

Striking deposition of toxic eosinophil major basic protein in mucus: Implications for chronic rhinosinusitis

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Background: The mechanisms by which eosinophilic inflammation damages the epithelium and contributes to recurrent acute exacerbations in chronic rhinosinusitis (CRS) have not been fully elucidated.

Objective: We tested the hypotheses that eosinophils deposit toxic major basic protein (MBP) in the mucus and that MBP reaches concentrations able to damage the sinonasal epithelium.

Methods: Tissue specimens with mucus attached to the tissue were carefully collected from 22 patients with CRS and examined by using immunofluorescence staining for MBP. This immunofluorescence was digitally analyzed to determine the area covered by MBP and the intensity of the staining (estimating MBP concentration). Levels of MBP in extracts from nasal mucus were quantitated by means of RIA.

Results: Heterogeneous eosinophilia was evident within tissue and mucus specimens. All tissue specimens showed intact eosinophils, but diffuse extracellular MBP deposition, as a marker of eosinophil degranulation, was rare. In contrast, all mucus specimens showed diffuse MBP throughout and abundant diffuse extracellular MBP deposition within clusters of eosinophils. Digitized analyses of MBP immunofluorescence revealed increased area coverage ($P < .0001$) in mucus compared with that seen in tissue. Estimated concentrations of MBP within the clusters suggested toxic levels. MBP concentrations in mucus extract reached 11.7 $\mu\text{g/mL}$; MBP was not detectable in healthy control subjects.

Conclusion: In patients with CRS, eosinophils form clusters in the mucus where they release MBP, which is diffusely deposited on the epithelium, a process not observed in the tissue. Estimated MBP levels far exceed those needed to damage epithelium from the luminal side and could predispose

patients with CRS to secondary bacterial infections. (J Allergy Clin Immunol 2005;116:362-9.)

Key words: Eosinophils, chronic rhinosinusitis, mucus, degranulation, major basic protein

A recent survey by the National Center for Health Statistics reported that 14.2% (29.2 million patients) of the US adult population recalled a health professional's diagnosis of sinusitis.¹ Rhinosinusitis is now preferred to the previous term sinusitis "... because sinusitis is almost always accompanied by concurrent nasal airway inflammation. ..."² The economic effect of chronic rhinosinusitis (CRS) is huge; in the US the direct cost was estimated in 1996 at \$5.6 billion per year, and the indirect cost was estimated as more than 70 million lost activity days per year.³ Patients with CRS have long-term nasal congestion, thick mucus production, loss of sense of smell, and intermittent acute exacerbations secondary to bacterial infections; they also experience severe quality-of-life impairment.^{2,4} As an additional burden, CRS lacks a plausible cause. To date, the US Food and Drug Administration has not approved any drug or treatment for CRS; no medical intervention has ever been efficacious in a controlled clinical trial.

CRS is an inflammatory disease of the nasal and paranasal mucosa with persistent symptoms for longer than 3 months; its ultimate end stage is inflammatory mucosal thickening and, in a subset of patients, polypoid changes.^{2,5} The histologic hallmark of CRS is persistent underlying eosinophilic inflammation.⁵⁻⁷ Eosinophil granules contain several cytotoxic proteins,⁸ and eosinophil granule major basic protein (MBP) is directly toxic to extracellular microorganisms as well as host tissue, including respiratory mucosa.⁹ CRS specimens show epithelial damage that is colocalized with MBP deposition.^{6,7,10} *In vitro*, MBP directly damages respiratory and sinus epithelium in a time- and dose-dependent manner.^{11,12}

Recent histologic analyses of CRS specimens suggested that intact eosinophils migrate from the tissue into the mucus to form distinct and characteristic clusters.^{13,14} Therefore we tested whether eosinophils release MBP in the mucus, but not in the tissue, and whether MBP reaches concentrations capable of damaging the sinonasal epithelium in patients with CRS.

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Abbreviations used

CRS: Chronic rhinosinusitis
MBP: Major basic protein

METHODS

Patient selection

The diagnostic guidelines and criteria for CRS were consistent with those adopted at the recent Rhinosinusitis Consensus Conference.² All patients had symptoms consistent with CRS for longer than 3 months, inflamed mucosa on endoscopy, and a coronal computed tomographic scan demonstrating mucosal thickening of greater than 5 mm in more than 2 sinuses. Retention cysts and cystic fibrosis are differential diagnoses to CRS, and if these diseases were diagnosed, those patients were excluded from the study. Because complete immunologic evaluations were not performed in all patients, we have not excluded patients (if any) with immunodeficiencies. With regard to noneosinophilic inflammatory sinusitis, we found eosinophilia in tissues from all patients of our otherwise unselected patient population. Patients were not preselected for having eosinophilic CRS.

Histologic analyses of specimens

For the histologic analyses, specimens were collected from 22 consecutive patients with CRS undergoing endoscopic sinus surgery. During surgical intervention, we used Blakesley surgical forceps to carefully and gently collect the maximum amounts of tissue and mucus, and we ensured that the mucus remained attached to the tissue, which was immediately fixed in formalin. Four specimens from the ethmoid sinuses of healthy individuals (nonallergic and no asthma) undergoing septoplasty procedures served as negative controls. The Institutional Review Board of the Mayo Clinic approved the study.

Paraffin-embedded tissue blocks with attached mucus were cut in 5- μ m-thick serial sections, mounted on positively charged slides, and stained with the following: (1) hematoxylin and eosin; (2) antibody to eosinophil MBP using rabbit antihuman MBP¹⁵⁻¹⁷; (3) antibody to neutrophil elastase¹⁰ using rabbit antihuman elastase (IgG fraction; Cortex Biochem, San Leandro, Calif); and (4) negative control for MBP and elastase (normal rabbit IgG). All specimens were incubated in 10% normal goat serum to block nonspecific binding by the second-stage antibody and in 1% chromotrope 2R to block nonspecific binding of fluorescein dye to the eosinophils.⁶ Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was used as the secondary antibody.¹⁵⁻¹⁷ MBP was chosen to assess eosinophil infiltration and degranulation because it is the predominant eosinophil granule protein. It accounts for roughly 50% of the total protein mass in the eosinophil granule,¹⁸ and the release of MBP is highly correlated with the release of the other granule proteins.¹⁹ Elastase, the predominant neutrophil granule protein, was studied to assess neutrophil infiltration and degranulation.

Semiquantitative analysis of MBP and elastase immunofluorescence in tissue and mucus

Three examiners independently examined the entire specimen. To exclude artifacts from trauma caused through the removal of the specimen during surgery, only areas of untouched mucosa covered with mucus were evaluated. In contrast, areas with obvious tears or influx of red blood cells, indicating trauma with bleeding in the areas touched by the forceps, were excluded.

The tissue and the attached mucus were each evaluated with the following scoring system, which was previously used to describe eosinophil⁷ and neutrophil¹⁰ infiltration and degranulation. Because of the heterogeneity of the eosinophilic and neutrophilic inflammation, only those areas of tissue and attached mucus with the most prominent cellular infiltrate for these leukocytes were scored in this semiquantitative scheme. For each specimen, we calculated the means of the examiners' scores (0, not present; 1, few present/scattered; 2, many present/abundant) for tissue and for attached mucus using the criteria listed below:

- intact eosinophils or neutrophils;
- punctate staining (MBP or elastase within intact extracellular granules); and
- diffuse staining (extracellular MBP or elastase not in granules).

In addition, eosinophils forming clusters within the mucus (eosinophilic mucin) were noted as present (+) or absent (–), and diffuse MBP staining within the clusters was noted as present (+) or absent (–). Neutrophil clusters and elastase deposition were evaluated similarly.

Digital analyses of MBP and elastase immunofluorescence in tissue and mucus

Computer analysis of sections stained for MBP or elastase by means of immunofluorescence was performed to evaluate objectively the overall areas covered by MBP or elastase, as well as the intensities of the staining (as indirect markers for MBP or elastase concentrations).⁶ Briefly, we used a confocal microscope (LSM510 Confocal Microscope; Carl Zeiss, Inc, Oberkochen, Germany) to survey and select both the least and most intense areas of MBP or elastase staining in the tissue and the mucus. First, digital images (512 $\times$ 512 pixels, 400 $\times$ magnification, 488-nm excitation wavelength) of areas that showed maximal fluorescence staining (most intense accumulation of either eosinophils or neutrophils or diffuse extracellular MBP or elastase deposition) were obtained. Second, digital images of the corresponding areas on the serial section, which was stained with normal rabbit IgG, as the negative control, were recorded. Third, by using image-analysis software (KS400 Image Analysis System, Carl Zeiss, Inc), the threshold for each negative control image was calibrated to a baseline value that showed no positive pixels. Fourth, this background threshold was used to analyze the corresponding area on the MBP or elastase immunofluorescence-stained specimen. Any pixels recorded were quantitated as a percentage of an area (512 $\times$ 512 pixels) positive for MBP or elastase. The image-analysis software then compared the different areas within the specimen and determined the area with the highest percentage of positive pixels; this percentage indirectly indicated the area of maximal inflammatory eosinophilic or neutrophilic infiltrate (tissue) or maximal MBP or elastase deposition (mucus). A similar survey was made to find the area in the MBP or elastase immunofluorescence specimen with the least fluorescence in the tissue and in the mucus; once located, these areas were compared with the respective corresponding areas in the negative control serial section and analyzed as above.

Electron microscopy and immunogold labeling for MBP

We also used electron microscopy to investigate the morphology of eosinophils and eosinophil granules and to localize MBP in tissues from 3 patients with CRS, as described earlier.²⁰ After the primary antibody, affinity-purified antibody to MBP, we used a secondary antibody conjugated to 15 nm colloidal gold particles.¹⁷

Quantitative analysis of MBP in mucus

Nasal mucus was collected by means of direct trap suctioning from the nasal cavity (middle meatus region) and from one maxillary sinus under endoscopic guidance with a sinus secretion collector (Xomed Surgical Products, Jacksonville, Fla) from 12 additional patients with CRS who met the same diagnostic criteria as described above and from 9 healthy control subjects. The mass of each mucus specimen was obtained, and a 3-fold excess of normal saline (0.15 M NaCl) was added. After vigorous vortexing for 10 seconds ($\times 3$), the mucus suspension was centrifuged, and the resulting supernatant fluid was frozen at -70°C . (This vortexing step probably did not release the intracellular intragranular MBP, which requires more severe conditions. Specifically, to extract MBP, eosinophils need to be incubated for 30 minutes at room temperature in 0.5% NP-40 [Sigma-Aldrich, St Louis, Mo] and 0.01 M HCl⁸). Finally, MBP levels in the mucus supernatant fluid were determined by means of RIA, essentially as described earlier.²¹

Statistical analysis

The groups were compared with a 2-sided Student *t* test or the Wilcoxon matched-pairs signed-rank test, and a *P* value of less than .05 was considered significant. Data are presented as means ($\pm$ SD) or medians (ranges).

RESULTS

Patient demographics

Patient demographics have been previously described.⁶ Briefly, the mean age of the 22 patients with CRS was 47 years (range, 16–86 years); 11 were women; the mean number of sinus operations was 1.8 (range, 0–7); the mean duration of disease was 8.6 years (range, 2–27 years); the incidence of aspirin idiosyncrasy was 41%; 11 had increased serum levels of total IgE (>128 U/mL, 2 SDs above the mean value of healthy adult control subjects); and 10 were considered allergic, as defined by a positive skin prick test response to at least one allergen from a panel of 16 common aeroallergens. The incidence of physician-diagnosed asthma was 68% (15/22); the other 7 patients underwent a methacholine challenge, and 5 had positive responses.

Eosinophil and neutrophil infiltration and degranulation in tissue and mucus

Although intact eosinophils were abundant in numerous areas in the tissue (Fig 1, A and B), the most striking observation was the abundance of diffuse MBP staining (not in cells and not in granules) in the mucus compared with its absence in the tissue (Fig 1, A–D). Eosinophils formed cell clusters in the mucus, and diffuse MBP staining was observed within and around these clusters (Fig 1, A–D and F). Punctate staining, indicating intact extracellular eosinophil granules, was frequently observed in both tissue and mucus (Fig 1, E and F). Compared with MBP staining, only isolated areas in tissue and mucus stained for neutrophil elastase (results not shown).

As summarized in Fig 2 (top), tissue in all specimens showed abundant intact eosinophils (22/22) compared with mucus (11/22). The numbers of patient specimens

showing various amounts of punctate staining were similar between tissue and mucus. Diffuse MBP deposition in the mucus was abundant in all (22/22) CRS specimens but was not observed in the tissue, except in one small area from 1 specimen (1/22). Intact neutrophils were evident in both tissue (10/22) and mucus (14/22) specimens. In contrast to the numerous areas showing diffuse MBP deposition in the mucus of all 22 specimens (Fig 2, A), isolated areas of diffuse elastase deposition were noted in the mucus of only 9 of 22 patients. The majority of specimens showed a virtual absence of diffuse elastase in the mucus (Fig 2, B).

Eosinophils forming clusters within the mucus (eosinophilic mucin) were present in 22 of 22 specimens, and diffuse deposition of MBP in and around these clusters was seen in 22 of 22 specimens. In contrast, neutrophil cluster formation was observed in only 5 of 22 specimens, and elastase deposition in these clusters was observed in only 4 of 22 specimens. None of the specimens from healthy control subjects (0/4) were positive for intact eosinophils or neutrophils or punctate or diffuse staining for MBP or elastase (results not shown).

Digital analyses of MBP and elastase immunofluorescence

In the tissue the maximum area positive for MBP immunofluorescence staining had a median of 18.35% (range, 0.64% to 56.63%); by contrast, the maximum area positive for elastase immunofluorescence was significantly less (median, 3.11%; range, 0.05% to 46.2%; $P < .002$; Fig 3). In the mucus the maximum area positive for MBP immunofluorescence staining had a median of 93.28% (range, 3.62% to 100%); by contrast, the maximum area positive for elastase immunofluorescence was also significantly less (median, 29.30%; range, 0.10% to 85.42%; $P < .001$; Fig 3). Furthermore, the maximum area positive for MBP immunofluorescence in the mucus (median, 93.28%) was significantly increased compared with the maximum mean area in tissue (median, 18.35%; $P < .0001$).

Electron microscopy and immunogold labeling for MBP

In tissue from a patient with CRS, both an intact eosinophil containing the characteristic electron-dense granule cores with MBP and extracellular granules are visible (Fig 4, A). However, the immunogold MBP stain shows MBP confined within the intact granules (Fig 4, B), corresponding with the punctate staining in MBP immunofluorescence (see Fig 1, E). Diffuse MBP immunogold staining is not seen in the tissue (Fig 4, B), which is consistent with the lack of diffuse MBP immunofluorescence staining seen in the tissue (see Fig 1, E).

Measurement of MBP in the mucus

As shown in Fig 5, the mean concentration of detectable MBP in mucus extracts from the maxillary sinuses in patients with CRS was 4.2 $\mu\text{g/mL}$ ($\pm$ 3.1 $\mu\text{g/mL}$; range,

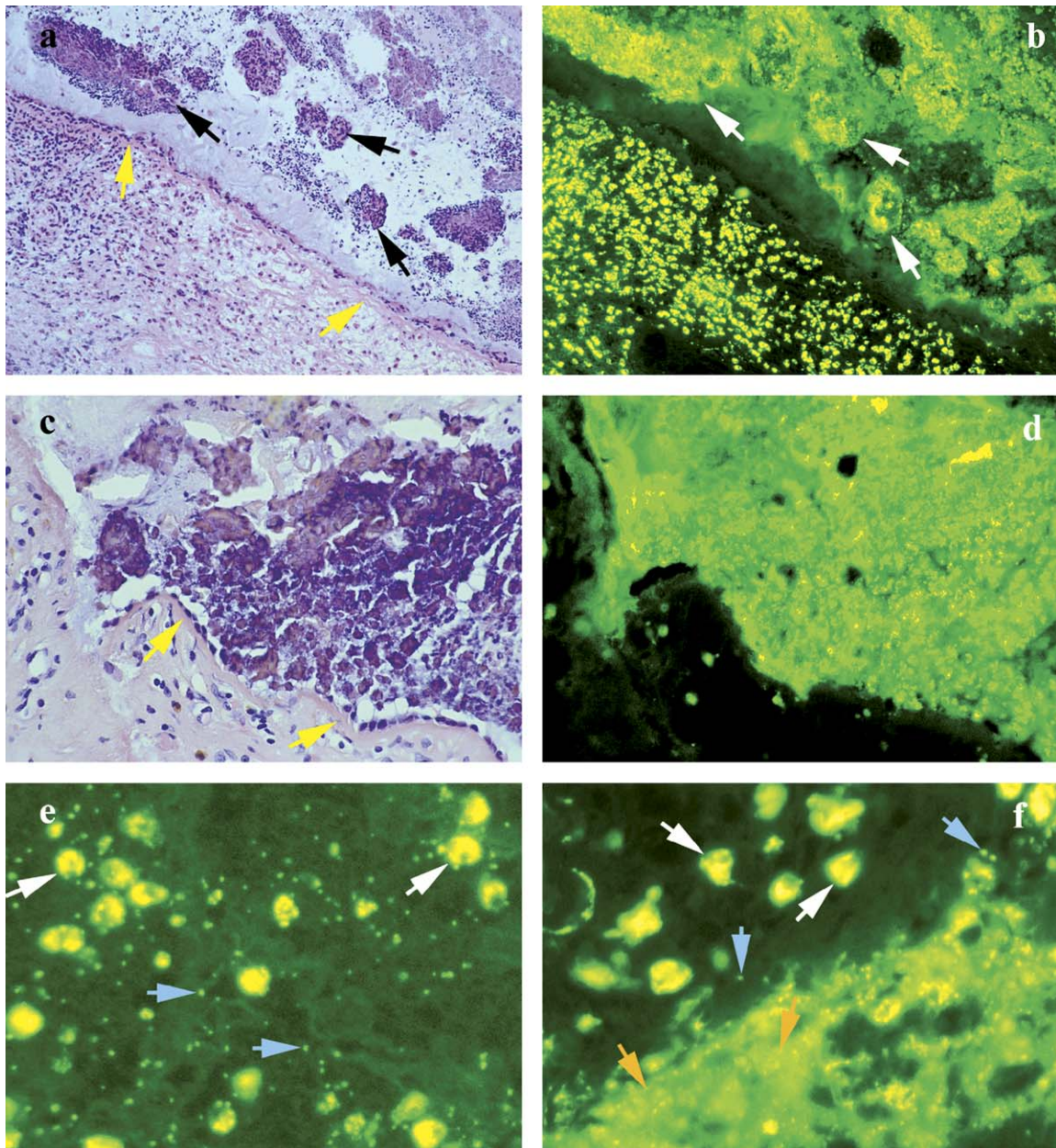


FIG 1. Photomicrographs of CRS specimens stained for MBP by means of immunofluorescence or stained with hematoxylin and eosin. Panel **a** demonstrates eosinophilic inflammation in tissue, eosinophil clusters (*black arrows*) in mucus, subepithelial basement membrane thickening, and damaged epithelium (*yellow arrows*) (hematoxylin and eosin counterstain of Panel **b**; original magnification, 160 $\times$). Panel **b** shows MBP in tissue is contained within the cells or in intact granules (punctate staining) outside the cells. In mucus, diffuse MBP staining is in eosinophil clusters (*white arrows*) and outside of clusters (anti-MBP; original magnification, 160 $\times$). Panel **c** shows minimal tissue eosinophilia, massive eosinophilia in mucus, subepithelial basement membrane thickening, and the damaged epithelium (*yellow arrows*) (hematoxylin and eosin; original magnification, 400 $\times$). Panel **d** (serial section of Panel **c**) shows few intact eosinophils in tissue, intense diffuse MBP deposition within the mucus, and MBP adjacent to the epithelial surface (anti-MBP; original magnification, 400 $\times$). Panels **e** (tissue) and **f** (mucus) show intact eosinophils (*white arrows*) and free granules (punctate staining, *blue arrows*); diffuse extracellular MBP staining (*orange arrows*) appears unique to mucus (anti-MBP; original magnification, 1400 $\times$). Serial sections stained with normal rabbit IgG were negative (results not shown).

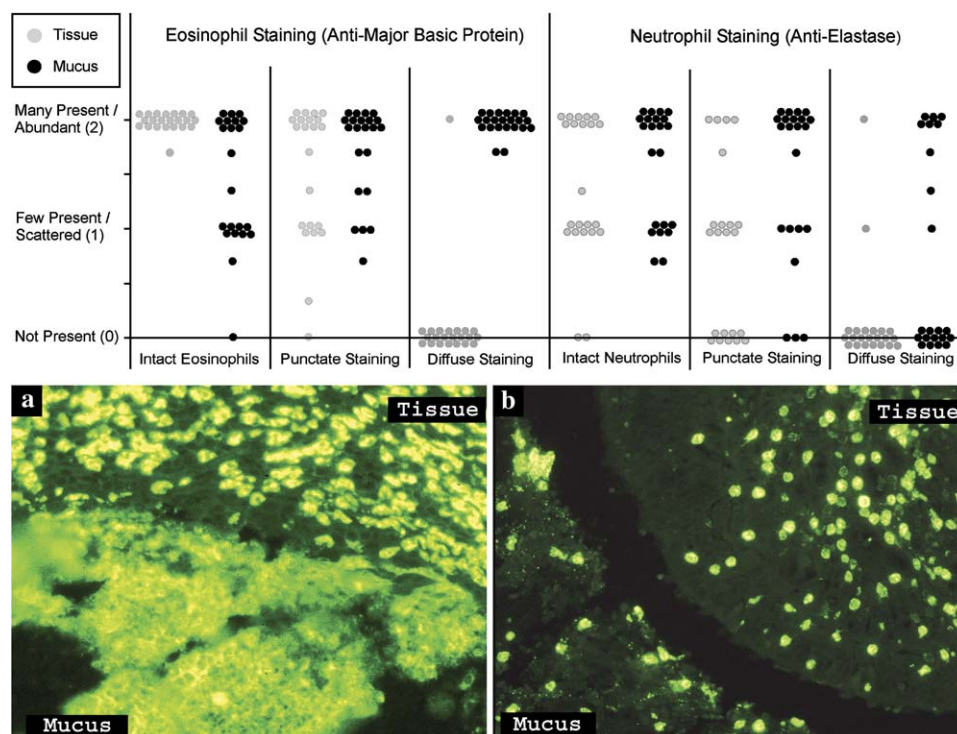


FIG 2. Comparisons of eosinophilic and neutrophilic inflammation in tissue versus mucus. The graphs show the mean MBP and elastase immunofluorescence scores for intact eosinophils and neutrophils, punctate staining (MBP and elastase within intact extracellular granules), and diffuse staining (extracellular MBP and elastase not in granules) in tissue and mucus from 22 patients with CRS. Each dot represents the mean score of 3 independent examiners for each patient (scoring on vertical axis follows the grading system presented in the Methods section). Panel **a** shows a CRS specimen stained with anti-MBP; note the abundant diffuse MBP in mucus that is absent in tissue. Panel **b** shows a CRS specimen stained with anti-elastase; note the virtual absence of diffuse elastase in both mucus and tissue (original magnification, 400 $\times$).

0.3–11.7 $\mu\text{g/mL}$; $n = 12$). In 9 of 12 patients with CRS, we were also able to harvest sufficient mucus from the nasal cavity to detect a mean MBP concentration of 4.1 $\mu\text{g/mL}$ (± 2.6 $\mu\text{g/mL}$; range, 0–8.0 $\mu\text{g/mL}$), which did not differ significantly from the mean concentration in the maxillary sinuses. In contrast, in mucus specimens from the nasal cavities of the healthy control subjects, no MBP could be detected above the sensitivity of the assay (0.010 $\mu\text{g/mL}$). Thus even the lowest MBP concentration detected in mucus from the maxillary sinus of a patient with CRS (0.31 $\mu\text{g/mL}$) was at least 30-fold greater than that of healthy control subjects.

DISCUSSION

The underlying eosinophilic inflammation is increasingly recognized to play an important role in the pathogenesis of CRS, and its association with epithelial damage has been suspected.^{6,7} How the eosinophil actually mediates the pathophysiology, such as damaging the epithelium, remains unclear. Earlier studies in patients with CRS used tissue biopsy specimens without mucus^{7,10} and showed MBP deposition within damaged sinus epi-

thelium; in contrast, our specimens were carefully collected to ensure that the mucus remained attached to the harvested tissue. Thus we could document the extent, localization, and degranulation pattern of the heterogeneous eosinophilia and neutrophilia in both the tissue and the mucus. Eosinophil, rather than neutrophil, inflammation was predominant in both tissue and mucus. Striking extracellular deposition of diffuse MBP (especially in clusters) was unique to the mucus in all 22 patients with CRS and was not found in the tissue (with the exception of one small area in 1 patient). In contrast, extracellular deposition of diffuse elastase was found in less than half of the patients (9/22) and only in isolated areas.

The pattern of eosinophil degranulation described in this study could explain the sinonasal epithelial erosion observed in patients with CRS.⁶ As seen in Fig 1, A and C, the outer layers of the epithelium are eroded away, but a layer of basal epithelial cells still remains. This observation, as well as the marked deposition of toxic eosinophil granule MBP in the mucus, suggests that the damage to the epithelium occurs from the outside (luminal side). The damaged epithelium might provide an entry port for colonizing bacteria that are present in the sinuses of patients with CRS, as well as healthy control subjects.^{22,23} Thus

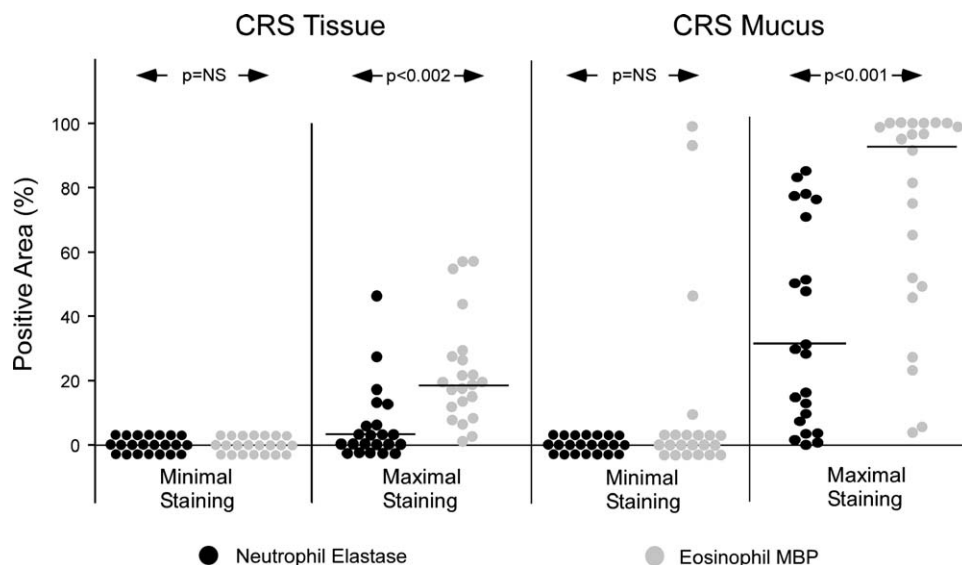


FIG 3. Comparison of digitized areas of minimal and maximal MBP or elastase staining in the tissue and mucus of patients with CRS. To demonstrate the heterogeneity within the 22 specimens, these data points represent the minimal and maximal percentages of area positive for MBP or elastase immunofluorescence. The horizontal lines show the median values in each group.

the release of toxic eosinophil granule proteins, such as MBP, in the mucus could be a crucial predisposing factor for the secondary bacterial infections that likely mediate the acute exacerbations of CRS. In healthy control subjects, the absence of MBP in the mucus might also explain the lack of epithelial damage and, consequently, the lack of bacterial infections, despite the presence of bacteria.^{22,23} Taken together, these findings might explain the complex clinical course of CRS with its occasional bacterial exacerbations; indeed, the numbers of infiltrating tissue neutrophils have been directly correlated to the numbers of bacteria present.²⁴ Overall, these exacerbations are superimposed on the persistent and underlying eosinophilic inflammation uniformly seen in all patients.

Although we used an RIA for MBP in extracts from mucus specimens, this procedure likely underestimated the actual local concentrations for the following reasons. First, we attempted to extract MBP from the thick mucus through vortexing in saline, and thus only the MBP that actually dissolved in the saline could be measured. The remaining mucus and probably a large amount of undissolved MBP had to be discarded. Second, the concentration of MBP was based on the entire volume of the mucus specimen; because portions of the mucus do not contain MBP (Fig 3), our results underestimate the local maximal MBP concentrations.

To address this potential underestimation, we calculated the approximate MBP concentration in an eosinophil to be 33 mg/mL or 2.1×10^{-3} M (8.98×10^{-9} mg [mass MBP/eosinophil]/ 2.68×10^{-10} mL [volume/eosinophil]).⁸ Although the immunogenic epitopes might not be equally available for MBP in mucus compared with MBP in the granules of intact cells, we used digital

analyses to compare the brightness of anti-MBP staining in confocal microscopic images. Overall, 17 of 22 specimens showed brighter immunofluorescence staining for MBP in mucus compared with that seen in tissue; no brightness differences were noted for intact cells in tissue, blood vessels, or mucus. Thus we estimate that areas of diffuse MBP in the mucus might exceed 33 mg of MBP/mL. Because MBP is toxic to and causes erosion of the epithelium at concentrations less than 10 μ g/mL,¹² our MBP immunofluorescence results suggest that MBP reaches local concentrations in the mucus of patients with CRS far exceeding those necessary to mediate epithelial damage. In addition, inspissation (dessication) of mucus at mucosal surfaces *in vivo* might lead to a further increase in the local MBP concentration. We observed striking MBP deposition directly adjacent to the damaged epithelium (Fig 1, A-D).

In the tissue, extracellular MBP is confined to intact eosinophil granules (ie, punctate staining), as shown by means of MBP immunofluorescence staining (Fig 1, B, D, and E) and by means of electron microscopy and immunogold labeling for MBP (Fig 4). The apparent lack of subepithelial tissue damage, as assessed with hematoxylin-and-eosin staining of specimens from patients with CRS, suggests that intact granules containing MBP might not have physiologic or damaging actions in the tissue. The accumulation of diffuse MBP immunofluorescence in the mucus (and not in the tissue) suggests that the cells found in the tissue are in transit toward their final target in the mucus, with ongoing deposition of MBP (ie, the cluster of eosinophils surrounded by the diffuse cloud of MBP). This pattern of cluster-forming eosinophils appears strikingly similar to the eosinophils' role in their immune

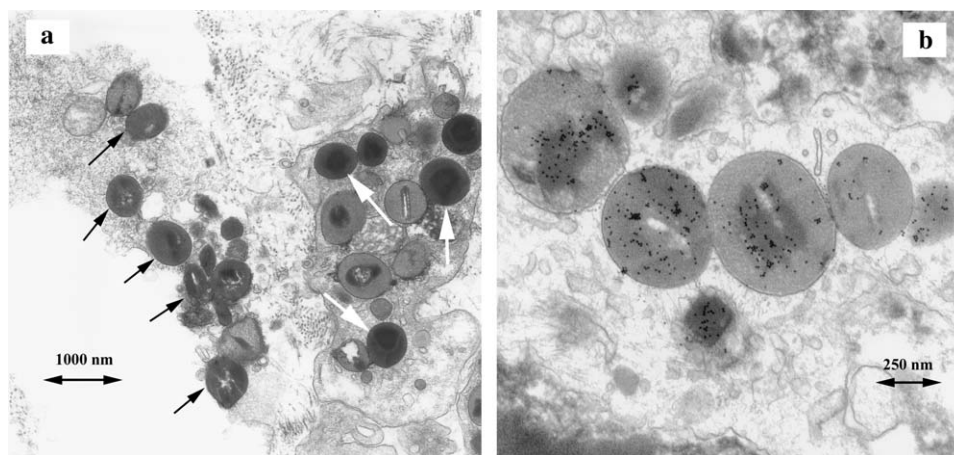


FIG 4. Transmission electron micrographs of sinus tissue from a patient with CRS. Panel **a** shows the characteristic electron-dense secondary granules within an intact cell (*white arrows*) and intact extracellular granules in the tissue (*black arrows*; original magnification, 10,000 $\times$). Panel **b** shows immunogold labeling (*black dots*) for MBP and demonstrates that MBP is localized within the intact granules; note the lack of MBP labeling in the surrounding tissue (original magnification, 33,000 $\times$).

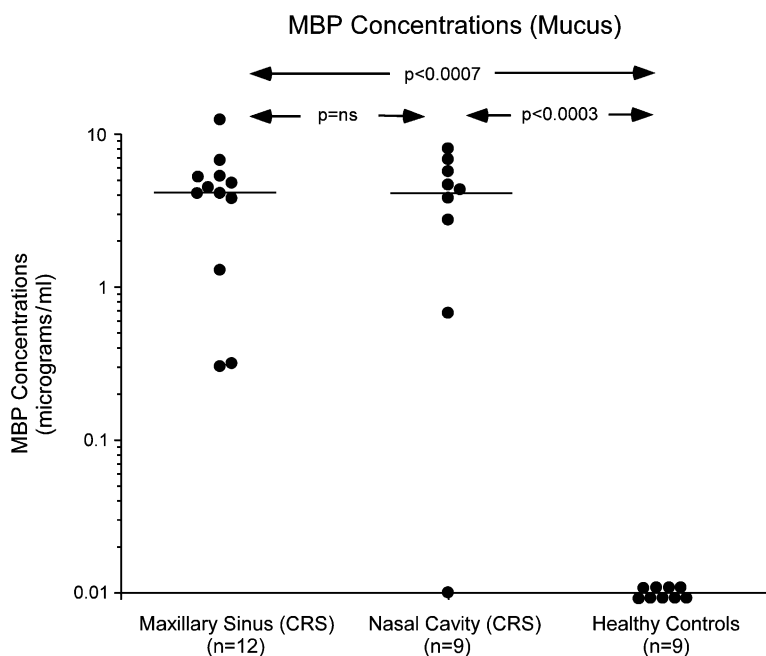


FIG 5. MBP concentrations in mucus specimens from patients with CRS and healthy control subjects. Mucus specimens were extracted with 0.15 M NaCl, and MBP was measured in the supernatants by means of RIA. MBP was detected in the maxillary sinus mucus and in the nasal cavity mucus of patients with CRS but not in mucus from the healthy control subjects. *Horizontal bars* indicate mean values for each group.

defense against parasites when they cluster around the organisms, subsequently releasing granular proteins (including MBP) that destroy the parasite.²⁵

It is generally assumed that eosinophil cytolysis and piecemeal degranulation are distinct mechanisms by which granules and, subsequently, granule proteins are released in diseased airway tissue.²⁶ Instead, we found that

eosinophils release cytotoxic MBP in the mucus, but not in the tissue, at concentrations likely exceeding those needed to damage the epithelium in patients with CRS. Therefore one might need to take not only the tissue but also the mucus into account when trying to understand the pathophysiology of CRS and probably other airway diseases. In addition, this new understanding suggests a beneficial

effect in therapies that target primarily the underlying and presumably damage-inflicting eosinophilic inflammation instead of the secondary bacterial infection.

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