. Since H1-R are widely distributed in blood vessels, gland cells, etc. (26), as well as in the sensory nerve endings, it seems difficult to explain the nasal hypersecretion from the results of H1-R density measured by the receptor binding assay alone. In order to solve the problems remaining in our study, we are planning to investigate the distribution and density of parasympathetic and sensory neurons in the nasal mucosa of the nasal allergic model by immunohistochemical demonstration of choline acetyltransferase and calcitonin gene-related peptide. When such experiments are added to our present study, the relationship between impaired function of the parasympathetic or sensory nervous system and nasal hypersecretion will be made clearer.

In conclusion, one of the factors to induce the nasal mucosal hypersensitivity and nasal hypersecretion is assumed to be an increase in the density of m-ACh-R though an immunological mechanism is of importance in the onset of nasal allergy.

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