

Glandular mast cells with distinct phenotype are highly elevated in chronic rhinosinusitis with nasal polyps

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Background: Although chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP) is characterized by T_H2 inflammation, the role of mast cells is poorly understood.

Objective: The objective of this study was to investigate the presence, localization, and phenotype of mast cells in patients with CRS.

Methods: We collected nasal tissue and nasal lavage fluid from patients with CRS and control subjects. We analyzed mRNA for the mast cell proteases tryptase, chymase, and carboxypeptidase A3 by using real-time PCR and measured mast cell protease proteins by using ELISA, immunohistochemistry, and immunofluorescence.

Results: Tryptase mRNA was significantly increased in nasal polyps (NPs) from patients with CRSwNP ($P < .001$) compared with uncinate tissue from patients with CRS or control subjects. Tryptase protein was also elevated in NPs and in nasal lavage fluids from patients with CRSwNP. Immunohistochemistry showed increased numbers of mast cells in epithelium and glands but not within the lamina propria in NPs. The mast cells detected in the epithelium in NPs were characterized by the expression of tryptase and carboxypeptidase A3 but not chymase. Mast cells expressing all the 3 proteases were abundant within the glandular epithelium of NPs but were not found in normal glandular structures.

Conclusions: Herein we demonstrated a unique localization of mast cells within the glandular epithelium of NPs and showed that mast cells in NPs have distinct phenotypes that vary by tissue location. Glandular mast cells and the diverse subsets of mast cells detected may contribute to the pathogenesis of CRSwNP. (*J Allergy Clin Immunol* 2012;■■■:■■■-■■■.)

Key words: Chronic rhinosinusitis, nasal polyps, mast cells, tryptase, chymase, carboxypeptidase A3 (CPA3)

Abbreviations used

CRS:	Chronic rhinosinusitis
CPA3:	Carboxypeptidase A3
CRSsNP:	CRS without nasal polyps
CRSwNP:	CRS with nasal polyps
ECP:	Eosinophil cationic protein
GUSB:	β -Glucuronidase
MC-T:	Mast cell-tryptase
MC-TC:	Mast cell-tryptase/chymase
NPs:	Nasal polyps
SCF:	Stem cell factor
SP:	Substance P
UT:	Uncinate tissue

Chronic rhinosinusitis (CRS) is one of the most common chronic diseases in adults in the United States and affects up to 15% of the population.^{1,2} The prevalence and medical costs of CRS are increasing and have become important social issues. This disease is typically classified into 2 types: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). The etiology and pathogenesis of CRS remain controversial, but bacteria, viruses, and fungi have all been implicated in the establishment of an inflammatory process.³⁻⁵ Particularly, CRS is often comorbid with asthma and is resistant to therapeutic interventions.⁶ Surgical intervention is frequently necessary to clear the nasal and sinus passages and repeat endoscopic sinus surgery is often required. Recent studies suggest that CRSwNP is characterized by a T_H2-skewed eosinophilic inflammation characterized by significantly elevated levels of IL-5, IL-13, eotaxin, and eosinophil cationic protein (ECP).⁷⁻⁹ Taken together, these results point to a critical role for eosinophils in the pathophysiology of CRSwNP and further suggest that factors triggering eosinophil degranulation may also be associated with the formation of nasal polyps (NPs).¹⁰

The potential importance of mast cells has been considered in CRS disease pathogenesis because they produce an abundance of cytokines that activate eosinophils, molecules that directly promote tissue remodeling and chemical mediators that can produce profound tissue edema.¹¹ Mast cells play key roles in host defense, homeostasis, tissue repair, and mechanisms of allergic inflammation.¹² In the case of human mast cells, 2 subtypes have been recognized by variable expression of granular neutral proteases: mast cell-tryptase (MC-T) and mast cell-tryptase/chymase (MC-TC). MC-T-type predominantly express high levels of tryptase but not chymase or carboxypeptidase A3 (CPA3),

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This study was supported by National Institutes of Health grants R37HL068546-27, R01HL078860, and R01AI072570 and the Ernest S. Bazley Trust.

Disclosure of potential conflict of interest: R.C. Kern and R.P. Schleimer received research support from the National Institutes of Health. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication August 16, 2011; revised February 20, 2012; accepted for publication February 21, 2012.

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0091-6749/\$36.00

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doi:10.1016/j.jaci.2012.02.046

TABLE 1. Subjects' characteristics

	Control			CRSsNP			CRSwNP			CRSwNP polyp
Total no. of subjects	n = 42 (17 M/25 F)			n = 70 (26 M/44 F)			n = 91 (56 M/35 F)			—
Age (y), median (range)	37 (16-63)			36 (18-69)			44 (22-75)			—
	Y	N	U	Y	N	U	Y	N	U	—
Atopy	2	32	8	35	27	8	45	24	22	—
Asthma	0	35	7	17	46	7	44	44	3	—
Methodology used										
Tissue RNA	n = 16 (7 M/9 F)			n = 26 (10 M/16 F)			n = 33 (21 M/12 F)			n = 34 (22 M/12 F)
Age (y), median (range)	45 (16-62)			35 (20-59)			38 (23-67)			39 (23-67)
Nasal scraping RNA	n = 7 (4 M/3 F)			n = 15 (8 M/7 F)			n = 10 (6 M/4 F)			n = 13 (7 M/6 F)
Age (y), median (range)	47 (29-62)			37 (18-61)			54 (27-73)			48 (22-73)
Tissue extract	n = 7 (5 M/2 F)			n = 19 (7 M/12 F)			n = 16 (9 M/7 F)			n = 19 (13 M/6 F)
Age (y), median (range)	49 (19-64)			36 (26-69)			46 (26-73)			45 (29-71)
Nasal lavage	n = 15 (5 M/10 F)			n = 14 (5 M/9 F)			n = 15 (9 M/6 F)			—
Age (y), median (range)	31 (21-63)			38 (24-64)			46 (27-73)			—
Immunohistochemistry	n = 10 (3 M/7 F)			n = 10 (4 M/6 F)			n = 10 (6 M/4 F)			n = 10 (7 M/3 F)
Age (y), median (range)	28 (16-62)			31 (21-58)			45 (26-75)			50 (30-75)

F, Female; M, male; N, no; U, unknown; Y, yes.

whereas MC-TC express all the 3 proteases.¹³ MC-TC are essentially the exclusive type of mast cell found in normal skin and account for a small minority of mast cells in the lung. MC-T predominate in the alveolar wall and epithelium of the lung, whereas MC-TC favor the bronchial smooth muscle of patients with asthma and correlate with their bronchial hyperreactivity.¹⁴ MC-T numbers in the respiratory epithelium increase during the pollen season in sensitive subjects.^{15,16} These distinct tissue distributions and disease associations suggest a purposeful presence for each type of mast cell. Recent studies have demonstrated increased levels of mast cells in the presence of highly eosinophilic allergic inflammatory diseases such as asthma and eosinophilic esophagitis.^{17,18} Moreover, mast cells in these studies have been shown to have a unique protease phenotype (tryptase and CPA3 high and chymase low).^{19,20} The expression, phenotype, regulation, and protease expression patterns of mast cells in CRS have not been explored in detail.

In this study, we examined the distribution and phenotype of mast cells in uncinate tissue (UT) from control as well as from patients with CRS, including both CRSsNP and CRSwNP; we also evaluated NPs in patients with CRSwNP. We used quantitative RT-PCR, ELISA, and immunohistochemistry to determine the expression and distribution of mast cells as well as mast cell tryptase, chymase, and CPA3 in subjects with CRS. We detected a profound elevation of mast cells located within glands in NPs, an observation not previously described.

METHODS

Patients

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.¹

Patients with an isolated antrochoanal polyp, cystic fibrosis, or unilateral NPs were excluded from the study. Details of subjects' characteristics are included in Table 1 and in the Methods section of this article's Online Repository at www.jacionline.org. 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.

Real-time RT-PCR

Total RNA from sinus tissue was extracted by using QIAzol (Qiagen, Valencia, Calif), and the quality of total RNA from sinus tissue was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif). Semiquantitative real-time RT-PCR was performed by using the TaqMan method. The mRNA expression levels were normalized to the median expression of the housekeeping gene β -glucuronidase (GUSB). Further details can be found in the Methods section of this article's Online Repository.

ELISA

Tryptase was assayed by using a commercially available assay kit (Usen Life Science, Inc, Wuhan, China). The color intensity was measured with a Bio-Rad Spectrophotometer Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, Calif). Concentrations of tryptase protein were normalized to the concentration of total protein. Details can be found in the Methods section of this article's Online Repository.

Immunohistochemistry

Immunohistochemistry was performed as described previously.²¹ Tissue sections were incubated with mouse antihuman tryptase mAb (Thermo Scientific, Fremont, Calif) or mouse antihuman chymase mAb (Thermo Scientific) or rabbit antihuman CPA3 polyclonal antibody (HPA008689, Sigma, St Louis, Mo) overnight at 4°C. Sections were rinsed and then incubated in biotinylated secondary horse antimouse or goat antirabbit 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 (Vector Laboratories, Burlingame, Calif) for 1 hour. Sections were rinsed again and incubated in DAB reagent (Invitrogen, Carlsbad, Calif) and then counterstained with hematoxylin. Slides were blined, and 10 pictures were randomly taken from each slide. The number of positive cells in epithelium, glands, and submucosa was counted by 2 independent observers. Details of the methods for immunofluorescence and immunohistochemistry are described in the Methods section of this article's Online Repository.

Statistical analysis

All data are reported as the median. Differences between groups were analyzed by using the 1-way ANOVA Kruskal-Wallis test. Correlations were assessed by using the Spearman rank correlation. A *P* value of less than .05 was considered statistically significant.

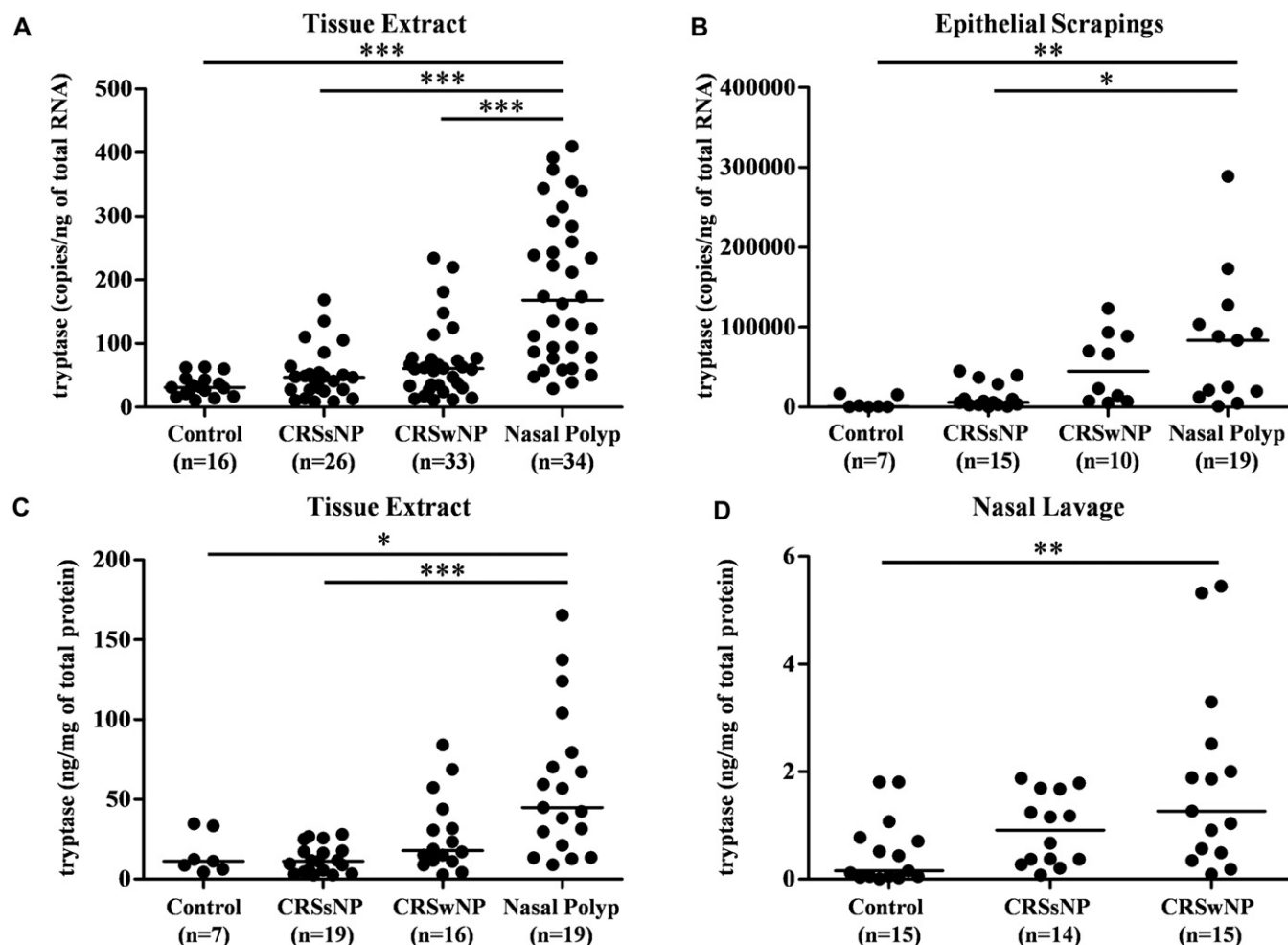


FIG 1. Increased expression of tryptase in NPs. The gene expression of tryptase in UT and NPs (**A**) and nasal scrapings (**B**) was measured by using real-time PCR. The concentration of tryptase in tissue homogenates of UT and NPs (**C**) and nasal lavage (**D**) was measured by using ELISA. Tryptase concentration was normalized to the concentration of total protein. * $P < .05$, ** $P < .001$, *** $P < .0001$.

RESULTS

Mast cell expression and distribution in patients with CRS

Sinonasal and polyp tissues were collected from 70 subjects with CRSsNP, 91 subjects with CRSwNP, and 42 control subjects to determine the levels of expression of mast cells and their products in patients with CRS. Subject characteristics are shown in Table I.

To estimate the levels of mast cells in nasal mucosa, we first assessed the expression of mRNA for tryptase in UT from patients with CRSsNP, patients with CRSwNP, and controls, as well as in NPs from patients with CRSwNP. The expression of the house-keeping gene GUSB was not significantly different among the 4 groups (data not shown). Tryptase mRNA was significantly increased in NPs from patients with CRSwNP ($P < .0001$) in comparison with UT from either patients with CRS or control subjects (Fig 1, A). Likewise, the levels of tryptase mRNA were significantly elevated in NPs in 17 patients from which we had matched sets of both polyp and UT for RT-PCR evaluation ($P < .05$ data not shown). To assess the gene expression level in epithelium, we used nasal scraping-derived epithelial cells. Tryptase mRNA was significantly increased in scrapings from NPs in comparison

with scrapings of the UT from either patients with CRSsNP or control subjects (Fig 1, B). To confirm this observation at the protein level, we made detergent extracts from homogenates of UT and NPs and then measured the concentration of tryptase by using ELISA. Tryptase protein was significantly increased in NPs ($P < .05$) compared with UT from either patients with CRSsNP or control subjects (Fig 1, C). We also determined tryptase protein expression in nasal lavage fluids collected from a separate group of subjects. Tryptase protein was significantly increased in lavage fluids from patients with CRSwNP ($P = .0079$) than in lavage fluids from control subjects (Fig 1, D).

To further characterize the mast cells in the nasal mucosa of patients with CRS, we performed immunohistochemistry by using surgical samples from control subjects and patients with CRS to detect tryptase-expressing cells. Consistent with the real-time PCR and ELISA data, increased numbers of 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

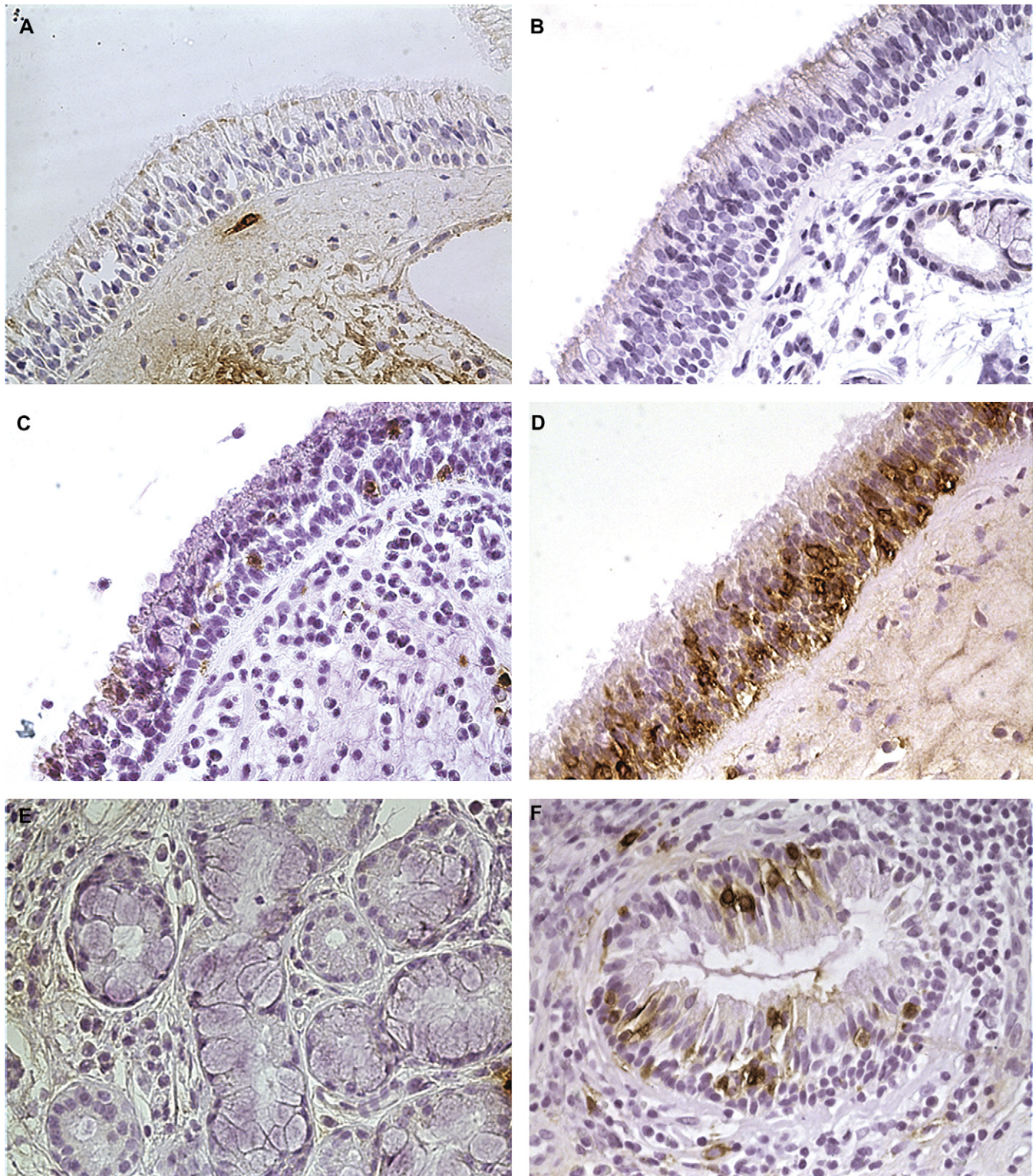


FIG 2. Immunohistochemical detection of tryptase was performed by using antihuman tryptase mAb. Representative immunostaining for tryptase within the epithelium of UT from a control subject (A), a patient with CRSsNP (B), a patient with CRSwNP (C), and an NP (D). Tryptase-positive cells in the glands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

CRSwNP compared with either UT from control or patients 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

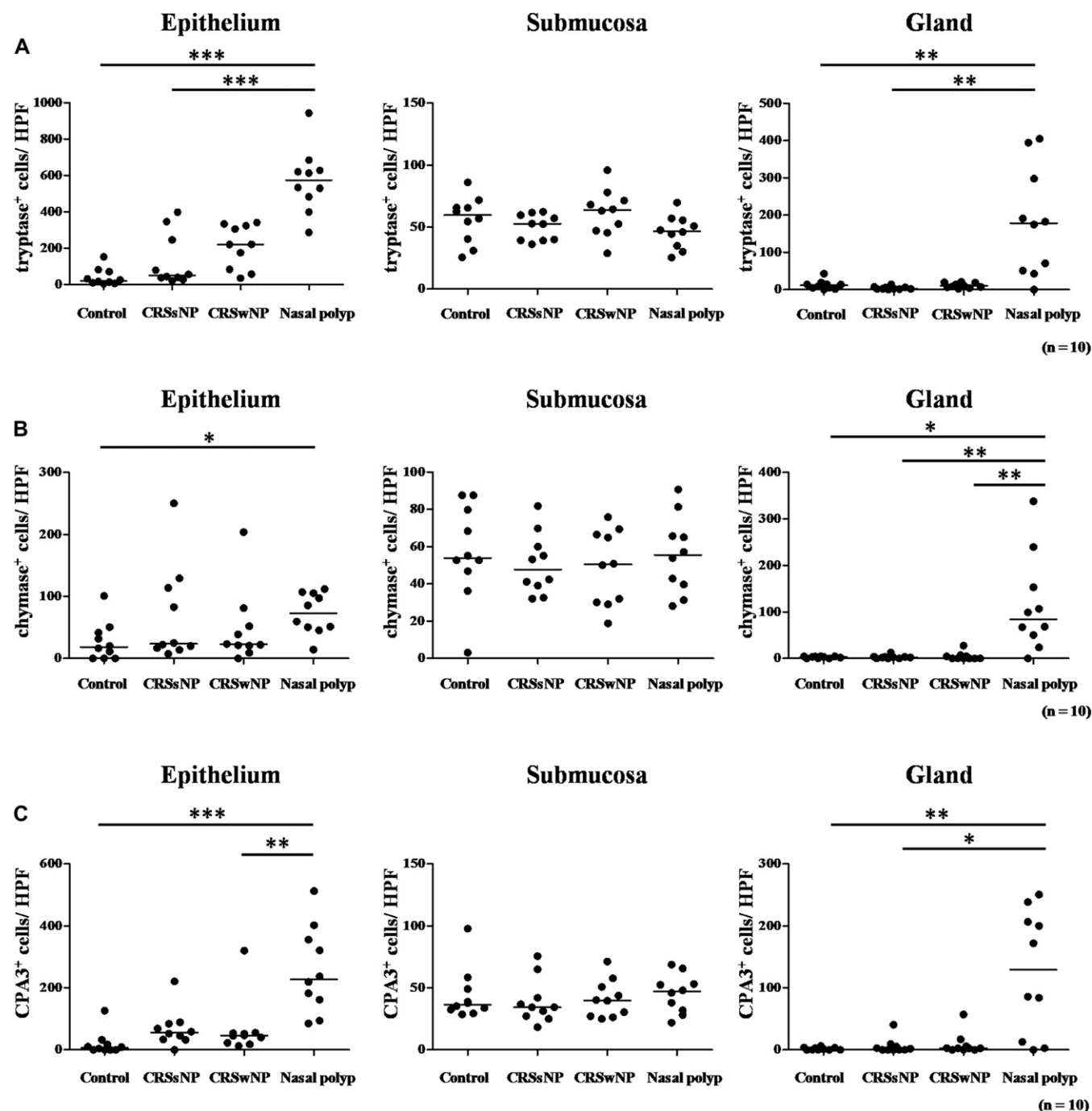


FIG 3. Quantitation of mast cell protease-positive cells in nasal mucosal tissue from a control subject and patients with CRS and distribution in epithelium, submucosa, and glands. Immunostaining for tryptase (A), chymase (B), and CPA3 (C). * $P < .05$, ** $P < .001$, *** $P < .0001$.

with 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 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

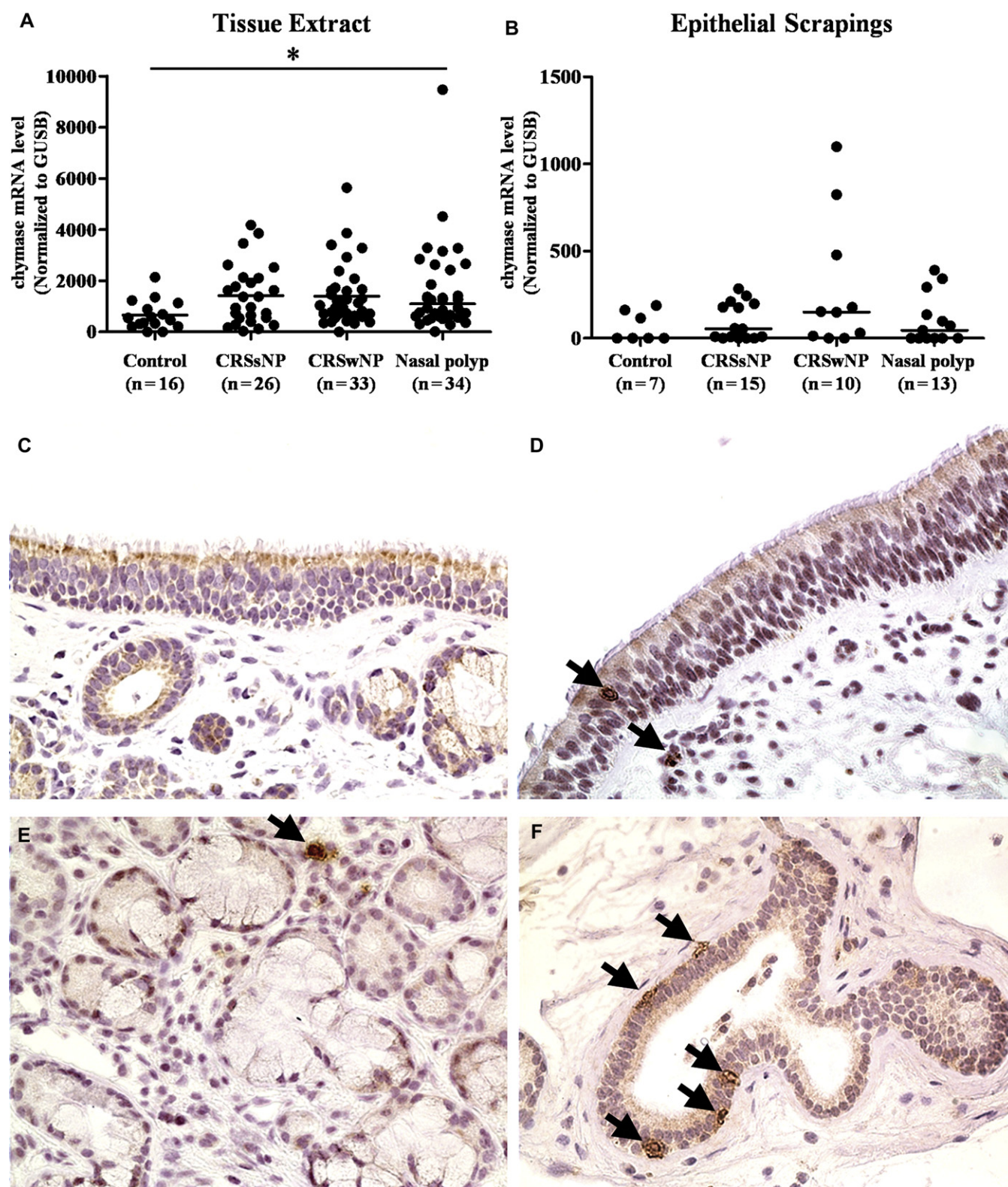


FIG 4. The expression of chymase in NPs. The gene expression of chymase in tissue (**A**) and nasal scrapings (**B**) was measured by using real-time PCR. Representative immunostaining for chymase within the epithelium of UT from a control subject (**C**) and an NP (**D**) and within the glands of UT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

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 CRSwNP 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, $74.8\% \pm 3.7\%$; $n = 10$) 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, $84.2\% \pm 2.9\%$; $n = 10$) (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, $79.5\% \pm 4.0\%$; $n = 10$; Fig 6, C) and most mast cells expressed both tryptase and CPA3 (mean and SEM double positive, $87.7\% \pm 2.7\%$; $n = 10$; 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 secretagogue 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 elevated²⁵⁻²⁸ 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 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.

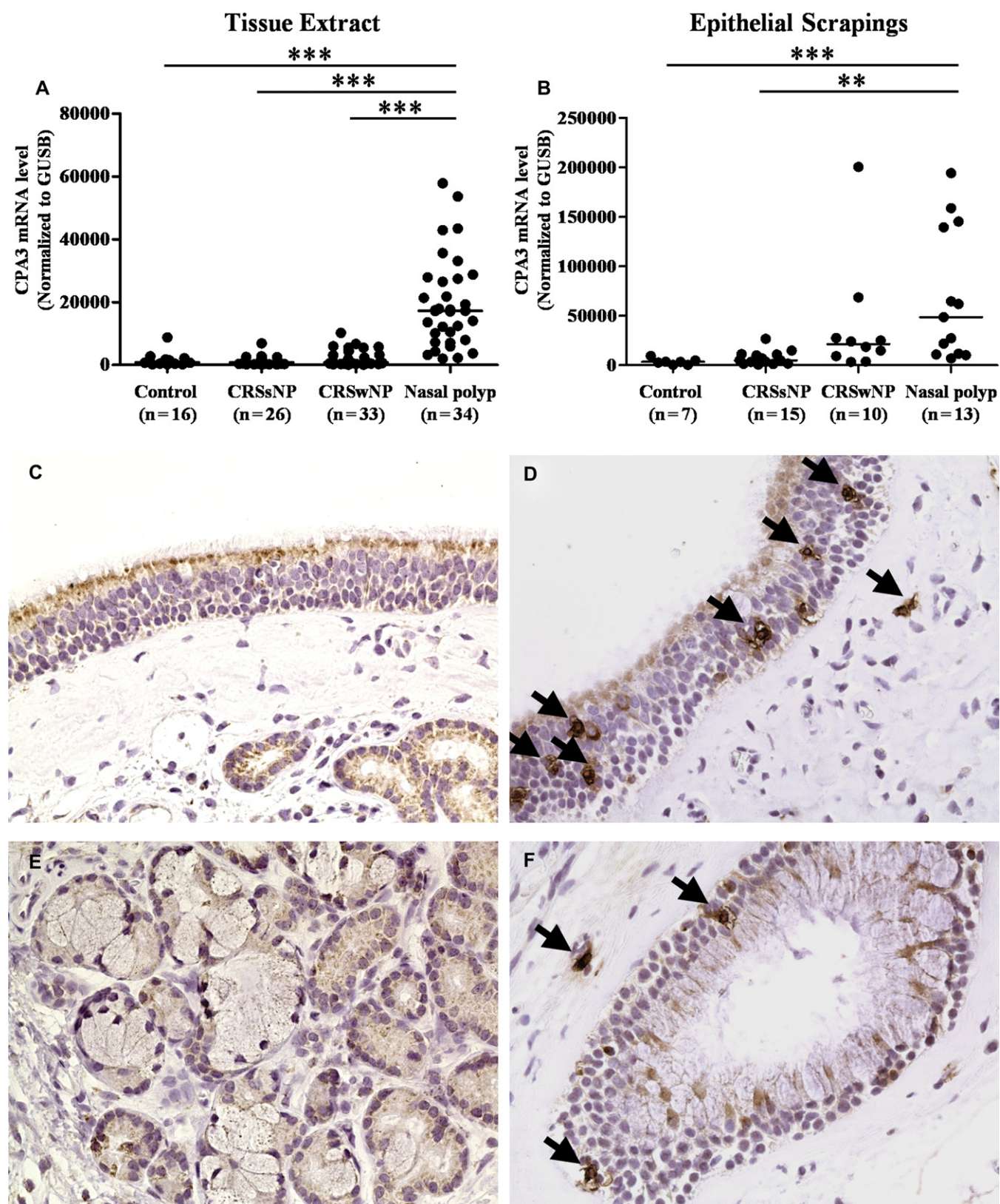


FIG 5. The expression of CPA3 in NPs. The gene expression of CPA3 in tissue (**A**) and nasal scrapings (**B**) was measured by using real-time PCR. Representative immunostaining for CPA3 within the epithelium of UT from a control subject (**C**) and an NP (**D**) and within the gland of UT from a control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

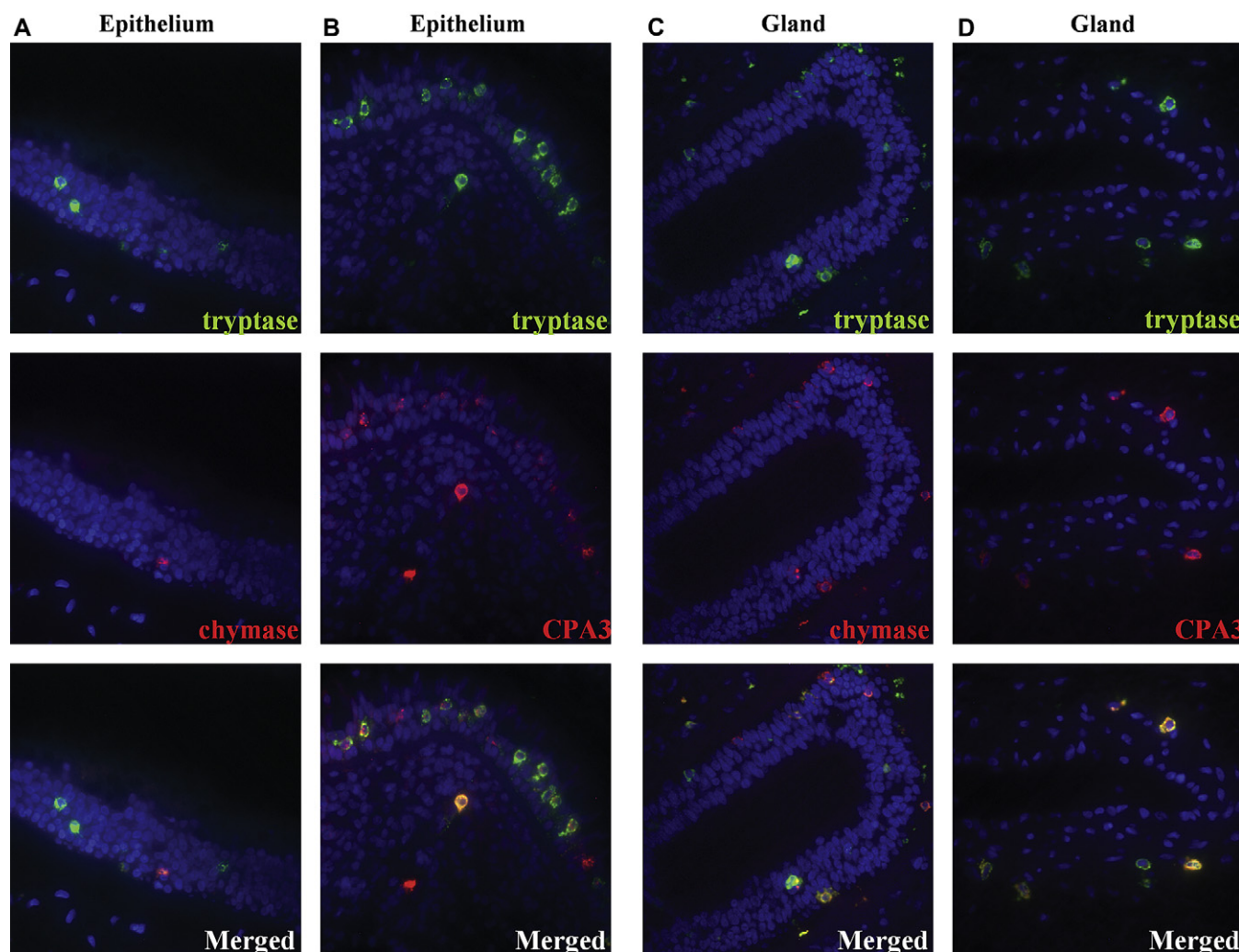


FIG 6. Immunofluorescence of mast cell proteases in NPs. Immunofluorescence assay was performed with antitryptase (green fluorescence) and antichymase (red fluorescence) (A), and anti-CPA3 (red fluorescence) (B) in epithelium and antitryptase (green fluorescence), and antichymase (red fluorescence) (C),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

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 lipoxigenase 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.

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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 $2\times$ 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'-CTGCTTCCAATACAGCCA-CAAG-3'; antisense, 5'-GAGCAGCTGTACTGGTGAATTCA; 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, IgG₁; Thermo Scientific, Fremont, Calif) at a dilution of 1:40,000 or mouse anti-human chymase mAb (clone: CC1, IgG₁; 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 $\times 400$. 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 $\mu\text{g/mL}$ Alexa Fluor 488-conjugated goat antimouse IgG (Invitrogen) and 4 $\mu\text{g/mL}$ 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 Fluor 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 μ g of each antibody was prepared in PBS solution and incubated with 5 μ L of the Zenon mouse IgG labeling reagent for 5 minutes at room temperature, followed by addition of 5 μ L 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 μ g/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 $\times 40$ 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).

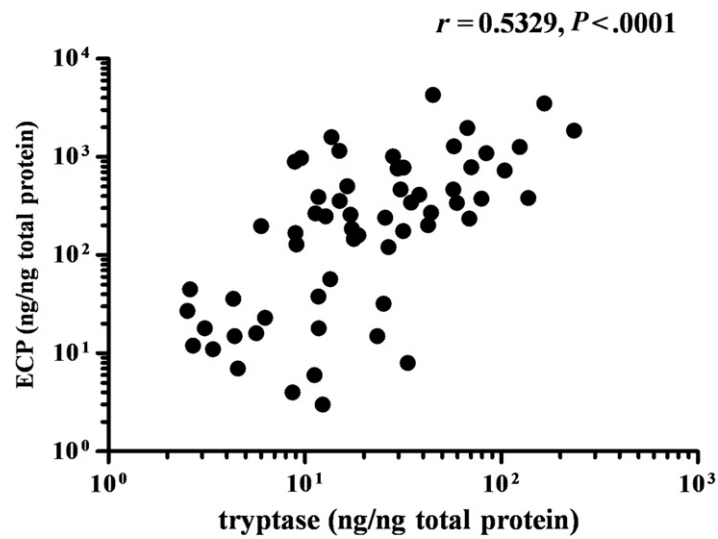


FIG E1. The relationship of tryptase and ECP in UT was evaluated by using ELISA. The correlation was assessed by using the Spearman rank correlation test.

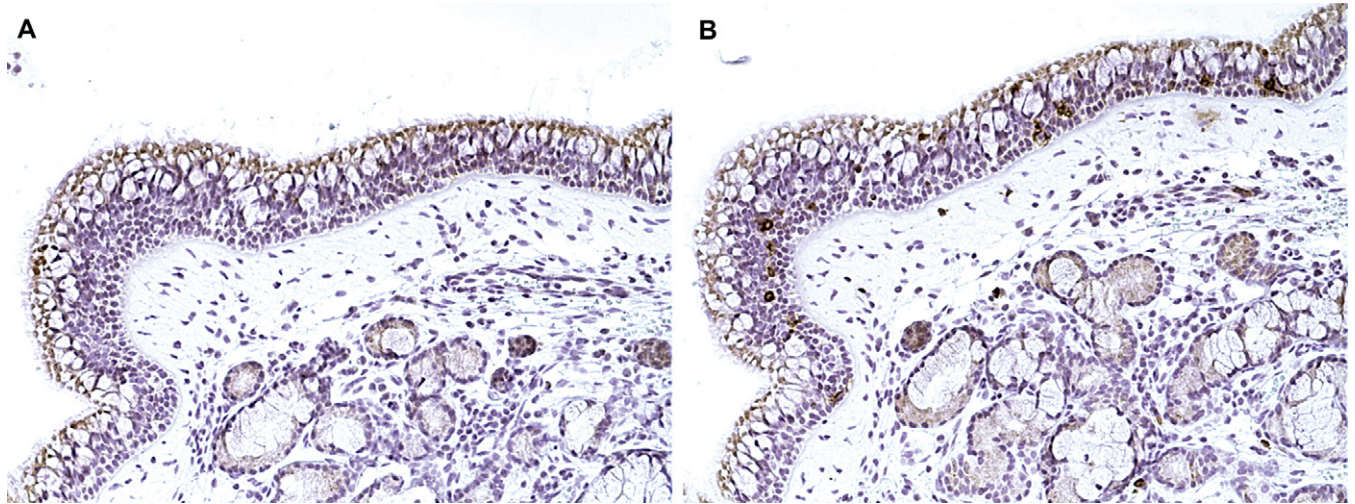


FIG E2. Negative control antibody staining in NPs tissue from a patient with CRSwNP. **A,** Normal rabbit IgG. **B,** Anti-human CPA3 antibody.

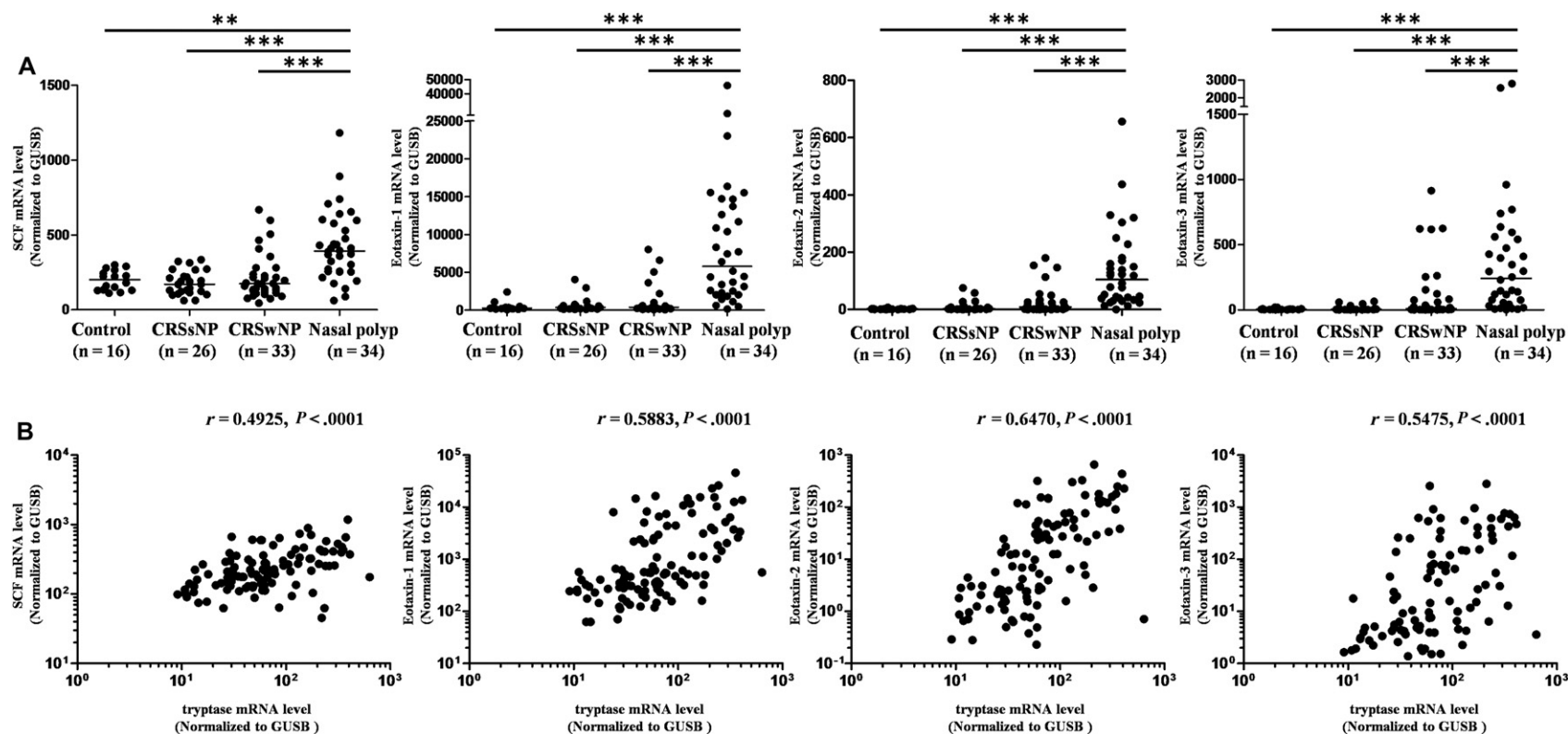


FIG E3. Correlation of tryptase with candidate mediators for elevation of mast cells. Total RNA was extracted from UT from control subjects ($n = 16$), patients with CRSsNP ($n = 26$), patients with CRSwNP ($n = 33$), and NPs ($n = 34$). **A**, The expression of tryptase and candidates for mast cell 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. $**P < .001$, $***P < .0001$.