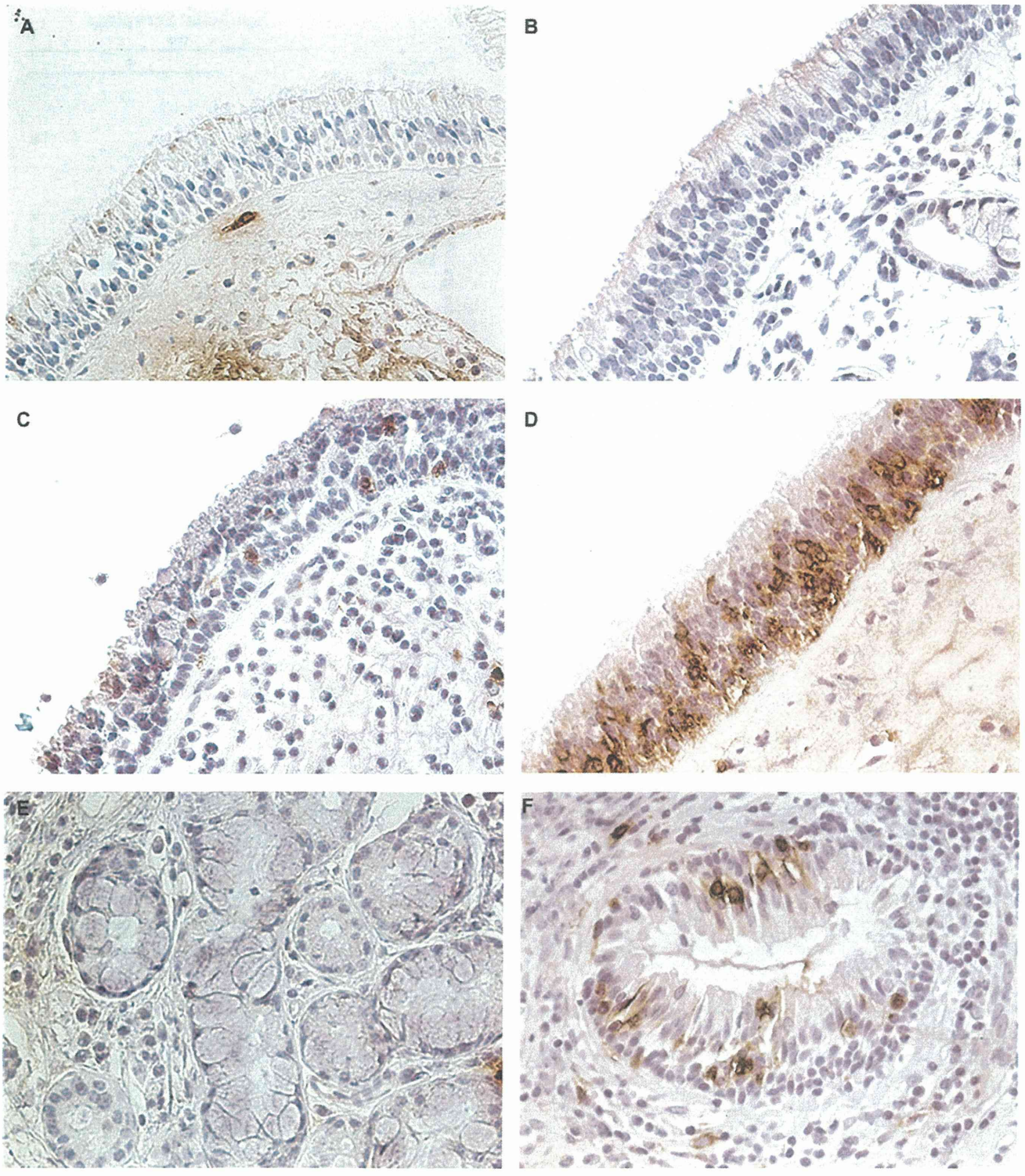

FIG 2. Immunohistochemical detection of tryptase was performed by using antihuman tryptase mAb. Representative immunostaining for tryptase within the epithelium of UT from a control subject **(A)**, a patient with CRSsNP **(B)**, a patient with CRSwNP **(C)**, and an NP **(D)**. Tryptase-positive cells in the glands of UT from a control subject **(E)** and an NP **(F)**. Magnification ×400.

tryptase-positive cells were found in the epithelium and glands of NPs from patients with CRSwNP (Fig 2). We also counted the number of tryptase-positive cells by using a semiquantitative method. Tryptase-positive cells were significantly elevated ($P < .001$) in both the epithelium and glands of NPs from patients with CRSwNP compared with either UT from control or patients

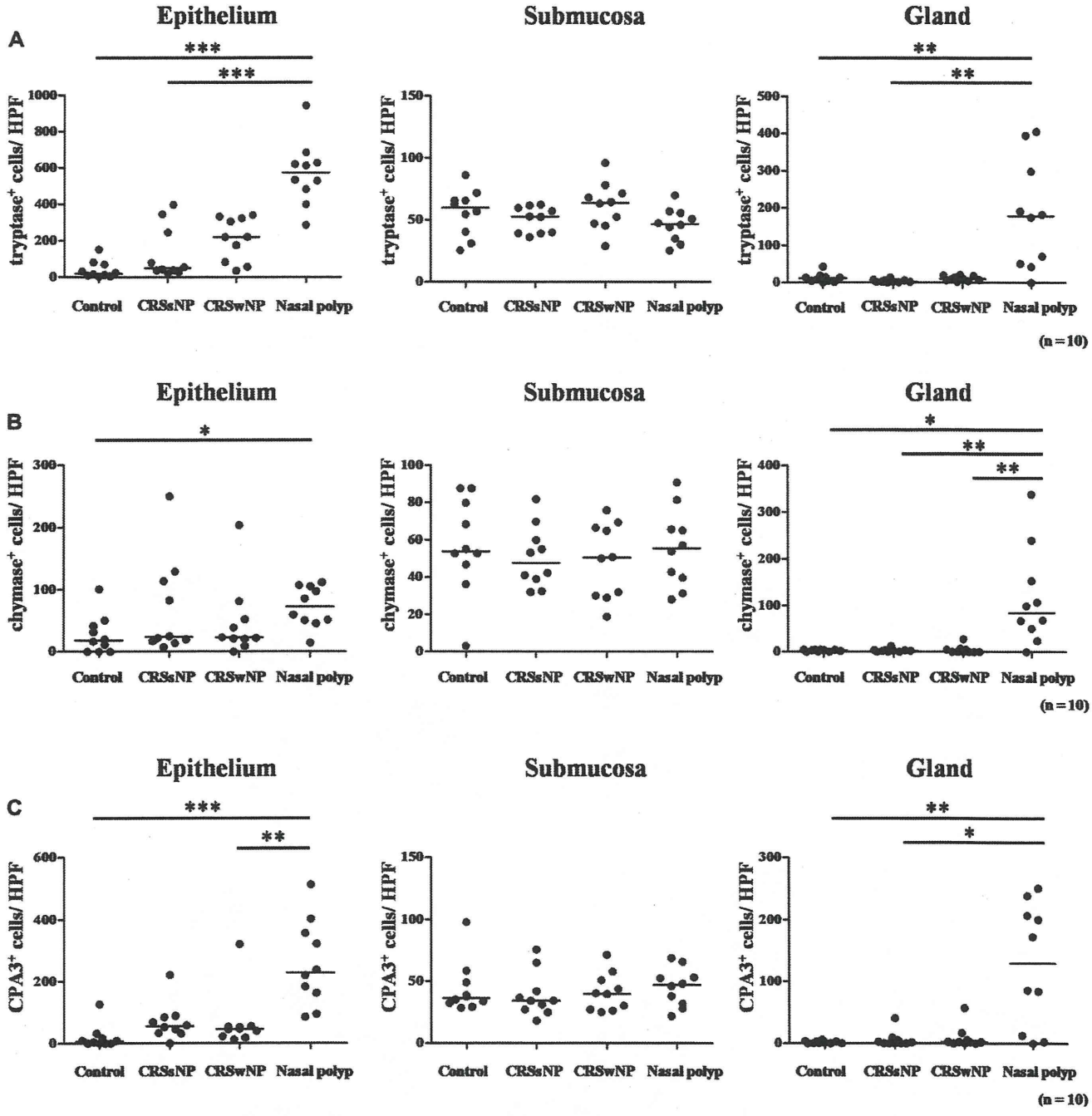

FIG 3. Quantitation of mast cell protease-positive cells in nasal mucosal tissue from a control subject and patients with CRS and distribution in epithelium, submucosa, and glands. Immunostaining for tryptase **(A)**, chymase **(B)**, and CPA3 **(C)**. $*P < .05$, $**P < .001$, $***P < .0001$.

with CRSsNP (Fig 3, *A*, left). We did not observe a significant difference in the number of tryptase-positive cells in the submucosa (excluding those within the glands) among the 4 groups of subject tissues (Fig 3, *A*, center). These results suggest that mast cells were significantly increased in NPs but not in UT from patients with either form of CRS. Importantly, the increase in the mast cells was localized to only within the epithelium and the epithelium of submucosal glands within NPs.

Chymase expression and distribution in patients with CRS

To determine the phenotype of mast cells in nasal mucosa, we assessed the expression of chymase. The expression of mRNA for chymase was elevated in subjects with NPs than in control UT, but there were no differences among CRS subjects (Fig 4, *A*). Likewise, the level of chymase was not different between UT and NPs in 17 patients for whom we had matched sets of both polyp

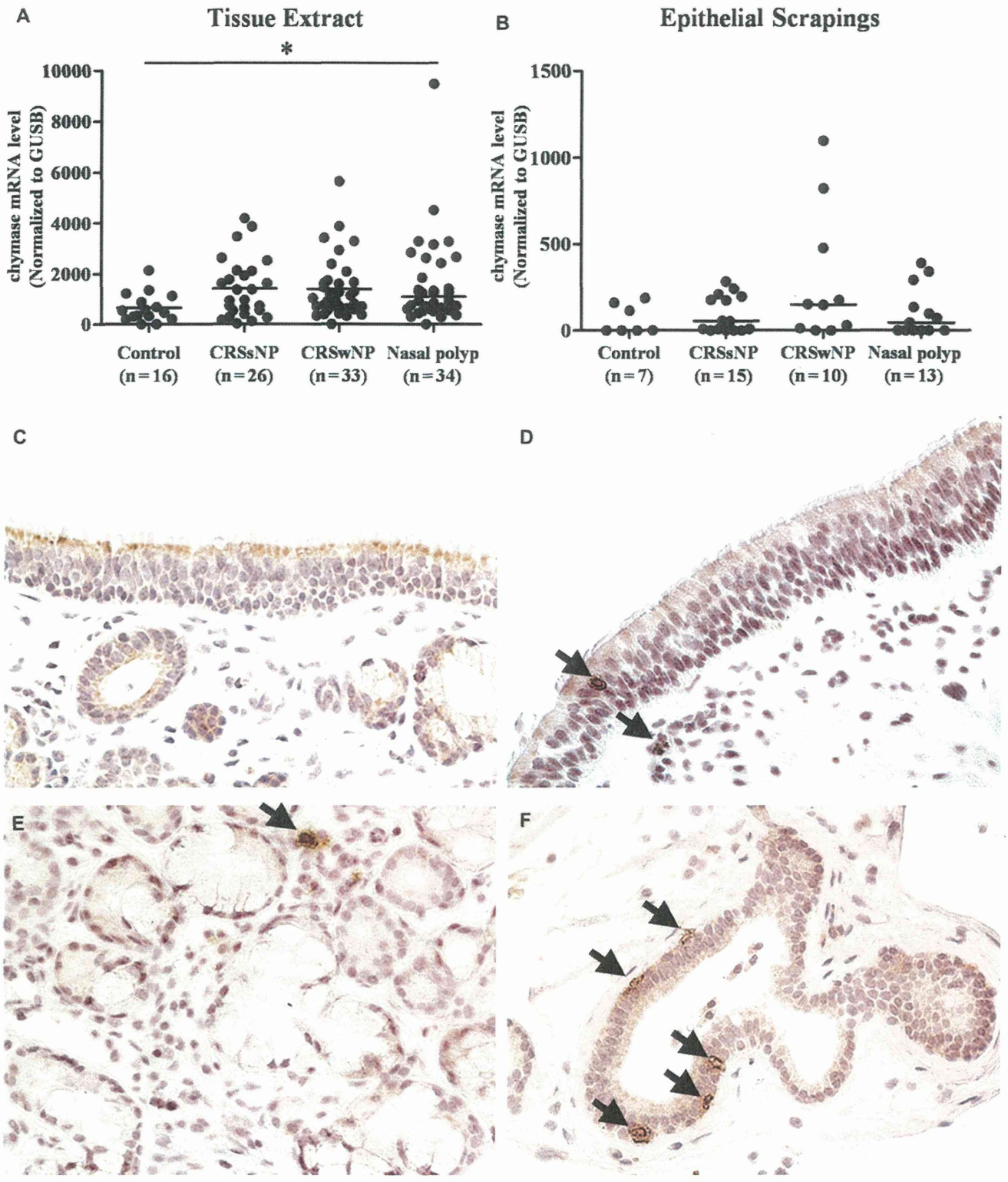

FIG 4. The expression of chymase in NPs. The gene expression of chymase in tissue **(A)** and nasal scrapings **(B)** was measured by using real-time PCR. Representative immunostaining for chymase within the epithelium of UT from a control subject **(C)** and an NP **(D)** and within the glands of UT from a control subject **(E)** and an NP **(F)**. Magnification ×400. *$P < .05$.

and UT for RT-PCR evaluation (data not shown). The expression of mRNA for chymase in nasal scraping samples was not different among these groups (Fig 4, *B*). To examine the chymase-positive mast cells in the nasal mucosa of patients with CRS, we performed semiquantitative immunohistochemical evaluation by using UT from control subjects and patients with CRS (Fig 4, *C-F*, and Fig 3, *B*). In epithelium, chymase-positive cells were slightly increased in NPs than in control UT, but there were no differences among CRS groups (Fig 3, *B*, left panel). On the other hand, chymase-positive mast cells located in glands were profoundly elevated in NPs from patients with CRSwNP (Fig 3, *B*, right panel). When we counted cells outside of the glands but still within the submucosa, we did not observe a significant difference among these 4 groups of tissue samples (Fig 3, *B*, middle panel).

CPA3 expression and distribution in patients with CRS

We next assessed the expression of CPA3. The expression of mRNA for CPA3 was significantly increased in NPs from patients with CRSwNP ($P < .0001$) in comparison with UT from either patients with CRS or control subjects (Fig 5, *A*). Levels of CPA3 mRNA were significantly elevated in NPs in 17 patients with matched polyp and UT ($P < .05$, data not shown). To measure the gene expression in epithelium, we evaluated nasal scraping samples and found that CPA3 mRNA was significantly increased in the epithelium from NPs in comparison with the epithelium from the UT of either patients with CRSsNP or control subjects ($P < .001$). To further examine the CPA3-positive mast cells in the nasal mucosa of patients with CRS, we performed immunohistochemistry in samples from control subjects and patients with CRS. As shown in Fig 5, *C* to *F*, CPA3-positive cells were found in the mucosal and glandular epithelium within NPs from patients with CRS. Lack of staining in NP with a negative control antibody is shown in Fig E2 (see this article's Online Repository at www.jacionline.org). We also counted the number of CPA3-positive cells by using a semiquantitative method and found that CPA3-positive cells were significantly elevated in both the epithelium ($P < .0001$) and glands ($P < .001$) of NPs compared with the levels seen in those locations in UT from control subjects. We did not observe a significant difference in CPA3-positive cells among these 4 groups of samples in submucosal locations outside of the glands (Fig 3, *C*).

Colocalization of mast cell serine proteases

To determine whether the 3 mast cell proteases under investigation are present in the same or different mast cells, we utilized dual-label immunofluorescence and characterized the mast cells localized within the epithelium or glands of NPs from patients with CRSwNP. As illustrated in Fig 6, *A*, of the tryptase-positive mast cells, most did not express chymase (mean and SEM tryptase single positive, 74.8% ± 3.7%; n = 10) in the epithelium. On the other hand, most mast cells within the epithelium that expressed tryptase were also CPA3 positive (mean and SEM double positive, 84.2% ± 2.9%; n = 10) (Fig 6, *B*).

We also determined the coexistence of these proteases in the mast cells found in glands of NPs. As shown in Fig 6, most mast cells in glands within NPs expressed both tryptase and chymase (mean and SEM double positive, 79.5% ± 4.0%; n = 10; Fig 6, *C*) and most mast cells expressed both tryptase and CPA3 (mean and SEM double positive, 87.7% ± 2.7%; n = 10; Fig 6, *D*). These results suggest that the mast cells found within submucosal glands of NPs were primarily double and triple positive for these proteases. In contrast, mast cells contained within the mucosal epithelium predominantly expressed high levels of tryptase and CPA3 but not chymase. Thus, the main distinction between the mast cells found located within the glands and those found elsewhere was that the glandular mast cells were chymase positive. We note that the immunohistochemistry counts suggest that there are approximately 50% as many chymase of CPA3-positive cells as tryptase-positive cells. However, using immunofluorescence, the percentage of tryptase-positive cells that were positive for chymase or CPA3 was approximately 80% or 90%. This apparent discrepancy may reflect the different sensitivities of immunofluorescence and immunohistochemical approaches.

DISCUSSION

The current study provides the first in-depth description of the expression, distribution, and phenotype of mast cells in NPs from patients with CRSwNP. We found increased levels of mast cells in NPs than in UT from control subjects and patients with CRS (Fig 1). We made the striking observation that mast cells were present within glandular tissues contained in NPs but were almost never detected within glands in UT from either patients or controls. Another important original finding was that these mast cells within glands of NPs, unlike mast cells in control tissue or outside of NPs, expressed the potent glandular secretagog chymase, along with tryptase and/or CPA3.

Mast cells are classically important in acute IgE-mediated reactions as observed in anaphylaxis, asthma, and rhinitis. However, recently, considerable evidence suggests that the roles of mast cells in health and disease are complex, and extend to include innate and adaptive immune responses as well as the control of physiological and homeostatic responses.[11] Previous studies demonstrated that the mucosa of NPs exhibits a high degree of tissue eosinophilia as well as T cells demonstrating skewing toward T_H2 cytokine expression.[3] Mast cells can produce an abundance of cytokines that activate eosinophils such as IL-5, granulocyte macrophage colony-stimulating factor, eotaxin, and RANTES.[22] In addition, ECP can activate mast cell degranulation and cytokine production.[23,24] In this study, we found a strong correlation between the levels of tryptase and ECP in nasal tissue extracts (see Fig E1 in this article's Online Repository at www.jacionline.org). It is thus reasonable to speculate that either mast cells and eosinophils employ the same pathways for recruitment or they may play a role in the accumulation and activation of one another.

Previous studies of mast cell abundance and distribution in NPs have reported contradictory findings; it is described that the number of epithelial mast cells in NPs is elevated[25-28] or that there is no difference in the number of mast cells in NPs compared with that in control tissue.[8,29,30] One possible explanation may be that most studies used inferior turbinate tissue as a control tissue. We know that turbinate and UT differ with respect to the expression of numerous host defense and inflammatory molecules (Tieu et al unpublished observations). NPs usually arise from mucous membranes in the middle nasal meatus and thus we contend that UT or other ethmoid tissue represents a better control than inferior turbinate. In this study, we used UT resected at skull base surgery as a control tissue and compared it with NPs. To our knowledge, this is the first report

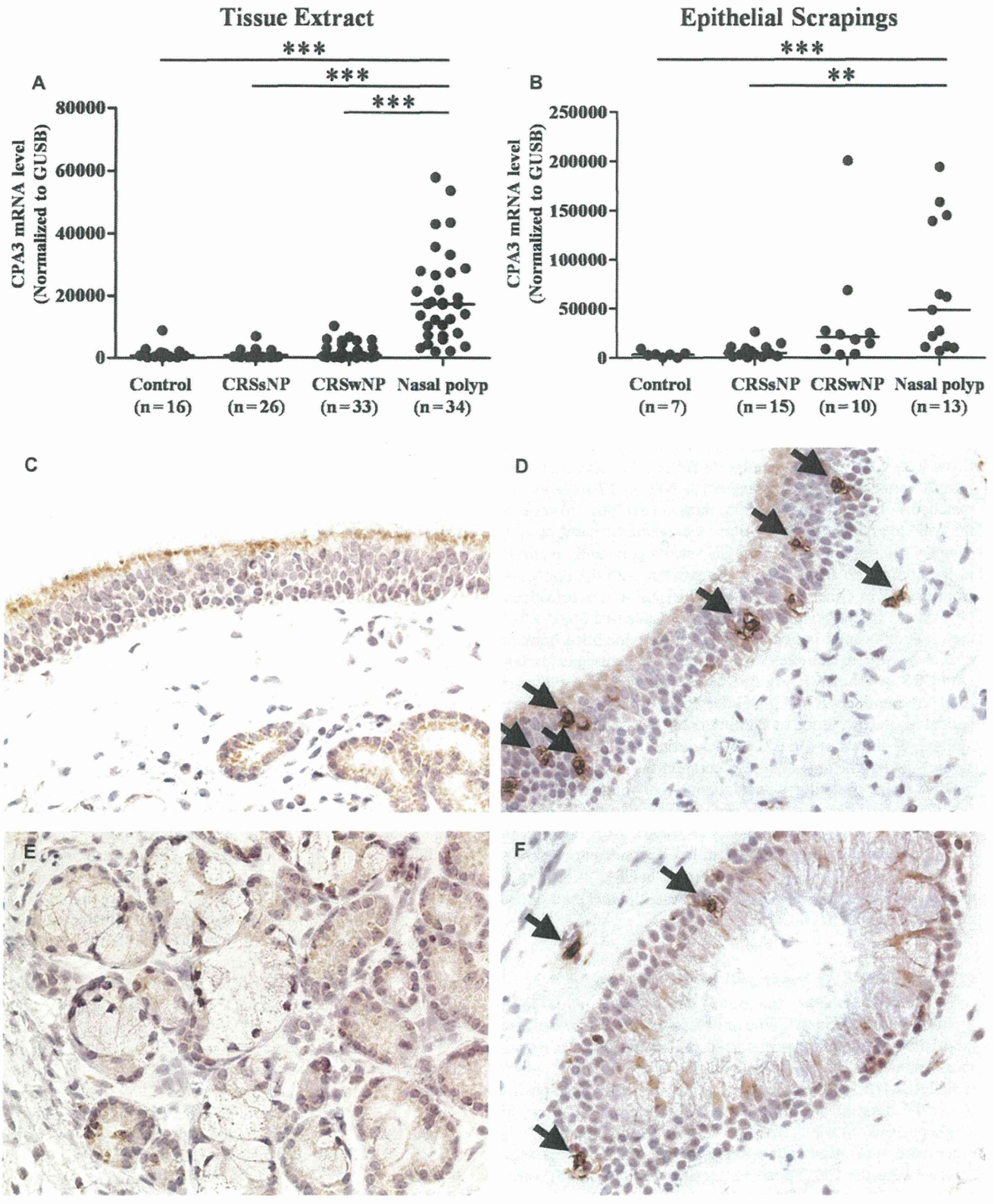

FIG 5. The expression of CPA3 in NPs. The gene expression of CPA3 in tissue **(A)** and nasal scrapings **(B)** was measured by using real-time PCR. Representative immunostaining for CPA3 within the epithelium of UT from a control subject **(C)** and an NP **(D)** and within the gland of UT from a control subject **(E)** and an NP **(F)**. Magnification ×400. $^{**}P < .001$, $^{***}P < .0001$.

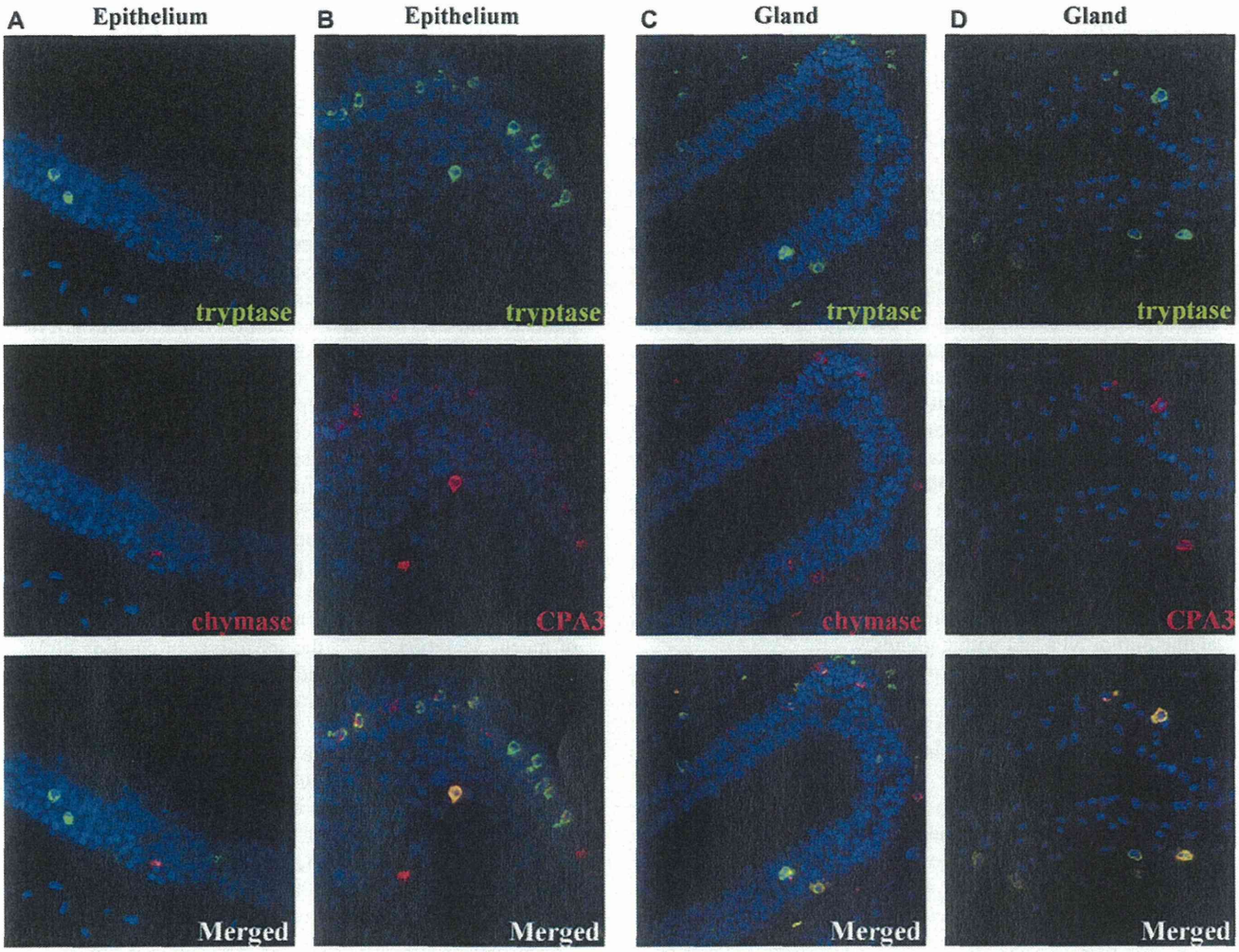

FIG 6. Immunofluorescence of mast cell proteases in NPs. Immunofluorescence assay was performed with antitryptase (*green* fluorescence) and antichymase (*red* fluorescence) **(A)**, and anti-CPA3 (*red* fluorescence) **(B)** in epithelium and antitryptase (*green* fluorescence), and antichymase (*red* fluorescence) **(C)**, and CPA3 (*red* fluorescence) **(D)** in glands. Nuclei ware counterstained with 4′,6-diamidino-2-phenylindole (*blue* fluorescence). The results are representative of 5 separate subjects.

to use UT as a control for studying mast cells in CRS. We also utilized numerous complementary methods, including RT-PCR, ELISA, and double immunofluorescence, which have not been previously employed to study mast cells in CRS.

We observed unequivocally increased numbers of mast cells in epithelium and glands but not submucosa in NPs by immunohistochemistry (Fig 2). These results were reinforced by findings utilizing real-time PCR and ELISA assays in nasal epithelial scraping samples (Fig 1). Although there is no statistically significant difference between NPs and UT from patients with CRSwNP, we observed the same trend. Previous studies in allergic rhinitis and asthma support a model in which deeper tissue mast cells migrate to the mucosal surface during the allergy season, while the total content of mast cells (as measured by total tissue histamine) does not increase greatly.[31] In our studies, we show a very clear expansion of the total number of mast cells within NPs, as measured by several techniques. This suggests that local mechanisms are promoting the expansion of mast cells within NPs, either by recruitment or proliferation or both mechanisms.

The mechanism for this accumulation of mast cells remains unclear. Airway epithelial cell–derived stem cell factor (SCF) has been shown to be both chemoattractant and essential survival factor for mast cells.[20] Previous reports demonstrated a significant increase in the expression of SCF in the epithelium of NPs compared with normal nasal mucosa.[27,32] T_H2 cytokine-induced epithelial eotaxins could also lead to mast cell recruitment and activation by CCR3.[33] In this study, we found an upregulation of mRNA for SCF and eotaxin-1, eotaxin-2, and eotaxin-3 in NPs from patients with CRSwNP in comparison with UT from either patients with CRS or control subjects (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). We also found strong correlations between each of these cytokines and the level of tryptase in nasal tissue (Fig E3, *B*). Although these correlations do not prove cause and effect, it is reasonable to speculate that mast cells may be recruited via CCR3 active chemokines and their survival maintained via SCF expressed locally in sinonasal tissue. Other possible mediators of the mast cell response could include neuropeptides such as substance P (SP)

and vasoactive intestinal polypeptide or leukotriene B4, all of which have been shown to influence mast cell responses.[34-36]

Clearly, further study will be required to determine the mechanism of mast cell accumulation within the epithelium of NPs, both at the mucosal surface and within submucosal glands.

Although we did not directly establish mast cell activation (eg, by using electron microscopy), we detected elevated tryptase in nasal lavage fluids, suggesting that mast cell activation had been taking place in patients with NPs. There are several possible mechanisms that might activate mucosal mast cells in CRS. Staphylococcal colonization is common in CRS and staph enterotoxin may act as an allergen, along with aeroallergens, to induce the activation of mast cells.[37,38] Neuropeptides can also activate mast cells independent of crosslinking of FcεRI. A recent study from our laboratories demonstrated that SP and vasoactive intestinal polypeptide induce degranulation and chemokine production in human mast cells.[39] SP can also upregulate Toll-like receptor 2 on human mast cells and increase Toll-like receptor 2–dependent production of leukotriene C4 and IL-8.[34] In the rat, 80% of neurons projecting to the nasal epithelium were positive for SP.[40] It is thus worthy of consideration that neuropeptides may play an important role in the activation of mast cells in the epithelium of NPs and within glands.

Human mast cells can also be activated by thymic stromal lymphopoietin, an epithelial cell–derived cytokine present in CRS, resulting in production by mast cells of high levels of T_H2 cytokines in the presence of proinflammatory cytokines, IL-1 and tumor necrosis factor.[41]

Activated mast cells can release a variety of preformed mediators and *de novo* synthesized proinflammatory mediators that might contribute to the development of NPs. One such preformed mediator is histamine, which can promote vasodilatation and increased vascular permeability. Mast cell–derived chemokines may play a role in the recruitment of eosinophils and other cells found within NPs.[22]

In the current study, we found that mast cells within the epithelium of NPs were tryptase and CPA3 double positive and chymase negative by using double immunofluorescence (Fig 6). This pattern of mast cell protease expression is not consistent with the patterns observed for either MC-T or MC-TC. Recently, a similar unique phenotype of mast cells was reported in the epithelium of T_H2 high asthma and eosinophilic esophagitis.[19,20] In contrast, severe asthma is associated with a predominance of MC-TC in the airway epithelium and submucosa and increased prostaglandin D2.[42] It has also been reported that arachidonic acid metabolism in MC-TC preferentially uses the cyclooxygenase pathway.[43] The unique tryptase and CPA3 double-positive, chymase-negative mast cell type thus might utilize the lipoxygenase pathway as opposed to the mast cells reported in severe asthma. We did not find any difference in mast cell populations in NPs when comparing CRS patients with or without asthma (data not shown), suggesting that our findings do not simply reflect a large subpopulation of patients in our cohort who have T_H2 high asthma. To our knowledge, this is the first report showing the upregulation of a unique population of mast cells in the epithelium of NPs.

The underlying mechanism by which the tryptase-positive/CPA3-positive/chymase-negative cells develop is unknown. It has been previously reported that mast cells grown in coculture with epithelial cells downregulate chymase and the conditioned medium from IL-13–stimulated epithelial cells downregulates chymase while maintaining tryptase and CPA3 expression in cultured mast cells.[20,44] There are many potential sources of IL-13 in NPs, including T_H2 cells, innate type 2 cells, and even mast cells themselves.[41] We speculate that T_H2 skewing, and IL-13 in particular, might be involved in the development of the unique tryptase-positive/CPA3-positive/chymase-negative phenotype of mast cells in the lungs in T_H2 asthma, in the esophagus in eosinophilic esophagitis, and in the mucosal epithelium of NPs.

We are particularly intrigued by the increase in mast cells within submucosal glands in CRS, as this occurred only in patients with CRSwNP and within the NPs. Interestingly, most of the mast cells were triple positive, that is, traditional MC-TC. Hypersecretion of mucus is a hallmark feature of CRS, and mucus glands may thus play an important role in the pathogenesis of NPs.[45] The expression of mucin gene *MUC5B* was reported to be upregulated in submucosal glands of NPs compared with normal nasal mucosa and numerous inflammatory mediators can upregulate these genes.[46] It has been reported that the expression of prostaglandin D2 was upregulated in NPs compared with normal nasal mucosa, which, in turn, induced MUC5B overproduction.[47] Since MC-TC contribute to the increase in prostaglandin D2 levels,[42] and since chymase, but not tryptase, is a potent inducer of mucus secretion,[20,48,49] the specific accumulation of MC-TC within the submucosal glands of NPs should be suspected to play a role in the activation of the secretory response in these glands.

In summary, we report here a profound increase in mast cells in the epithelium and glands of NPs as compared with UT from control subjects and patients with CRS. The mast cells that have accumulated in the epithelium of NPs have a recently recognized unique phenotype, displaying the upregulation of tryptase and CPA3 but not chymase. On the other hand, those in glands have the typical MC-TC phenotype in which all the 3 proteases are expressed. Our results raise the exciting hypothesis that the expansion of distinct populations of mast cells within the mucosal epithelium and the submucosal glands may play an important role in the pathophysiology and development of NPs.

Clinical implications: A unique phenotype of mast cells in nasal polyp glands may have a pathogenic role in chronic rhinosinusitis.

REFERENCES

1. Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, et al. Rhinosinusitis: establishing definitions for clinical research and patient care. J Allergy Clin Immunol 2004;114:155-212.
2. Hamilos DL. Chronic rhinosinusitis patterns of illness. Clin Allergy Immunol 2007;20:1-13.
3. Van Zele T, Claeys S, Gevaert P, Van Maele G, Holtappels G, Van Cauwenberge P, et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. Allergy 2006;61:1280-9.
4. Bachert C, Gevaert P, van Cauwenberge P. *Staphylococcus aureus* superantigens and airway disease. Curr Allergy Asthma Rep 2002;2:252-8.
5. Shin SH, Ponikau JU, Sherris DA, Congdon D, Frigas E, Homburger HA, et al. Chronic rhinosinusitis: an enhanced immune response to ubiquitous airborne fungi. J Allergy Clin Immunol 2004;114:1369-75.
6. Schleimer RP, Kato A, Peters A, Conley D, Kim J, Liu MC, et al. Epithelium, inflammation, and immunity in the upper airways of humans: studies in chronic rhinosinusitis. Proc Am Thorac Soc 2009;6:288-94.
7. Van Bruaene N, Perez-Novo CA, Basinski TM, Van Zele T, Holtappels G, De Ruyck N, et al. T-cell regulation in chronic paranasal sinus disease. J Allergy Clin Immunol 2008;121:1435-41, 1441.e1-3.

8. Polzehl D, Moeller P, Riechelmann H, Perner S. Distinct features of chronic rhinosinusitis with and without nasal polyps. Allergy 2006;61:1275-9.
9. Perez-Novo CA, Claeys C, Van Cauwenberge P, Bachert C. Expression of eicosanoid receptors subtypes and eosinophilic inflammation: implication on chronic rhinosinusitis. Respir Res 2006;7:75.
10. Gevaert P, Lang-Loidolt D, Lackner A, Stammberger H, Staudinger H, Van Zele T, et al. Nasal IL-5 levels determine the response to anti-IL-5 treatment in patients with nasal polyps. J Allergy Clin Immunol 2006;118:1133-41.
11. Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. Nat Rev Immunol 2010;10:440-52.
12. Beaven MA. Our perception of the mast cell from Paul Ehrlich to now. Eur J Immunol 2009;39:11-25.
13. Schwartz LB. Analysis of MC(T) and MC(TC) mast cells in tissue. Methods Mol Biol 2006;315:53-62.
14. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. N Engl J Med 2002;346: 1699-705.
15. Gibson PG, Allen CJ, Yang JP, Wong BJO, Dolovich J, Denburg J, et al. Intraepithelial mast-cells in allergic and nonallergic asthma—assessment using bronchial brushings. Am Rev Respir Dis 1993;148:80-6.
16. Juliusson S, Pipkorn U, Karlsson G, Enerback L. Mast-cells and eosinophils in the allergic mucosal response to allergen challenge—changes in distribution and signs of activation in relation to symptoms. J Allergy Clin Immunol 1992;90:898-909.
17. Balzar S, Fajt ML, Comhair SA, Erzurum SC, Bleecker E, Busse WW, et al. Mast cell phenotype, location, and activation in severe asthma: data from the severe asthma research program. Am J Respir Crit Care Med 2011;183:299-309.
18. Hsu Blatman KS, Gonsalves N, Hirano I, Bryce PJ. Expression of mast cell–associated genes is upregulated in adult eosinophilic esophagitis and responds to steroid or dietary therapy. J Allergy Clin Immunol 2011;125:1307-8.
19. Abonia JP, Blanchard C, Butz BB, Rainey HF, Collins MH, Stringer K, et al. Involvement of mast cells in eosinophilic esophagitis. J Allergy Clin Immunol 2010;126:140-9.
20. Dougherty RH, Sidhu SS, Raman K, Solon M, Solberg OD, Caughey GH, et al. Accumulation of intraepithelial mast cells with a unique protease phenotype in T(H)2-high asthma. J Allergy Clin Immunol 2010;125:1046-53.
21. Kato A, Peters A, Suh L, Carter R, Harris KE, Chandra R, et al. Evidence of a role for B cell-activating factor of the TNF family in the pathogenesis of chronic rhinosinusitis with nasal polyps. J Allergy Clin Immunol 2008;121:1385-92.
22. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol 2005;6:135-42.
23. Piliponsky AM, Gleich GJ, Bar I, Levi-Schaffer F. Effects of eosinophils on mast cells: a new pathway for the perpetuation of allergic inflammation. Mol Immunol 2002;38:1369-72.
24. Zheutlin LM, Ackerman SJ, Gleich GJ, Thomas LL. Stimulation of basophil and rat mast cell histamine release by eosinophil granule-derived cationic proteins. J Immunol 1984;133:2180-5.
25. Pawankar R. Mast cells in allergic airway disease and chronic rhinosinusitis. Chem Immunol Allergy 2005;87:111-29.
26. Kowalski ML, Grzegorczyk J, Pawliczak R, Kornatowski T, Wagrowska-Danilewicz M, Danilewicz M. Decreased apoptosis and distinct profile of infiltrating cells in the nasal polyps of patients with aspirin hypersensitivity. Allergy 2002;57: 493-500.
27. Kowalski ML, Lewandowska-Polak A, Wozniak J, Ptasinska A, Jankowski A, Wagrowska-Danilewicz M, et al. Association of stem cell factor expression in nasal polyp epithelial cells with aspirin sensitivity and asthma. Allergy 2005; 60:631-7.
28. Di Lorenzo G, Drago A, Pellitteri ME, Candore G, Colombo A, Gervasi F, et al. Measurement of inflammatory mediators of mast cells and eosinophils in native nasal lavage fluid in nasal polyposis. Int Arch Allergy Immunol 2001;125: 164-75.
29. Patou J, Holtappels G, Affleck K, Gevaert P, Perez-Novo C, Van Cauwenberge P, et al. Enhanced release of IgE-dependent early phase mediators from nasal polyp tissue. J Inflamm (Lond) 2009;6:11.
30. Ediger D, Sin BA, Heper A, Anadolu Y, Misirligil Z. Airway inflammation in nasal polyposis: immunopathological aspects of relation to asthma. Clin Exp Allergy 2005;35:319-26.
31. Enerback L, Karisson G, Pipkorn U. Nasal mast cell response to natural allergen exposure. Int Arch Allergy Appl Immunol 1989;88:209-11.
32. Otsuka H, Kusumi T, Kanai S, Koyama M, Kuno Y, Takizawa R. Stem cell factor mRNA expression and production in human nasal epithelial cells: contribution to the accumulation of mast cells in the nasal epithelium of allergy. J Allergy Clin Immunol 1998;102:757-64.
33. Price KS, Friend DS, Mellor EA, De Jesus N, Watts GF, Boyce JA. CC chemokine receptor 3 mobilizes to the surface of human mast cells and potentiates immunoglobulin E-dependent generation of interleukin 13. Am J Respir Cell Mol Biol 2003;28:420-7.
34. Tancowny BP, Karpov V, Schleimer RP, Kulka M. Substance P primes lipoteichoic acid- and Pam3CysSerLys4-mediated activation of human mast cells by up-regulating Toll-like receptor 2. Immunology 2010;131:220-30.
35. Maintz L, Wardelmann E, Walgenbach K, Fimmers R, Bieber T, Raap U, et al. Neuropeptide blood levels correlate with mast cell load in patients with mastocytosis. Allergy 2011;66:862-9.
36. Weller CL, Collington SJ, Brown JK, Miller HRP, Al-Kashi A, Clark P, et al. Leukotriene B4, an activation product of mast cells, is a chemoattractant for their progenitors. J Exp Med 2005;201:1961-71.
37. Van Zele T, Gevaert P, Holtappels G, van Cauwenberge P, Bachert C. Local immunoglobulin production in nasal polyposis is modulated by superantigens. Clin Exp Allergy 2007;37:1840-7.
38. Verbruggen K, Van Cauwenberge P, Bachert C. Anti-IgE for the treatment of allergic rhinitis and eventually nasal polyps? Int Arch Allergy Immunol 2009;148:87-98.
39. Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP. Neuropeptides activate human mast cell degranulation and chemokine production. Immunology 2008;123:398-410.
40. Hunter DD, Dey RD. Identification and neuropeptide content of trigeminal neurons innervating the rat nasal epithelium. Neuroscience 1998;83:591-9.
41. Allakhverdi Z, Comeau M, Ziegler SF, Sarfati M, Delespesse G. Thymic stromal lymphopoietin (TSLP) is released by human epithelial cells in response to microbes and potently activates mast cells. J Allergy Clin Immunol 2007;119:253-8.
42. Balzar S, Fajt ML, Comhair SAA, Erzurum SC, Bleecker E, Busse WW, et al. Mast cell phenotype, location, and activation in severe asthma: data from the Severe Asthma Research Program. Am J Respir Crit Care Med 2011;183:299-309.
43. Boyce JA. Mast cells and eicosanoid mediators: a system of reciprocal paracrine and autocrine regulation. Immunol Rev 2007;217:168-85.
44. Hsieh FH, Sharma P, Gibbons A, Goggans T, Erzurum SC, Haque SJ. Human airway epithelial cell determinants of survival and functional phenotype for primary human mast cells. Proc Natl Acad Sci U S A 2005;102:14380-5.
45. Tos M, Mogensen C. Mucous glands in nasal polyps. Arch Otolaryngol-Head Neck Surg 1977;103:407-13.
46. Ali MS, Wilson JA, Bennett M, Pearson JP. Mucin gene expression in nasal polyps. Acta Otolaryngol 2005;125:618-24.
47. Choi YH, Lee SN, Aoyagi H, Yamasaki Y, Yoo JY, Park B, et al. The extracellular signal-regulated kinase mitogen-activated protein kinase/ribosomal S6 protein kinase 1 cascade phosphorylates cAMP response element-binding protein to induce *MUC5B* gene expression via D-prostanoid receptor signaling. J Biol Chem 2011; 286:34199-214.
48. Sommerhoff CP, Caughey GH, Finkbeiner WE, Lazarus SC, Basbaum CB, Nadel JA. Mast cell chymase: a potent secretagogue for airway gland serous cells. J Immunol 1989;142:2450-6.
49. Nadel JA. Role of enzymes from inflammatory cells on airway submucosal gland secretion. Respiration 1991;58:3-5.

METHODS

Patients and biopsies

Patients with CRS were recruited from the allergy and otolaryngology clinics at Northwestern University and the Northwestern Sinus Center. Sinonasal and polyp tissues were obtained from routine functional endoscopic sinus surgery in patients with CRS. All subjects met the criteria for CRS as defined by the Sinus and Allergy Health Partnership.

The presence of sinusitis or bilateral nasal NPs was confirmed by office endoscopy and computed tomography imaging. Nasal lavage was collected from patients and control subjects without history of allergic rhinitis, CRS, or asthma. Specimens from patients without CRS were obtained during the performance of skull base tumor excision, facial fracture repair, lacrimal duct surgery, and orbital decompression surgery. All patients scheduled for surgery had previously failed to respond to adequate trials of conservative medical therapy (prolonged antibiotic regimens, nasal steroid sprays, oral steroids, saline irrigations, and decongestants) for control of symptoms. Patients with an isolated antrochoanal polyp, cystic fibrosis, or unilateral NPs were excluded from the study. Disease-free control subjects undergoing procedures to correct anatomical defects without history of CRS or asthma were recruited from the otolaryngology clinic at Northwestern University. All subjects signed informed consent, and the protocol and consent forms governing procedures for the study were approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine. A portion of each sample for isolation of RNA was transferred in RNA later (Ambion, Austin, Tex) and stored at −20°C.

Real-time PCR

Total RNA from sinus tissue was extracted with QIAzol (Qiagen, Valencia, Calif) and was cleaned and treated with DNase I by using NucleoSpin RNA II (MACHEREY-NAGEL, Bethlehem, Pa) according to the manufacturer's instructions. Total RNA from nasal scrapings was extracted by using NucleoSpin RNA XS (MACHEREY-NAGEL) according to the manufacturer's instructions. The quality of total RNA from sinus tissue was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif) by using a RNA 6000 Nano LabChip (Agilent Technologies). Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif) and random primers. Semiquantitative real-time RT-PCR was performed with a TaqMan method by using an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif) in 15 μL reactions (7.5 μL of 2× TaqMan Master mix [Applied Biosystems], 400 nmol/L of each primer, and 200 nmol/L of TaqMan probe plus cDNA). Primer and probe sets for GUSB (human GUSB endogenous control, PN; 4326320E), tryptase (Hs02576518_gH), chymase (Hs00156558_m1), CPA3 (Hs00157019_m1), eotaxin-1 (Hs00237013_m1), eotaxin-2 (Hs00171082_m1), SCF (Hs01030228_m1), and eotaxin-3 (sense, 5′-CTGCTTCCAATACAGCCACAAG-3′; antisense, 5′-GAGCAGCTGTTACTGGTGAATTCA; FAM/black hole quencher 1-labeled probe, 5′-CTTCCCTGGACCTGGGTGCGAA-3′) were purchased from Applied Biosystems or Integrated DNA Technologies. A primer and probe set for GUSB was chosen as the reference housekeeping gene because there was no difference in the expression of this gene among patients and controls. To determine the exact copy number of the target genes, quantified aliquots of purified PCR fragments of the target genes were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 10 ng of total RNA were used for real-time PCR. The mRNA expression levels were normalized to the median expression of the housekeeping gene GUSB.

Measurement of tryptase and ECP in tissue homogenates

Freshly obtained tissue specimens were weighed, and 1 mL of PBS supplemented with 0.05% Tween 20 (Sigma-Aldrich, St Louis, Mo) and 1% protease inhibitor cocktail (Sigma-Aldrich) was added for every 100 mg of tissue. The tissue was then homogenized with a Bullet Blender Blue (Next Advance, Averill Park, NY) at setting 7 for 8 minutes at 4°C. After homogenization, the suspension was centrifuged at 4000 rpm for 20 minutes at 4°C, and supernatants were stored at −80°C until analyzed. Nasal lavage was performed with PBS without calcium and magnesium. Nasal lavage fluids were centrifuged in 15-mL conical tubes for 10 minutes at 3000 rpm. The resulting supernatants were then transferred and concentrated with an Amicon Ultra-4 Centrifugal Filter Device (10,000-d molecular weight cutoff) and centrifuged at 3000 rpm for 5 to 10 minutes at 4°C or until the sample was concentrated 2-fold. The concentrated supernatants were placed in aliquots in tubes and stored at −20°C until use. The protein concentrations for tissue extracts and nasal lavage fluids were determined by using the BCA Protein Assay Kit (Pierce/Thermo Scientific, Rockford, Ill). Before analysis, samples were thawed at room temperature and vortexed to ensure a well-mixed sample. Tryptase and ECP were assayed with commercially available assay kits (Uscn Life Science, Inc, Wuhan, China, and MBL, Woburn, Mass). The minimal detection limit for these kits is 0.156 ng/mL and 0.4 ng/mL, respectively. According to the manufacturer of the former kit, the standard used in the kit is recombinant human tryptase alpha/beta-1 (aa31-275). The capture antibody is an mAb, and the detection antibody is a polyclonal antibody. Both antibodies have been raised against the recombinant tryptase alpha/beta-1. Both protryptase and mature tryptase are recognized by this kit. The color intensity was measured with a Bio-Rad Spectrophotometer Model 680 Microplate Reader (Bio-Rad, Hercules, Calif) with associated software applied to the sandwich enzyme immunoassay technique. Concentrations of tryptase and ECP in the tissue homogenate and nasal lavage fluid were normalized to the concentration of total protein.

Immunohistochemistry

Nasal tissue was dehydrated, infiltrated, and embedded with paraffin, and tissue was sectioned at 3 μm by using a Leica RM2245 Cryostat (Leica Microsystems, Inc, Bannockburn, Ill). Sections were rehydrated and endogenous peroxidase activity blocked with 3% H_2O_2/methanol. After rinsing, nonspecific binding was blocked with 3% goat serum/0.3% Tween-20/PBS. Tissue sections were then incubated with mouse anti-human tryptase mAb (clone: AA1, IgG_1; Thermo Scientific, Fremont, Calif) at a dilution of 1:40,000 or mouse anti-human chymase mAb (clone: CC1, IgG_1; Thermo Scientific) at a dilution of 1:5000 or rabbit anti-human CPA3 polyclonal antibody (rabbit antibody; Sigma, St Louis, Mo) at a dilution of 1:1000 overnight at 4°C. The same concentrations of isotype control IgG were used in control experiments. Sections were rinsed and then incubated in biotinylated secondary goat anti-mouse or rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa) at a 1:500 dilution for 1 hour at room temperature. After another rinse, sections were incubated in ABC reagent (avidin-biotin-horseradish peroxidase complex; Vector Laboratories, Burlingame, Calif) for 1 hour at room temperature. Sections were rinsed again and incubated in diaminobenzidine reagent (Invitrogen) for 10 minutes at room temperature. They were then rinsed in deionized H_2O, counterstained with hematoxylin, dehydrated, cleared, mounted, and coverslipped by using Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, Mich) in preparation for microscopic analysis. Microscopic analysis was performed with an Olympus IX71 inverted research microscope (Olympus, Center Valley, Pa) and MicroFire AR digital microscope camera (Optronics, Goleta, Calif). The number of positive cells in epithelium, glands, and submucosa was counted at a magnification of ×400. Each section was randomly selected, and diagnosis was unknown to the observer.

Immunofluorescence staining

Immunofluorescence staining was used to visualize the localization of mast cell proteases. Sections were prepared the same way as for immunohistochemistry and were blocked for nonspecific binding with 3% donkey serum/0.3% Tween-20/PBS. To stain tryptase and CPA3, tissue sections were then incubated with tryptase mAb or CPA3 polyclonal antibody at the dilution mentioned above overnight at 4°C. After washing with PBS, cells were incubated with 4 μg/mL Alexa Fluor 488-conjugated goat antimouse IgG (Invitrogen) and 4 μg/mL Alexa Fluor 568-conjugated goat antirabbit IgG (Invitrogen) for 1 hour at room temperature in the dark. After final washing with PBS, coverslips were mounted onto slides by using SlowFade Gold antifade reagent with 4′, 6-diamidino-2-phenylindole (Invitrogen) and the slides were stored in the dark at 4°C. For staining of tryptase and chymase, the mouse IgG1 Zenon Alexa Fluo 488 labeling reagent was combined with

tryptase mAb, and the mouse IgG1 Zenon Alexa Fluo 568 labeling reagent was combined with chymase mAb, according to the manufacturer's instructions (Invitrogen). Briefly, 1 μg of each antibody was prepared in PBS solution and incubated with 5 μL of the Zenon mouse IgG labeling reagent for 5 minutes at room temperature, followed by addition of 5 μL of the Zenon-blocking reagent into the mixture and incubation for 5 minutes at room temperature. The labeled complexes were diluted with PBS (to the working concentration of 50 μg/mL) and used immediately. Sections were incubated with conjugated antibodies that were diluted with PBS (0.01 mol/L, pH 7.4) at a working concentration (tryptase 1:10,000, chymase 1:2000) and added onto each section followed by incubation for 2 hours at room temperature. Each slide was washed and mounted as described above.

Images from immunofluorescence slides were obtained with an Olympus IX71 inverted research microscope using ×40 objective lens and were collected by using SlideBook software (Olympus). Slides were blinded, and 5 pictures were randomly taken from each slide for analysis by a blinded observer.

In the submucosa, the intensity of staining for mast cell proteases was strong and homogeneous. However, within the epithelium, staining was heterogeneous, due to the presence of some weakly staining mast cells (perhaps due to degranulation?). Regarding the specificity of the anti-CPA antibody, we observed weak staining in some structural cells on occasion, but this was not the norm, and the staining was not intense enough to cause these cells to be deemed positive. In fact, most (greater than 95%) of the CPA3-positive cells were also positive for tryptase, whereas of the tryptase-positive cells, 84.2% were also positive for CPA3 (mentioned in the Results section), indicating that the CPA3 antibody was nearly exclusively recognizing mast cells. To clarify the specificity of this antihuman CPA3 antibody, we added negative control antibody staining in NP tissue from a patient with CRSwNP (Fig E2).

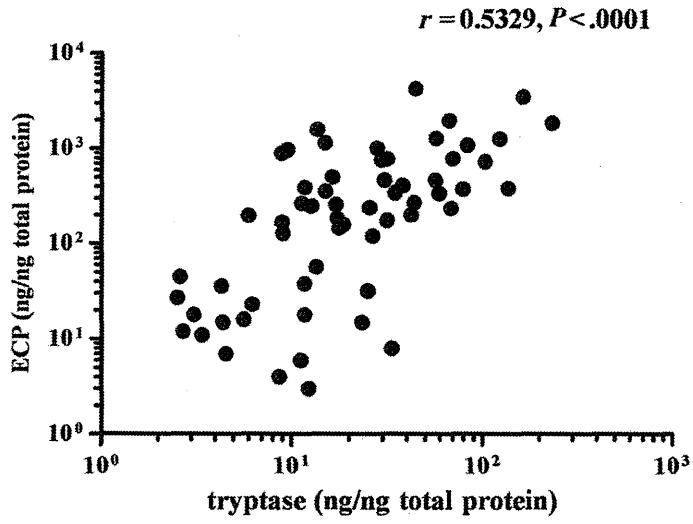

FIG E1. The relationship of tryptase and ECP in UT was evaluated by using ELISA. The correlation was assessed by using the Spearman rank correlation test.

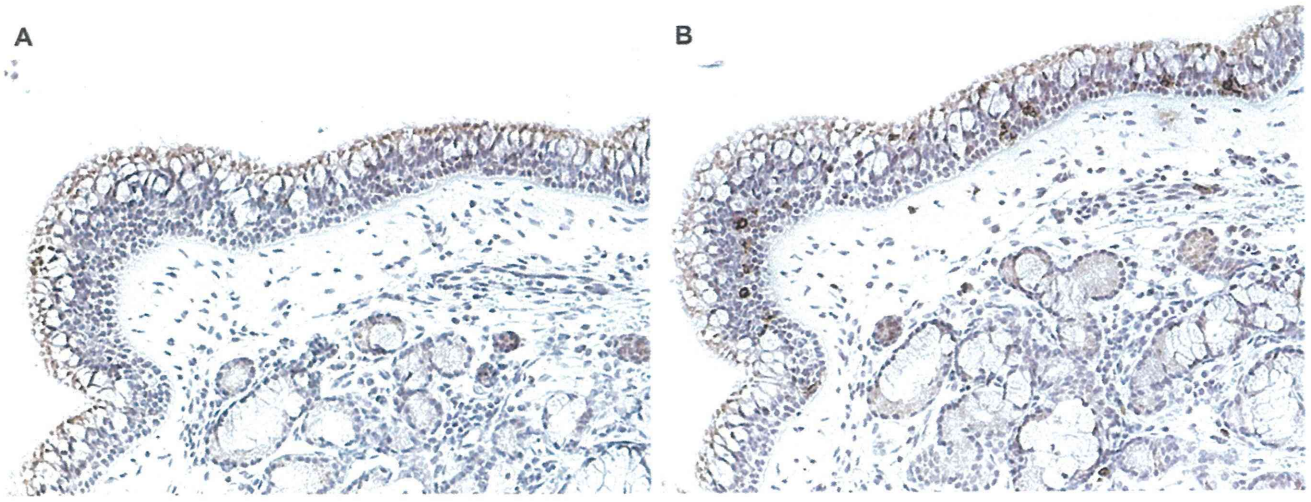

FIG E2. Negative control antibody staining in NPs tissue from a patient with CRSwNP. **A,** Normal rabbit IgG. **B,** Anti-human CPA3 antibody.

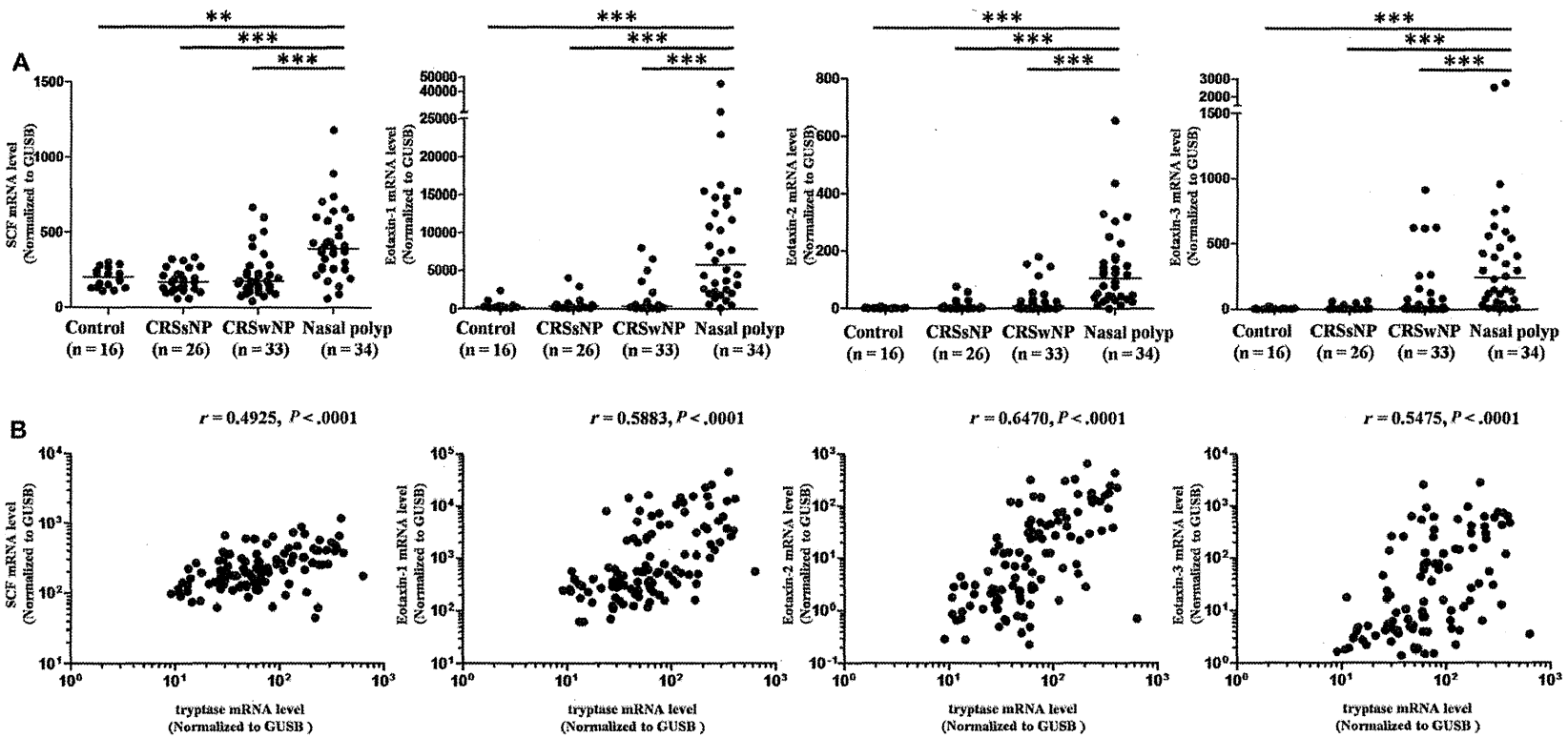

FIG E3. Correlation of tryptase with candidate mediators for elevation of mast cells. Total RNA was extracted from UT from control subjects (n = 16), patients with CRSsNP (n = 26), patients with CRSwNP (n = 33), and NPs (n = 34). **A,** The expression of tryptase and candidates for mast cell chemoattractant, SCF, eotaxin-1, eotaxin-2, and eotaxin-3, was analyzed by using real-time PCR. **B,** The correlations in nasal tissues were assessed by using Spearman rank correlation. $^{**}P < .001$, $^{***}P < .0001$.

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Prevalence of inhaled antigen sensitization and nasal eosinophils in Japanese children under two years old

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ABSTRACT

Objective: The increasingly younger age of onset of allergic rhinitis (AR) has recently become a problem. This study examined the prevalence of inhaled antigen sensitization and nasal eosinophils in children younger than two years old, with measurement of the serum concentrations of aeroallergen-specific IgE antibodies to house dust mites, cat fur, and Japanese cedar pollen, measurement of nasal eosinophil counts, and a questionnaire administered to the children's parents.
Methods: The subjects were a group of healthy children undergoing 18-month infant health checks provided by the local government, and sick children younger than two years old at the pediatric hospital.
Results: Among 408 healthy infants, 44 (10.7%) had antigen-specific IgE antibodies, 29 (7.1%) had nasal eosinophils, and eight (2.0%) had both specific IgE antibodies and nasal eosinophils. Nasal assessment revealed that 125 children had rhinorrhea. Of the infants who showed both sensitization to antigens and nasal eosinophils, six (1.5%) had confirmed rhinorrhea. Among 186 sick children younger than two years old at the pediatric hospital, aeroallergen-specific IgE antibodies were detected in five (2.6%). The presence of nasal eosinophils was confirmed in six children (3.2%), which percentage was smaller than that of the healthy group. No infant had either sensitization to antigens or nasal eosinophils.
Conclusion: The findings described above indicate that the minimum prevalence of AR might be 1.5% in 18-month-old children and that around 10% of affected children have aeroallergen-specific IgE antibodies in Japan. The incidence of AR in young children might increase further.

1. Introduction

Allergic rhinitis (AR) is an important global public health problem because of its markedly increased prevalence worldwide. Our recent study revealed that over 35% of the Japanese population aged 20–50 years old suffers from allergies during the Japanese cedar pollen (JCP) season [1]. Infantile allergic disease has been described as the 'allergic march' and is believed to begin with atopic dermatitis accompanied by infantile asthma, with subsequent progression to house dust mite-induced perennial AR. Otitis media with effusion is also included in allergic march among children 2–6 years. From around elementary school age onwards, seasonal allergic rhinitis (SAR) occurs [2]. Other authors have suggested that AR can occur earlier and can progress to bronchial asthma [3,4]. Despite the lack of definite evidence, a younger age of onset for AR has recently become a problem in Japan. However, because infants (unlike adults) develop AR with diverse symptoms, it is often difficult for pediatricians to make a diagnosis of AR. Therefore, even when symptoms of rhinitis are present, the contribution of AR is not strongly suspected in many cases. These points might explain why the incidence of AR and the sensitization rate have not been determined in children younger than two years old. In a clinical setting, however, it is important to have exact data for these parameters.

The accuracy of most studies performed to date is open to question because they have relied on parents' completed questionnaires to assess the incidence of AR. More recently, some studies have combined the results of surveys with measurement of antigen-specific IgE [5,6]. Nevertheless, other diagnostic procedures are also required, such as identification of symptoms with

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duration of at least two weeks and the skin prick test. According to Allergic Rhinitis and its Impact on Asthma (ARIA), for the diagnosis of AR, information about the existence of symptoms and identification of inhalant antigens must be obtained [7]. Furthermore, the Japanese Guidelines for Allergic Rhinitis (JG-AR) specify as requirements for the diagnosis the presence of nasal eosinophils or positive result of nasal provocation test, in addition to the above-mentioned ARIA's requirements [8]. Consequently, we combined a conventional questionnaire survey of AR completed by parents with direct observation by an ear, nose and throat specialist, measurement of the nasal eosinophil count, and qualitative measurements of aeroallergen-specific IgE antibodies. The subjects were a control group of healthy 18-month-old children and a group of children younger than two years for whom hospital care had been sought.

2. Methods

2.1. Subjects

In Japan, 18-month-old children undergo a free health check by a pediatrician and vaccination status. In this study, children undergoing this infant health check in Fukui City during September–November 2008 were enrolled as group 1. Of 539 children underwent the infant health check, 408 (220 boys, 188 girls, mean age of 18.4 months) participated in this study.

Group 2 comprised 186 infants younger than two years old (109 boys, 77 girls, with mean age of 10.9 months) who received care at the Department of Pediatrics at Fukui Aiiku Hospital during September–October, 2008. They were grouped by age: 0–5 months, $n = 53$; 6–11 months, $n = 49$; 12–17 months, $n = 46$; and 18–23 months, $n = 38$. Informed consent was obtained from the parents of all participating children. The Ethics Review Board of the University of Fukui approved this study.

2.2. Questionnaire survey

Each child's parents were asked about siblings, the past history of allergic disease, exposure to environmental tobacco smoke, family history of allergy, and pets (Table 1). To exclude 'diagnosis' of the allergic state by the parents, the item 'cured' was included in the questionnaire to exclude any temporary condition that differed from constitutional allergic disease.

2.3. Assessment of nasal eosinophils

Nasal scraping from both inferior nasal turbinates was performed with a disposable swab and sampled onto a glass slide. Each sample was stained with Wright–Giemsa stain to confirm the presence or absence of eosinophils. Each child was classified as negative or positive. Positive means that more than 10 eosinophils were found under 200× magnification.

Table 1
Questionnaire.

Q1: About your baby

The baby has _____ siblings, and the baby's birth order is _____th.

Has the baby ever been diagnosed as having an allergic disease at a hospital or clinic?

Please check the baby's disease(s) and mark the condition with a circle if "Yes".

No Yes

( ) Atopic dermatitis (From _____month, treated cured wait and see)

( ) Bronchial asthma (From _____month, treated cured wait and see)

( ) Allergic rhinitis (From _____month, treated cured wait and see)

( ) Allergic conjunctivitis (From _____month, treated cured wait and see)

( ) Food allergy (From _____month, treated cured wait and see)

Q2: About the baby's family

Please check the disease(s) and mark the person(s) with a circle if the baby has a family member(s) with an allergy diagnosed at a hospital or clinic.

( ) Atopic dermatitis (Brothers, Sisters, Father, Mother, Others)

( ) Bronchial asthma (Brothers, Sisters, Father, Mother, Others)

( ) Allergic rhinitis (Brothers, Sisters, Father, Mother, Others)

( ) Allergic conjunctivitis (Brothers, Sisters, Father, Mother, Others)

( ) Food Allergy (Brothers, Sisters, Father, Mother, Others)

Is there a smoker at home?

No Yes (Smoker: Father, Mother, Other(s))

Q3: About pets

Are there any pets at home?

No Yes (Kind:)

2.4. Identification of inhaled antigens

After finger prick with a needle, blood was collected into a pipette, and specific IgE antibodies for house dust mites, cat fur, and JCP were measured (IMMFAST Check J1; Mitsubishi Chemical Medience Corp., Tokyo, Japan). This test contains reagents to detect allergen-specific IgE antibodies using immunochromatography and corresponded to a Class 2 level or higher result according to the CAP-RAST method [9].

2.5. Intranasal observation

To check the existence of nasal discharge and observed inferior turbinate, each child was examined by an otorhinolaryngology and allergy specialist (Y. Osawa) using an anterior rhinoscope before the blood collection. Intranasal observation was defined as positive when rhinorrhea and inferior conchal swelling were noted. This criterion enabled us to detect more patients than when the diagnoses were based on AR symptoms, and led to identification of numerous children.

After that, if a thick deposit of mucus was obtained on the disposable cotton swab at the time of nasal eosinophil measurement, then intranasal observation was regarded as positive.

2.6. Statistical analysis

Statistical analysis was conducted using Fisher's exact test. Differences were considered statistically significant for $p < 0.05$.

3. Results

3.1. Group 1

The results are presented in Table 2. Of the children who showed positivity for antigen-specific IgE antibodies, 31 (7.6%) were positive for house dust mites, 12 (2.9%) for cat fur, and five (1.2%) for JCP. Some children had antibodies for more than one antigen. Therefore, a total of 44 (10.7%) had one or more positive antibodies for these antigens. Among the 29 children (7.1%) who had nasal eosinophils, eight (2.0%) also had antigen-specific IgE antibody for house dust mite (seven children) or cat fur (one child).

Table 2
Details of children undergoing an 18-month infant health check (Group 1) who had antigen-specific IgE antibody or nasal eosinophils.

	Antigen-specific IgE				Total
	Mite	Cat	JCP	Negative	
Group 1					
Eosinophils in nasal smear					
Positive	7	1	0	21	29
Negative	24	11	5	343	379
Total	31	12	5	364	408

Demographic and social data are presented in Table 3. In 30 children, a pet cat was significantly associated with positivity for specific IgE antibodies to house dust mite, cat fur, and JCP. The same trend was apparent for the presence of any pet (68 children), irrespective of the species. The other hand, a significant correlation was found between the presence of nasal eosinophils and a 'positive paternal history of allergic disease', as well as having three or more siblings.

Intranasal observation revealed rhinorrhea in 125 of the 408 children (30.6%). Among the children who were positive for antigen-specific IgE antibodies, an intranasal observation revealed rhinorrhea in 12 where the antigen was house dust mites, six where it was cat fur, and one where it was JCP.

Table 4 presents details of eight children who had evidence of both sensitization to antigens and positivity for nasal eosinophils. Of these eight children, six (1.5%) were positive for intranasal rhinorrhea, who had definite AR according to the criteria of JG-AR [8]. Of these eight children, four (50%) had close contact with pets. A diagnosis of AR had been made for only one (Case 3) of the eight children described above. The simultaneous questionnaire survey results revealed that 11 children (2.7%) had been diagnosed with AR. However only one of 11 children had sensitization to antigens or none had nasal eosinophils.

Table 3
Demographic and social data—group 1 (*/**/***: there are overlapping cases).

	Number	Antigen-specific IgE positive cases						Eosinophils in nasal smear	
		Mite	*P* value	Cat	*P* value	JPC	*P* value	Positive	*P* value
Past history of allergic diseases									
No	334	23		9		4		22	
Yes—any allergic diseases*	74	8	0.2340	3	0.4624	1	>.9999	7	0.4509
Yes—atopic dermatitis*	29	1	0.2547	1	0.5698	1	0.3241	3	0.4371
Yes—asthma*	12	2	0.2120	0	>.9999	0	>.9999	3	0.0475
Yes—food allergy *	29	2	0.2616	2	0.2164	0	>.9999	3	0.4371
Family history of allergic diseases									
No	207	13		7		3		12	
Yes-maternal**	136	12	0.4009	4	>.9999	2	>.9999	13	0.2073
Yes-paternal**	125	12	0.2879	3	0.2667	1	>.9999	17	0.0257
Number of brothers (sisters)									
Only one	219	14		8		4		12	
Two	131	13	0.3004	3	0.5467	1	0.6543	9	0.6449
Three or more	58	4	>.9999	1	0.69	0	0.5828	8	0.0429
Passive smoking									
No	181	14		6		3		11	
Yes—by mother	29	2	>.9999	1	>.9999	0	>.9999	3	0.4173
Yes—by other(s)	198	15	>.9999	5	0.7668	2	0.6792	15	0.7668
Pet(s) keeping									
No	340	21		5		2		23	
Yes—any pets***	68	10	0.023	7	0.0011	3	0.0345	6	0.6036
Yes—a cat***	30	6	0.015	7	<.0001	2	0.0345	2	>.9999
Yes—a dog***	40	5	0.1741	0	>.9999	1	0.2844	4	0.5093

Table 4
Details of eight children with antigen sensitization/nasal eosinophils in group 1.

Case	Age (months)	Sex	Antigen specific IgE	Eosinophils in nasal smear	Intranasal observation	Complications and past history of allergic disease	Family history of allergic disease	Siblings (order)	Pet(s)	Passive smoking
1	18	M	Mite	++	+	–	Father, mother	1 (1)	Cat, dog, bird	+
2	18	F	Mite	+++	+	–	Father, mother	1 (1)	Dog	+
3	19	M	Cat	+++	+	Asthma	–	2 (2)	Cat	–
4	19	F	Mite	+	–	Food allergy	–	1 (1)	–	–
5	20	M	Mite	+	+	–	–	2 (2)	Cat	–
6	18	M	Mite	+	+	–	–	2 (2)	–	+
7	18	F	Mite	+	+	–	Mother, sister	3 (3)	–	+
8	19	M	Mite	+	–	–	Father	2 (2)	–	–

3.2. Group 2

Of group 2, 150 (80.6%) had upper respiratory tract infections; 36 (19.4%) had viral infections of the digestive tract. No other severe illness was found or reported. Five children showed positivity for aeroallergen-specific IgE antibodies (2.6%) (Table 5). Among them, three (1.6%) had antibodies against house dust mites; two (1.1%) had antibodies against cat fur. They were classified according to age as follows: 6–11 months—one (0.5%, IgE antibody specific to cat fur), 12–17 months—one (0.5%, IgE antibody specific to house dust mite), and 18–23 months—three (two with IgE antibodies specific to house dust mites [5.3%], and one with IgE antibodies specific to cat fur [2.6%]). The youngest child, nine months old, resided with a cat. Although the presence of cat fur-specific IgE antibodies was confirmed, this infant was negative on intranasal observation and had no personal or family history of allergic disease.

The presence of nasal eosinophils was confirmed in six children (3.2%) (Table 5). These children were classified according to age: 0–5 months—two, 6–11 months—one, 12–17 months—one, and 18–23 months—two. The youngest child, for whom respiratory syncytial virus infection had been diagnosed, was only one month old (data not shown). In group 2, many children had infections, and the percentage of children in whom intranasal observation had detected rhinorrhea was high (63.4%, 118). Intranasal observation for rhinorrhea was positive in all six children with nasal eosinophils. These children were characterized by their 'positive family history of allergic disease' and having 'two or more siblings'; each was the youngest child in the family.

4. Discussion

This study showed that the prevalence of AR in health 18-month-old Japanese children might be at least 1.5% according to the criteria in the JG-AR [8]. Because this survey was performed outside the JCP season, the prevalence could be as high as 6.1% when the following children are included: five who had specific IgE antibodies for JCP, two who were negative for intranasal observation of rhinorrhea despite sensitization to antigens and positivity for nasal eosinophils, and 12 who had a combination of sensitization to antigens, negative nasal eosinophil test, and positive intranasal rhinorrhea. The incidence of specific IgE antibodies to three antigens (house dust mites, cat fur, and JCP) was as high as 10.7% at the age of 18 months. These findings suggest that the actual prevalence of AR at the age of 18 months might be higher than 1.5%.

Table 5
Details of children to hospital (group 2) who had antigen-specific IgE antibody or nasal eosinophils.

	Antigen-specific IgE				Total
	Mite	Cat	JCP	Negative	
Group 2					
Eosinophils in nasal smear					
Positive	0	0	0	6	6
Negative	3	2	0	175	180
Total	3	2	0	181	186

Results for group 2 show that no child younger than two years old had either antigen-specific IgE antibodies or nasal eosinophils. Furthermore, the positivity rate for specific IgE antibodies to the three antigens in group 2 was lower than in group 1. Classification of group 2 according to age showed that 38 were 18–23 months old. Because the overall positivity rate for antigen sensitization among group 1 was estimated as 10.7%, the corresponding incidence in group 2 would be approximately 10% of 38: four children. In fact, three were positive in the age group of 18–23 months (7.9%). Consequently, the positivity rates for sensitization to antigens are equivalent for group 1 and group 2.

Regarding previously reported epidemiological studies of AR in children younger than three years of age, the GINA study in Germany revealed that the positivity rate for sensitization to common inhaled antigens among 1290 infants aged 12 months was 2.3% [6]. In the Netherlands, 5.9% of 751 infants aged 12–24 months were sensitized [5]. According to a survey conducted on 562 infants aged 18 months in Denmark, persistent sensitization was noted in 10%, and 3% had a positive skin prick test [10]. Taken together with the results of the present study, these results indicate no difference in the positivity rate for sensitization to antigens or the prevalence of AR between Europe and Japan, as judged by the skin prick test.

Many previous investigations of infantile AR have been based solely on questionnaire surveys for the parents. Their reliability is open to question. According to this questionnaire survey, 11 children (2.7%) had a history of AR that had been diagnosed by a medical practitioner. Although this prevalence is intermediate between the percentages of 1.5% and 6.1% found in this study, sensitization to antigens was confirmed in only one of the 11 children. Furthermore, none of the 11 children had nasal eosinophils. These findings indicate that the diagnoses of AR in infants younger than two years old by medical practitioners or based on reports of AR from the parents are not accurate. Under the age of two years, reports of upper respiratory tract infections might be excessive in many cases because it is impossible for infants to describe their symptoms reliably.

In this study, nasal eosinophils were counted. Nasal eosinophil measurement has a long history as an important parameter for diagnosis of AR: its use has been reported since the 1970s. Malmberg reported that eosinophilia in nasal smears was correlated significantly with a history of AR, nasal mucosal swelling, and nasal discharge [11]. Thereafter, much work has been done to assess the usefulness of assessing nasal eosinophils in the diagnosis of AR. For example, Zeiger and Heller performed a prospective study of infants with a strong family history of allergic disease and showed that eosinophils were present at higher levels

in the nasal scrapings of allergic children between birth and four years of age, but were uncommon in children without allergic disease [12]. Crobach et al. reported that nasal smear eosinophilia were significantly associated with the diagnosis of AR, but the contribution of this parameter was small and clinically irrelevant [13]. Because of such difficulty in interpreting the clinical significance of nasal smear eosinophils, this test is not often performed for the diagnosis of AR. However, reviews of the importance of nasal eosinophilia in AR have confirmed that the eosinophil count is correlated with various symptoms and with an immunological marker. Therefore, nasal eosinophil measurement is useful [14–16].

In this study, 29 children of group 1 nasal eosinophils were positive (7.1%). Nasal eosinophilia was found in two of 46 children (12–17 months in group 2, 4.3%) and two of 38 children (18–23 months in group 2, 5.3%). We expected that positive rate of nasal eosinophils in group 2 would be higher than that of group 1 because viral and bacterial infection in the respiratory tract will induce eosinophils in nasal mucosa. The association between nasal eosinophils and the respiratory tract infection is now under investigation. Furthermore, for respiratory syncytial virus (RSV) infection, eosinophils appear in infantile rhinorrhea [16]. However, recently reports suggested local allergic rhinitis; allergic rhinitis with skin test and serum specific IgE negative [17,18]. Rondón et al. revealed local allergic rhinitis patients had local production of specific IgE and eosinophilic cationic protein induced by allergens [18]. These findings suggest that detecting the combination of specific IgE antibodies to inhaled antigens and nasal eosinophils supports a diagnosis of AR.

Demographic and social analyses of this study revealed that sensitization to inhaled antigens is correlated with the presence of pets, especially cats, which agrees with reports from western countries [19–21]. In contrastnasal eosinophilia was correlated in the present study with a family history (paternal) of AR and with the number of siblings. No comparisons can be made because of the paucity of literature describing nasal eosinophilia in infants, the influence of a family history of allergic disease, or the number of siblings [22]. It has been reported that nasal eosinophils even appear in infants with food allergy [22]. However, there was no significant correlation between the presence of specific IgE/nasal eosinophilia and a positive history of food allergy (Table 3).

Our aim in this study is to estimate the minimum prevalence rate of AR in Japanese children under two years old. The minimum prevalence of AR is 1.5% in Japanese healthy 18-month-old-children, although the maximum prevalence of AR might be 6–10%. Results show that AR can be assumed to occur from the age of 18 months onwards. Therefore, it is necessary to establish diagnostic criteria for AR in children younger than two years old to assist with prevention by early intervention and early diagnosis.

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References

[1] M. Sakashita, T. Hirota, M. Harada, R. Nakamichi, T. Tsunoda, Y. Osawa, et al., Prevalence of allergic rhinitis and sensitization to common aeroallergens in a Japanese population, Int Arch Allergy Immunol 151 (2009) 255–261.
[2] S. Illi, E. von Mutius, S. Lau, R. Nickel, C. Grüber, B. Niggemann, et al., The natural course of atopic dermatitis from birth to age 7 years and association with asthma, J Allergy Clin Immunol 113 (2004) 925–931.
[3] P.A. Pedersen, E.R. Weeke, Asthma and allergic rhinitis in the same patients, Allergy 38 (1983) 25–29.
[4] R.J. Settipane, G.W. Hagy, G.A. Settipane, Long-term risk factors for developing asthma and allergic rhinitis: a 23-year follow-up study of college students, Allergy Proc 15 (1994) 21–25.
[5] A. Baatenburg de Jong, L.D. Dikkeschei, P.L.P. Brand, High prevalence of sensitization to aeroallergens in children 4 yrs of age or younger with symptoms of allergic disease, Pediatr Allergy Immunol 20 (2009) 735–740.
[6] I. Brockow, A. Zutavern, U. Hoffmann, A. Grübl, A. von Berg, S. Koletzko, et al., Early allergic sensitizations and their relevance to atopic diseases in children aged 6 years: results of GINI study, J Investig Allergol Clin Immunol 19 (2009) 180–187.
[7] J. Bousquet, N. Khaltaev, A.A. Cruz, J. Denburg, W.J. Fokkens, A. Togias, et al., Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen), Allergy 63 (Suppl. 86) (2008) 8–160.
[8] K. Okubo, Y. Kurono, S. Fujieda, S. Ogino, E. Uchio, H. Odajima, et al., Japanese guideline for allergic rhinitis, Allergol Int 60 (2) (2011) 171–189.
[9] T. Ono, M. Kawamura, S. Arao, H. Nariuchi, A highly sensitive quantitative immunochromatography assay for antigen-specific IgE, J Immunol Methods 272 (2003) 211–218.
[10] H. Jøhnke, L.A. Norberg, W. Vach, A. Høst, K.E. Andersen, Patterns of sensitization in infants and its relation to atopic dermatitis, Pediatr Allergy Immunol 17 (2006) 591–600.
[11] H. Malmberg, Symptoms of chronic and allergic rhinitis and occurrence of nasal secretion granulocytes in university students, school children and infants, Allergy 34 (1979) 389–394.
[12] R.S. Zeiger, S. Heller, Development of nasal basophilic cells and nasal eosinophils from age 4 months through 4 years in children of atopic parents, J Allergy Clin Immunol 91 (1993) 723–734.
[13] M.J. Crobach, J. Hermans, A.A. Kaptein, J. Ridderikhoff, H. Petri, J.D. Mulder, The diagnosis of allergic rhinitis: how to combine the medical history with the results of radioallergosorbent tests and skin prick tests, Scand J Prim Health Care 16 (1998) 30–36.
[14] G. Ciprandi, A. Vizzaccaro, I. Cirillo, M. Tosca, A. Massolo, G. Passalacqua, Nasal eosinophils display the best correlation with symptoms, pulmonary function and inflammation in allergic rhinitis, Int Arch Allergy Immunol 136 (2005) 266–272.
[15] S.T. Chen, H.L. Sun, K.H. Lu, K.H. Lue, M.C. Chou, Correlation of immunoglobulin E, eosinophil cationic protein, and eosinophil count with the severity of childhood perennial allergic rhinitis, J Microbiol Immunol Infect 39 (2006) 212–218.
[16] M. Shinohara, H. Wakiguchi, H. Saito, K. Matsumoto, Presence of eosinophils in nasal secretion during acute respiratory tract infection in young children predicts subsequent wheezing within two months, Allergol Int 57 (2008) 359–365.
[17] C. Rondón, J.J. Romero, S. López, C. Antúnez, E. Martín-Casañez, M.J. Torres, et al., Local IgE production and positive nasal provocation test in patients with persistent nonallergic rhinitis, J Allergy Clin Immunol 119 (4) (2007) 899–905.
[18] C. Rondón, J. Fernández, S. López, P. Campo, I. Doña, M.J. Torres, et al., Nasal inflammatory mediators and specific IgE production after nasal challenge with grass pollen in local allergic rhinitis, J Allergy Clin Immunol 124 (5) (2009) 1005–1011.
[19] G. Anyo, B. Brunekreef, G. de Meer, F. Aarts, N.A. Janssen, P. van Vliet, Early, current and past pet ownership: associations with sensitization, bronchial responsiveness and allergic symptoms in school children, Clin Exp Allergy 32 (2002) 361–366.
[20] J.C. Celedon, A.A. Litonjua, L. Ryan, T. Platts-Mills, S.T. Weiss, D.R. Gold, Exposure to cat allergen, maternal history of asthma, and wheezing in first 5 years of life, Lancet 360 (2002) 781–782.
[21] B. Holscher, C. Frye, H.E. Wichman, J. Heinrich, Exposure to pet and allergies in children, Pediatr Allergy Immunol 13 (2002) 334–341.
[22] H. Shioda, T. Mishima, The significance of mast cells in nasal smears from patients with food allergy, Allergy 37 (1966) 321–328.

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◆特集・耳鼻咽喉科における抗ウイルス薬・ステロイドの効果的処方

好酸球性副鼻腔炎とアスピリン喘息の治療とステロイドの位置づけ

藤枝重治[*1]　坂下雅文[*2]

Abstract 好酸球性副鼻腔炎は，鼻内に鼻茸を有し，篩骨洞主体の病変で嗅覚障害を伴い，鼻粘膜や末梢血中で好酸球増加を伴う難治性副鼻腔炎である．保存的治療として経口ステロイドが有効であり，内視鏡下鼻副鼻腔手術を行っても再発が多い．現在，大学病院レベルで行われている内視鏡下鼻副鼻腔手術の28％が好酸球性副鼻腔炎であった．好酸球性副鼻腔炎は，鼻粘膜組織中の強い好酸球浸潤で診断されていたが，最近組織以外の臨床像で診断できるように検討されている．アスピリン喘息に伴う副鼻腔炎も好酸球浸潤が強く，好酸球性副鼻腔炎の範疇に含まれる．当然経口ステロイドが有効であるが，好酸球性副鼻腔炎よりもさらに難治性である．これらは，まだ発症機序もよくわかっておらず，今後発展を期待する分野である．とりわけステロイド以外の新しい治療法の開発が待たれている．

Key words 好酸球性副鼻腔炎(eosinophilic chronic sinusitis)，アスピリン喘息(aspirin-induced asthma)，鼻茸(nasal polyp)，難治性副鼻腔炎(intractable sinusitis)

好酸球性副鼻腔炎とは

本邦における副鼻腔炎は，ウイルス感染とそれに続発する細菌感染による好中球浸潤を主体とする病変が中心であった．治療として急性期に対する抗菌薬の開発と慢性期に対するマクロライド少量長期投与によって，治癒率が有意に向上しえた．その一方で，アレルギー疾患の増加とともに好酸球浸潤を主体とする難治性の副鼻腔炎が増えてきた．森山・春名は，病変が篩骨洞主体，嗅覚障害を主訴とし，鼻茸が存在し，鼻粘膜・血中好酸球増加を伴う好酸球性副鼻腔炎の疾患概念を提唱した[1]．この疾患は，これまでの炎症性疾患とは異なり，成人発症であり気管支喘息の合併率が高く，難治性かつ感覚神経障害(嗅覚神経)を伴う特徴があった．平成22年度厚生労働省難治疾患克服事業「好酸球性副鼻腔炎の疫学，診断基準作成等に関する研究」(研究代表者：藤枝重治)では，札幌医大，獨協医大，自治医科大学附属大宮医療センター，慈恵会医大，順天堂大学，横浜市大，大阪医大，岡山大学，和歌山日赤，広島大学，島根医大，福井大学の12施設および関連病院で，2007年1月1日～2009年12月31日の3年間に行った病理組織が確実にある慢性副鼻腔炎手術症例を抽出し，臨床データを解析した．合計3,014例が集まり，うち好酸球性副鼻腔炎は822例(27.3％)であった．これまで本邦での慢性副鼻腔炎手術症例中，約30％程度が好酸球性副鼻腔炎と言われていたが，ほぼ一致した結果であった．

3,014例のデータを単ロジスティック解析した．血中好酸球率は3％以上5％未満，5％以上8％未満，8％以上に分類すると好酸球性副鼻腔炎であるリスクは，0％以上3％未満に比較してそれぞれ3.7倍，6.9倍，11.6倍増加した．臨床像では，

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表 1. 春名らによる好酸球性副鼻腔炎の診断基準(案)

絶対条件
1）成人発症
2）両側性副鼻腔病変
3）CT所見で上顎洞よりも篩骨洞の陰影が優位
4）主訴のなかに嗅覚障害がある
5）内視鏡所見で鼻ポリープを認める
6）血中好酸球6%(300個/ml)以上もしくは副鼻腔組織中好酸球100個以上で好酸球優位

付帯条件
1）ステロイド，特に経口ステロイドが臨床所見の改善に有効
2）気管支喘息，アスピリン喘息を合併する*
3）内視鏡下鼻内副鼻腔手術後に経過不良を呈する
4）マクロライド療法の効果は不明
5）粘稠性分泌物が認められる

表 2. Sakuma，Ishitoyaらによる好酸球性副鼻腔炎の診断基準

1）血中好酸球6%以上
2）CT所見(嗅裂陰影スコア1以上，後部篩骨洞陰影スコア1以上)
3）気管支喘息合併の有無

表 3. 好酸球性副鼻腔炎研究班の診断基準に含める項目(案)

1）年齢
2）両側病変
3）嗅裂閉鎖
4）篩骨洞陰影優位
5）薬物アレルギーの合併
6）末梢血好酸球率

両側病変ありが5.2倍，鼻茸あり3.4倍，粘稠鼻汁あり4.3倍，嗅裂閉鎖あり3.5倍，篩骨洞陰性優位6.1倍，アスピリン喘息合併4.6倍，薬物アレルギー合併2.5倍，気管支喘息合併2.8倍が有意であったが，各種項目RAST陽性は1.4倍から1.9倍，アレルギー性鼻炎合併1.7倍であった．

現在，好酸球性副鼻腔炎の診断は，副鼻腔粘膜の好酸球浸潤の程度によって診断されている．我々は現在のところ，400倍視野で120個以上の好酸球浸潤を認めた場合，好酸球性副鼻腔炎を考慮すべきと考えている．組織診断に加え，血中好酸球数の上昇と上述の臨床症状を加味し，診断するようにしている．今回の研究では，病理診断を行わない診断基準が臨床的に有用と考え，これまでの森山・春名らの基準(表1)を参考に，新しい重み付けによる診断基準を作成中である．一方，Sakumaらは，血中好酸球の割合，CTでの後部篩骨洞陰影スコアと嗅裂の陰影スコアを用いると好酸球性診断において高い特異度が得られると報告している[2](表2，3)．

アスピリン喘息とは

アスピリン喘息は，鼻茸を伴う副鼻腔炎，気管支喘息，アスピリン過敏を3主徴とする疾患である．アスピリン喘息の病態や発症機序はまだ明確にはされていないが，現時点での共通認識は，アラキドン酸カスケードにおいて重要な酵素であるCyclooxygenase(COX)の機能低下が起こるため，ロイコトリエン(LT)の方向に代謝されていくことに問題があるとされている[3]．すなわち図1に示す，LTC_4，LTD_4，LTE_4(3つを合わせてシステニルロイコトリエン；CysLTと呼ぶ)が多く産生される．アスピリン喘息患者の尿中には，有意に多くのLTE_4が含まれていることが報告されている[4]．これらCysLTは気管支収縮作用，気道粘液分泌亢進作用を有する．一方で，プロスタグランディン(PG)の合成は阻害され，有意に減少している．PGE_2はLTとは逆に気管支拡張作用を有する．PGE_2の受容体であるEP_3のノックアウトマウスでは，IgEは変化しないが，炎症反応が増大しEP_3アゴニストでは炎症反応が抑制されることが報告された[5]．PGE_2の他の受容体であるEP_2，EP_3のノックアウトマウスでも炎症反応が増強し