

Overexpression of the anaphylatoxin receptors, complement anaphylatoxin 3a receptor and complement anaphylatoxin 5a receptor, in the nasal mucosa of patients with mild and severe persistent allergic rhinitis

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Background: Although the complement anaphylatoxin peptides, complement anaphylatoxin 3a (C3a) and complement anaphylatoxin 5a (C5a), are implicated in the inflammatory process in allergic rhinitis, a direct interaction between allergic mucosa and complement receptors has not been demonstrated. **Objective:** We investigated the expressional levels and distributional patterns of the C3a receptor (C3aR) and C5a receptor (C5aR) in normal, mild, and severe persistent allergic nasal mucosa.

Methods: Immunohistochemistry and Western blotting using C3aR and C5aR antibodies were applied to normal nasal, mild, and severe persistent allergic nasal mucosa.

Results: Complement anaphylatoxin 5a receptor was detected in the inflammatory cells of normal and allergic nasal mucosa, and its expression level was significantly higher in allergic nasal mucosa than normal nasal mucosa. C3aR in normal and allergic nasal mucosa was commonly expressed in nonmyeloid cells such as epithelial cells, submucosal glands, and nerve fibers. In addition, C3aR was expressed in the endothelium of cavernous sinuses and the surrounding perivascular muscle layer in severe persistent allergic nasal mucosa, but not in normal and mild allergic nasal mucosa. Western blotting demonstrated that the expression level of C3aR was significantly increased in severe persistent allergic nasal mucosa compared with normal and mild nasal mucosa.

Conclusion: On the basis of the location of C3aR and C5aR, C5aR may play a role in activation of inflammatory cells, whereas C3aR may mediate mucus secretion and mucosal swelling in allergic nasal mucosa, especially severe persistent allergic nasal mucosa. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: C3aR, C5aR, normal nasal mucosa, allergic nasal mucosa

Allergic rhinitis is one of the most prevalent health problems in industrialized countries and is considered to be a complex inflammatory disorder of the nasal airways characterized by various pathophysiologic features.¹ The clinical symptoms are nasal itching, sneezing, rhinorrhea, and nasal congestion. Conservative medical management usually alleviates these symptoms. However, in patients with persistent allergic rhinitis for many years, a severe drug-resistant hypertrophy may develop, which leads to constant nasal obstruction and watery nasal drip.² There have been reported a good number of surgical procedures to treat patients with drug-resistant nasal obstruction.^{3,4} Examination of tissue from patient with persistent allergic rhinitis refractory to medical treatment suggests that the relative proportion of venous sinusoids is increased significantly in all aspects of the hypertrophied mucosa.^{2,5} Epithelial denudation, fibrosis, inflammation, and engorged venous sinusoids are noted in hypertrophied turbinate mucosa.^{2,5} However, more information is needed to appreciate the pathogenetic characteristics of persistent allergic rhinitis.

Although various mediators and cytokines may be involved in allergic rhinitis, recent studies suggest that complement activation products, especially the anaphylatoxins, complement anaphylatoxin 3a (C3a) and complement anaphylatoxin 5a (C5a), contribute to the inflammation of allergic rhinitis.^{6,7} Further, many pathophysiologic features such as the recruitment of inflammatory cells and increased vascular permeability are consistent with the actions of C3a and C5a.⁸ C3a and C5a regulate inflammatory functions by interacting with their receptors, C3a receptor (C3aR) and C5a receptor (C5aR), both of which belong to the rhodopsin family of 7 transmembrane G protein-coupled receptors.⁹⁻¹⁶ Various studies have further demonstrated that C3aR and C5aR expression is upregulated in various organs under inflammatory conditions, including fatal asthma.¹⁷⁻²² Although all of these properties are relevant to the pathogenesis of allergic rhinitis, precise participation of these anaphylatoxins in allergic rhinitis has not been sufficiently evaluated. To elucidate the role of C3a and C5a in these diseases, it is necessary to define the expression and distributional pattern of C3aR and C5aR in allergic rhinitis. Establishing the location of these receptors is important to understand the role of complement in allergic rhinitis and to provide direction for future study of possible alterations in complement regulation or signaling that may play a pathophysiologic role in this condition.

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Abbreviations used

C3a: Complement anaphylatoxin 3a
 C3aR: Complement anaphylatoxin 3a receptor
 C5a: Complement anaphylatoxin 5a
 C5aR: Complement anaphylatoxin 5a receptor

In the current study, we investigated the level of expression and pattern of distribution of C3aR and C5aR in the normal nasal mucosa of patients undergoing augmentation rhinoplasty and in the nasal mucosa of patients with mild and severe persistent allergic rhinitis by using immunohistochemistry and Western blotting.

METHODS**Subjects**

Twelve patients with mild persistent allergic rhinitis, 12 patients with severe persistent allergic rhinitis, and 12 healthy control subjects were enrolled in this study. Patients undergoing augmentation rhinoplasty were considered healthy control subjects and were excluded if they had a history of nasal infection or allergy, smoking, or ongoing drug treatment. All patients with allergic rhinitis had typical symptoms of perennial nasal allergy, nasal eosinophilia, and a positive skin test or positive RAST result to house dust mites, as well as positive reactions on provocation tests. Patients with allergy and exacerbation of upper respiratory infection during the 6 weeks preceding the study were not included in the study. Other exclusion criteria included current smoking; a medical history of recurrent infections of the respiratory tract; and use of topical or oral corticosteroids, leukotriene modifiers, and antihistamines during the 8 weeks that preceded the study.

Twelve pieces of normal nasal mucosa were obtained from the inferior turbinates of 12 healthy control subjects (7 men and 5 women; age range, 20–35 years) who had normal nasal mucosa and were admitted for augmentation rhinoplasty.

Allergic nasal mucosa was obtained from 12 patients (8 men and 4 women; age range, 22–38 years) with severe persistent allergic rhinitis during septo-turbinate surgery, and their turbinate mucosa showed an edematous and congested appearance (Fig 1). These patients had been previously treated with medical management (antihistamine and intranasal steroid spray) without satisfactory improvement for at least 3 months. They had 1 or more of the following symptoms: sleep disturbance, impairment of daily activities, leisure and/or sports, and impairment of school or work.

Nasal biopsy specimens were also obtained from the inferior turbinate of 12 patients (9 men and 3 women; age range, 19–39 years) with mild persistent rhinitis with Blakesley forceps (Fig 1) under local anesthesia. Patients with mild persistent rhinitis had none of the symptoms mentioned. Before obtaining the tissue specimens, the protocols and informed consent form were approved by the institutional review boards for human beings at our institution, and all participants gave written informed consent.

All tissue samples were divided into 2 portions. One portion was frozen in liquid nitrogen and stored at -70°C for protein isolation. For immunohistochemistry, another sample was also frozen in liquid nitrogen and stored at -70°C for a subsequent procedure.

Immunohistochemical and Western blot analysis

Complement anaphylatoxin 3a receptor antibody is a rabbit polyclonal antibody raised against amino acids 183 to 482 mapping at the C-terminus of C3aR of human origin, and C5aR is directed against amino acids 251 to 350 of C5aR of human origin. These antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif). Immunohistochemical staining was performed by using a peroxidase-labeled streptavidin-biotin technique. Briefly, the specimens were embedded in optimal cutting temperature

compounds (Tissue Tek; Miles Laboratories, Elkhart, Ind) and snap-frozen in liquid nitrogen. Frozen sections (12 μm) were cut on a cryostat and placed on poly-L-lysine-coated glass slides. Sections were fixed in cold acetone solution, followed by PBS, and were treated with 0.3% H_2O_2 in methanol for 15 minutes to quench endogenous peroxidase activity. After washing in PBS, the sections were incubated with 10% normal goat serum to block nonspecific binding. Sections were then incubated overnight at 4°C with a 1:100 dilution of anti-C5aR (Santa Cruz Biotechnology, Inc) or a 1:50 dilution of anti-C3aR (Santa Cruz Biotechnology, Inc) primary polyclonal rabbit antibodies. After washing in PBS, the sections were treated with biotinylated goat antirabbit IgG and subsequently with a streptavidin-peroxidase conjugate (Vector Laboratories, Burlingame, Calif). The 0.02% diaminobenzidine hydrochloride containing 0.03% H_2O_2 is used as chromogen to visualize the peroxidase activity. Negative immunohistochemical control procedure included omission of the primary antibodies and replacement of the primary antibodies by normal rabbit IgG in appropriate concentrations. For peptide blocking studies, a series of 2 whole sections of inferior turbinate mucosa from patients with mild and severe persistent allergic rhinitis were incubated with each antibody, which had been preincubated for 1 hour with peptides 183 to 482 mapping at the C-terminus of C3aR and peptides 251 to 350 of C5aR.

Submucosal inflammatory cell staining for C5aR antibody was reported as the number of positively stained inflammatory cells per field. Sections were counted in a blind fashion by a single observer, with a random sample recounted by a second individual, with good reproducibility. Two sections and 4 random fields per section were analyzed for each subject. Further, to assess the staining intensity of C3aR, independent examination by 2 different investigators was used in all tissue sections. Intensity of C3aR expression was classified into 4 degrees as follows: (–), negative; (+), weakly positive; (++) , moderately positive; and (+++) , strongly positive. Quantitative analyses of this intensity for each patient were calculated as the mean of the total score in tissue specimen.

For Western blot analysis, samples frozen in liquid nitrogen were crushed into pieces and vigorously vortexed in a buffer solution containing 50 mmol/L TRIS-HCl (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100 (Sigma, St Louis, Mo), and 1 mmol/L phenylmethylsulfonyl fluoride. A total of 50 μg extracted protein was resuspended in SDS sample buffer and boiled for 5 minutes. Equal amounts of total protein was separated on 12 % SDS-polyacrylamide gels and transferred to Immobilon (Millipore, Bedford, Mass).

The blots were reacted with the anti-C5aR or anti-C3aR antibodies in TRIS-buffered saline (20 mmol/L TRIS-HCl pH 8.0, 150 mmol/L NaCl, 0.1% Triton X-100, and 0.5 % skim milk) for 2 hours at 4°C , with or without 10 and 100 $\mu\text{g}/\text{mL}$ synthetic peptides, and then reacted with a biotin-conjugated anti-rabbit IgG antibody (Vector Laboratories) in PBS for 1 hour. After several washings with TRIS-buffered saline, the membranes were incubated with the avidin-biotin-peroxidase complex. Antibody reactions were detected by using the ECL detection kit (Amersham Bioscience, Piscataway, NJ), followed by detection of chemiluminescence on x-ray film. As an internal control, β -actin expression was analyzed in parallel blots by using the β -actin antibody (Sigma, St Louis, Mo).

The intensity of detected bands was quantified by using scion image Beta 4.0.2 (Scion Corp, Frederick, Md). Relative intensities of C5aR and C3aR signals were obtained by dividing intensities of C5aR and C3aR signals by those of β -actin signals.

Statistical analysis

Age differences in the 3 groups were calculated by 1-way ANOVA, and the Bonferroni *post hoc* correction was applied. The duration of rhinitis and the number of C5aR positive inflammatory cells between the mild and persistent severe rhinitis groups were statistically evaluated by the Mann-Whitney *U* test. The Mann-Whitney *U* test was also used to compare the relative intensities of C5aR and C3aR signals between normal control and mild persistent allergic nasal mucosa, between normal control and severe persistent allergic nasal mucosa, and between the mild and severe persistent allergic nasal mucosa. The level of significance was set at $P < .05$.

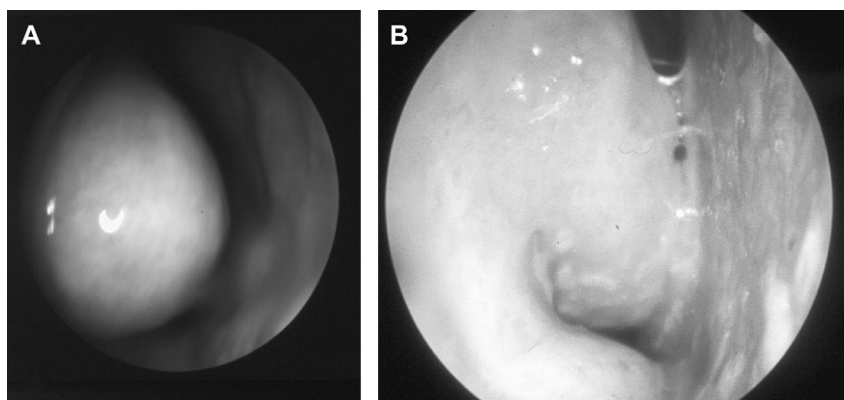


FIG 1. Endoscopic findings of the nasal cavity. **A**, Inferior turbinate of a patient with mild persistent allergic rhinitis. **B**, Inferior turbinate of a patient with severe persistent allergic rhinitis, showing an edematous and congested appearance.

TABLE I. Clinical characteristics of patients

Characteristic	Severe persistent rhinitis (n = 12)	Mild persistent rhinitis (n = 12)	Normal (n = 12)
Female/male	4/8	3/9	5/7
Age (y)	30.5 ± 5.63	31.2 ± 5.81	28.5 ± 4.6
Rhinitis duration (y)	13.74 ± 1.13	5 ± 1.73	NA
Inflammatory cell counts (cells per field)	20.1 ± 3.0	19.3 ± 2	0.3 ± 0.2
Size of wheal (mm)	6.82 ± 3.32	6.76 ± 2.63	NA
Size of flare (mm)	20.18 ± 12.26	19.78 ± 11.55	NA

Nasal provocation test was performed by using solutions containing extracts of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* (both 5000 biological units/mL; Allergopharma, Reinbek, Germany). The solutions were sprayed into the less congested nostril (100 μ L solution). Data are expressed as mean values $\pm$ SDs.

NA, Not applicable.

RESULTS

The characteristics of patients with mild and severe persistent rhinitis and normal controls are shown in Table I. Age was similarly distributed among the normal controls and the patients with mild and severe persistent allergic rhinitis (Table I). All patients with persistent severe rhinitis had an early onset of allergic rhinitis and showed disease duration significantly longer than patients with mild persistent rhinitis ($P = .0004$; Table I).

To examine the expression and pattern of distribution of C3aR and C5aR in normal and allergic nasal mucosa, sections from these tissues were analyzed by immunohistochemistry. Staining by immunohistochemistry showed expression of C3aR and C5aR protein in normal controls and patients with mild and severe persistent allergic nasal mucosa. As summarized in Table II, almost all C5aR-positive cells were myeloid cells, whereas C3aR was expressed exclusively in nonmyeloid cells. That is, C5aR was detected exclusively in infiltrating inflammatory cells in normal (Fig 2, A) and both types of allergic nasal mucosa (Fig 2, B and C). However, C5aR expression was not observed in nonmyeloid cells such as the epithelial cells, vascular smooth muscle or endothelial cells, and submucosal glands in each specimen. In comparison with normal nasal mucosa, the number of C5aR-positive myeloid cells was increased in both types of allergic nasal mucosa, which was verified by counting the number of positive cells ($P = .0005$; Table I) and analyzing Western blots ($P = .003$; $P = .0004$; Fig 3). However, there was no significant difference in the number of C5aR-positive cells between the mild and severe persistent allergic rhinitis mucosa ($P = .12$; Table I).

Immunohistochemical analysis for C3aR showed abundant signaling in sections of normal controls and both types of allergic nasal mucosa (Fig 2, D-I). C3aR was expressed in the epithelial cell layers, where strong immunopositivity was found in the whole layers (Fig 2, D-F). Next to staining of epithelial cells, submucosal glands exhibited C3aR immunopositivity (Fig 2, D-F). Within the glandular structures, C3aR immunopositivity was intensely expressed in some portions but not in other portions (Fig 2, D-F). Further, C3aR was found in some nerve fibers, which were distributed in the superficial and deep submucosa (Fig 2, E, F, and H). Interestingly, the endothelium of cavernous sinusoids with a perivascular muscle layer showed strong immunopositivity in severe persistent allergic nasal mucosa (Fig 2, I) but not in mild persistent allergic nasal mucosa and normal nasal mucosa (Fig 2, G and H), which was consistently noted in all nasal mucosa specimens examined in this study. These findings for C3aR and C5aR were completely inhibited when the antibodies were preincubated in the presence of synthetic peptides (Fig 4).

Western blot analysis also showed the presence of C5aR and C3aR in normal and allergic nasal mucosa (Fig 3). The levels of C5aR expression were increased in both types of allergic nasal mucosa compared with normal controls but were not significantly different between the mild and persistent allergic nasal mucosa. Further, the difference in the levels of C3aR expression was statistically significant between mild and severe persistent allergic nasal mucosa but did not reach statistical significance between normal controls and the mild persistent allergic rhinitis mucosa examined. Incubation with 10 and 100 μ g/mL of each synthetic

TABLE II. Distribution pattern of C3aR and C5aR in normal and allergic nasal mucosa

	Normal nasal mucosa		Allergic nasal mucosa			
	C5aR	C3aR	Mild persistent		Severe persistent	
			C5aR	C3aR	C5aR	C3aR
Epithelium	–	+++	–	+++	–	+++
Submucosal gland	–	++	–	++	–	++
Nerve	–	+	–	+	–	+
Sinusoidal vessel and perivascular muscle	–	–	–	–	–	+++
Inflammatory cells	+	–	+++	–	+++	–

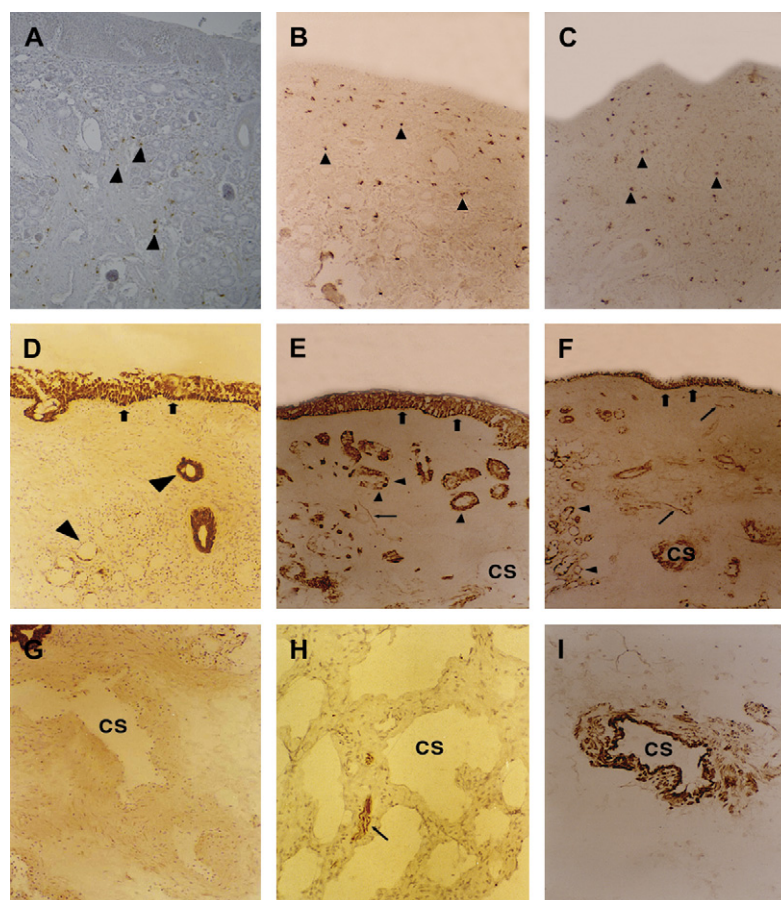


FIG 2. Localization of C5aR and C3aR in normal (A, D, G), mild persistent (B, E, H), and severe persistent allergic nasal mucosa (C, F, I). The C5aR (A–C) is exclusively located in the inflammatory cells (arrowhead) in normal, mild, and severe persistent allergic nasal mucosa, whereas C3aR (D–I) is mainly found in the whole epithelial layers (thick arrow), a portion of submucosal glands (arrowhead), nerve fibers (thin arrow), and the endothelium of cavernous sinusoid vessel (CS). A–F, Original magnification $\times 100$; G–I, original magnification $\times 200$.

peptide, together with the primary antibody, resulted in almost complete inhibition of the reaction (Fig 3).

DISCUSSION

The possible role of complement activation in the development of allergic symptoms has been reported by several authors.^{6,7,23} Andersson et al⁶ reported the generation of C3a and C5a after nasal allergen challenge concomitantly with the development of nasal symptoms in patients with allergic rhinitis. Hidvégi et al⁷ suggested that in patients with allergy, nasal challenge with

ragweed allergen induces a rise in complement activation in the nasal lavage fluid, highlighting the role of the complement system in the allergic inflammation on the nasal mucosal surface. Therefore, these data imply that anaphylatoxin receptors, C3aR and C5aR, may readily contribute to the biology of the nasal mucosa that is activated by inflammatory mediators. Nevertheless, no studies have yet defined the expression and significance of C3aR and C5aR in allergic nasal mucosa. This study documented, for the first time, the expression and distribution of C3aR and C5aR in normal nasal mucosa, and mild and persistent severe allergic nasal mucosa. The principal findings of these studies were

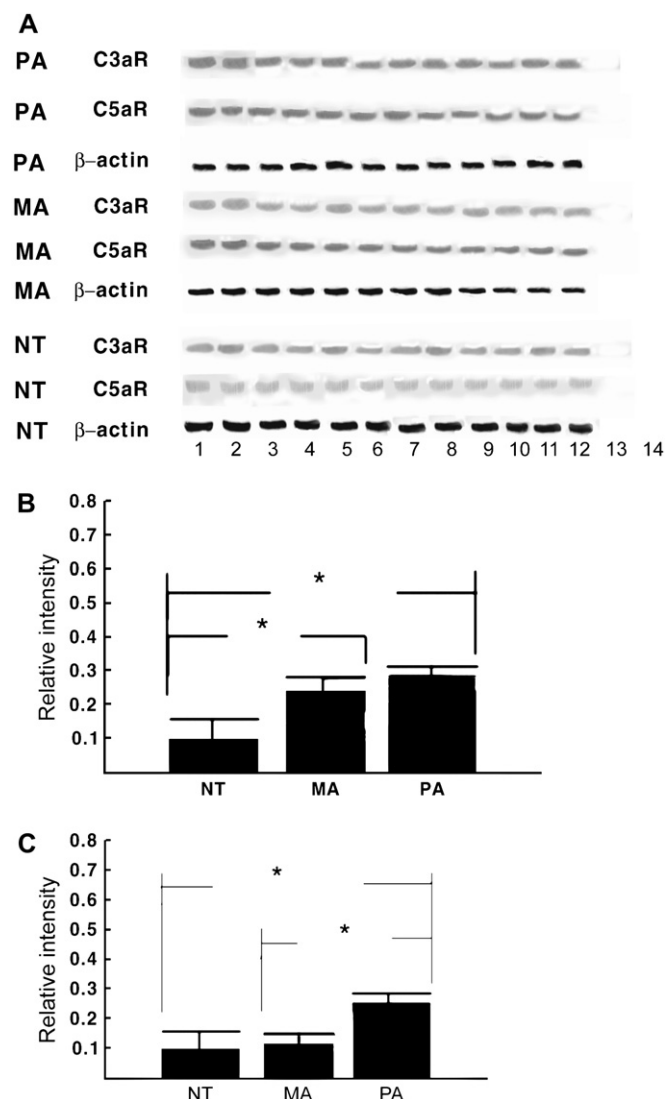


FIG 3. The levels of C5aR and C3aR expression analyzed by Western blot (**A**) and densitometry (**B**, C5aR, **C**, C3aR). Incubation with 10 and 100 μ g/mL synthetic peptide, together with the each primary antibody, resulted in almost complete inhibition of the reaction (lanes 13 and 14). MA, Mild persistent allergic nasal mucosa; NT, normal nasal mucosa; PA, severe persistent allergic nasal mucosa. * $P < .05$.

that the expression of C5aR appears to be restricted to the inflammatory cells infiltrated in normal nasal mucosa and both types of allergic nasal mucosa, and that C3aR was expressed in nonmyeloid cells in normal and allergic nasal mucosa. Interestingly, the high expression of C3aR in cavernous sinusoidal vessels was found only in severe persistent allergic nasal mucosa but was not noted in normal and mild persistent allergic nasal mucosa, suggesting an important role on mucosal swellings in severe persistent allergic rhinitis.

It is generally known that the complement system is an important component of both innate and adaptive immunity. The complement system helps to defend the host by initiating inflammatory and immunologic responses and by promoting the cell lysis and death of invading microorganisms.^{4,5} Complement can be activated through the classic, alternative, and lectin pathways. The third component of the complement system, C3, is

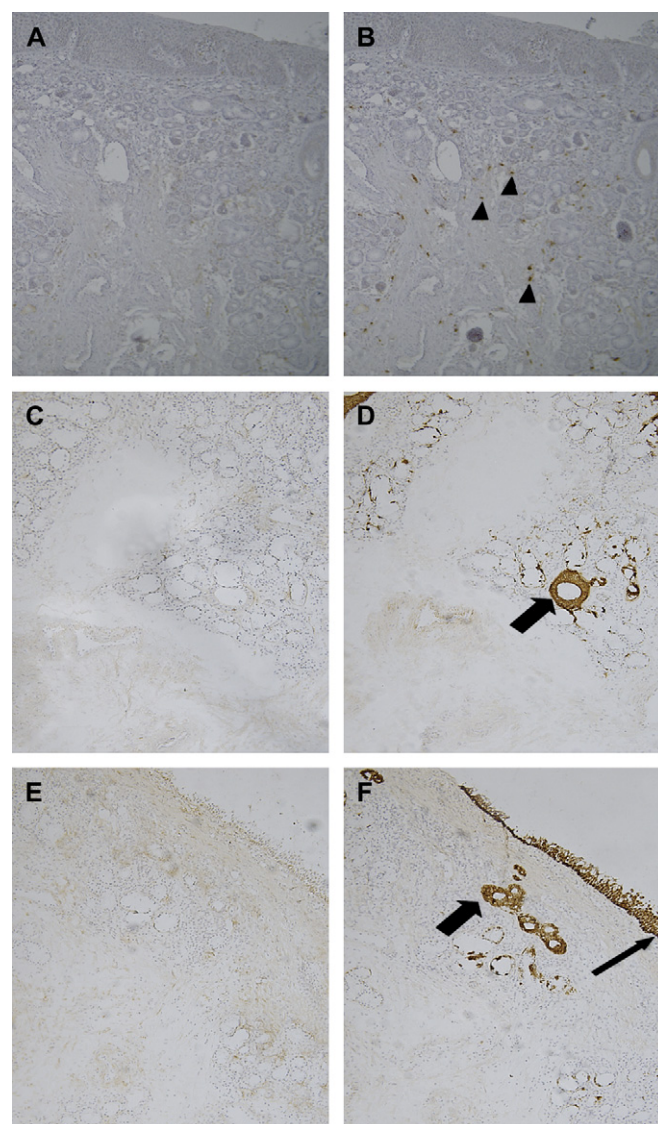


FIG 4. Immunohistochemical staining of serial sections of normal nasal mucosa with C5aR antibody with (**A**) and without (**B**) preincubation with C5aR peptide. Original magnification $\times 100$. Mild (**C**, **D**) and severe (**E**, **F**) persistent allergic rhinitis stained with C3aR antibody with (**C**, **E**) and without (**D**, **F**) preincubation with C3aR peptide. Arrowheads, Inflammatory cells. Thick arrow, Submucosal gland. Thin arrow, Epithelial layer. Original magnification $\times 200$.

cleaved on complement activation with generation of C3a and C3b, followed by the generation of C5a and C5b.²⁴ The anaphylatoxins, C3a and C5a, perform their functions by engaging specific receptors on target cells.²⁵ Traditionally, expression of C3aR and C5aR was thought to be restricted to cells of myeloid origin.⁶⁻⁸ With the cloning of the C3aR and C5aR cDNA, specific reagents for the detection of receptor expression could be generated, such as polyclonal and monoclonal antibodies. Using these antibodies, recent studies have demonstrated the presence of C3aR and C5aR on nonmyeloid cells, including human lung and liver, articular chondrocytes, and kidney tubular epithelial cells.¹¹⁻¹⁴ In the current study, the most unique finding was that the expression pattern of each complement receptor is different in normal and allergic nasal mucosa. That is, C5aR was expressed

in myeloid cells, whereas C3aR was found in nonmyeloid cells in normal and allergic nasal mucosa. Our current findings are in agreement with the previous results of Fregonese et al,²² who found the expression of C3aR in nonmyeloid cells and C5aR on myeloid cells in normal lung and mild intermittent and fatal asthmatic lung. The differential distribution of C3aR and C5aR was also found in various organs under pathologic conditions, including rheumatoid arthritis and glomerulonephritis.¹⁵⁻¹⁸

Considering the expression of C5aR in many inflammatory cells infiltrated into the nasal mucosa, and on the basis of the fact that C5a is a strong chemoattractant for various inflammatory cells and exerts its effects through the high-affinity C5aR, the results of the current study suggest that C5aR may play an important role in the activation of inflammatory cells in allergic rhinitis. In persistent severe allergic nasal mucosa, the expression of C3aR was also found in the endothelial cells of cavernous sinusoid and perivascular muscle layers. Interestingly, this finding was not found in normal and mild persistent allergic nasal mucosa. Therefore, these results provide evidence that C3aR is constitutively expressed in normal nasal mucosa, and the expression pattern is not altered in mild persistent allergic nasal mucosa. Our current results are consistent with the results showing the constitutive expression of C3aR in nonmyeloid cells of human lung and liver.¹¹ On the basis of the results that C3aR was expressed on the epithelial cell layers, submucosal glands, and nerve fibers, our data suggest that the C3a in normal and allergic nasal mucosa may mediate previously recognized functions, including mucus secretion and vascular permeability, by binding to tissue cells that express the C3aR. This hypothesis is supported by the results of *in vitro* experiment showing that C3a is a potent mucus secretagogue and possibly acts directly as a glandular stimulant.²⁶ The expression of C3aR receptor was also found in the endothelium of the cavernous sinus and perivascular muscle layer in severe persistent allergic nasal mucosa. This different expression pattern suggests that upregulation of C3aR expression may occur in severe persistent allergic rhinitis, which was confirmed by Western blot. The significance of this apparent differential distribution remains unclear, but it may reflect the exaggerated level of mucosal swellings in severe persistent allergic nasal mucosa, on the basis of the physiologic function of cavernous sinus vessel in the nasal mucosa. The nasal obstruction associated with allergic rhinitis probably is caused by the dilatation of the cavernous sinus and mucosal congestion by the increase in nasal microvascular permeability.²⁷ In perennial allergic rhinitis that failed to respond to medical treatment, the histopathology of the hypertrophic inferior turbinate showed that the relative surface area occupied by venous sinusoids was significantly greater than that of normal subjects.²

The nasal mucosa, as gatekeeper of the respiratory tract, is in immediate contact with ambient air, which contains known complement activators such as pollens, organic dusts, viruses, bacteria, and cigarette smoke.^{28,29} The nose is also continuously inhabited by various resident microorganisms that may increase the load of local complement activators. In addition to complement activators, complement itself is present in nasal airways in health and in disease.^{30,31} As expected from the presence of complement and its activators in the human nasal airways, activated complement is indeed found in the lining fluid of the nose in various respiratory tract diseases.^{6,32} When complement is activated, it is directed to protect the body from invading microorganisms and other insults, but at the same time, it may accidentally lyse

the host's normal cells, leading to tissue injury.³³ In addition to these findings and on the basis of our findings, we hypothesize that complement activation may be involved in the non-IgE chronic rhinitis and chronic sinusitis.

In conclusion, we have found distinctive staining patterns for C3aR and C5aR in normal and allergic nasal mucosa. These findings provide the basis for further studies on the role of complement activation and regulation in allergic rhinitis by providing a baseline of complement receptor distribution on the normal and allergic nasal mucosa.

Clinical implications: The interaction between complement anaphylatoxins and their receptors in normal and allergic nasal mucosa might play a role in activation of inflammatory cells, mucus secretion, and mucosal swelling.

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