

An immunologic test for chronic rhinosinusitis based on free intranasal eosinophilic major basic protein

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Background: A histologic hallmark of chronic rhinosinusitis (CRS) is an eosinophilic inflammation, present with and without nasal polyposis and independent of atopy. Eosinophils migrate through nasal tissue including the epithelium into the nasal airway mucus, where they form clusters and degranulate, releasing granule proteins including the toxic major basic protein (MBP). Specific biomarkers for CRS, which could be used as a diagnostic test for CRS with a high sensitivity and specificity, are presently lacking. Recently, an enzyme-linked immunosorbent assay (ELISA)-based test for MBP in nasal airway mucus received regulatory approval.

Methods: A new assay was specifically developed to detect released MBP in airway mucus. MBP levels in nasal mucus of 85 randomly selected CRS patients diagnosed by endoscopy, computed tomography (CT) scans and symptoms were compared to 13 healthy controls and 5 disease controls (allergic rhinitis).

Results: Overall, 92% (78/85) of CRS patients' mucus were positive for MBP (mean 7722 ng/mL) vs none of 13 healthy

controls and none of 5 allergic rhinitis patients (<7.8 ng/mL; $p < 0.000000000002$). In this study, the MBP ELISA had a 92% sensitivity and 100% specificity for CRS.

Conclusion: Free MBP in nasal mucus can be used as a biomarker to diagnose CRS. The MBP ELISA represents the first immunologically-based test to potentially distinguish CRS from the eosinophilic inflammation in allergic rhinitis.

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Key Words: rhinosinusitis; allergic rhinitis; major basic protein; ELISA; intranasal protein; eosinophil degranulation

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Chronic rhinosinusitis (CRS) is an inflammatory disease of the nasal and paranasal sinus mucosa. CRS patients have an impaired quality of life, with congestion, thick mucus production with postnasal drip, loss of sense of smell, facial pain and pressure, headaches, and susceptibility to secondary bacterial infections.¹ Patients with CRS are symptomatic for more than 3 months and have inflammatory mucosal thickening (or polypoid changes) seen on endoscopy or computed tomography (CT).^{1,2} CRS accounts for >90% of all rhinosinusitis cases and has a prevalence of 14% of the adult population in the United States, with an estimated direct cost of about \$5.8 billion and more than 73 million lost activity days per year.^{3,4} Antihistamines and antibiotics are usually ineffective in the long-term management of CRS,⁵ and systemic corticosteroids only provide temporary relief.

A histologic hallmark of CRS is an eosinophilic tissue inflammation, which is present with and without nasal polyposis and independent of atopy.^{6–9} On the other hand, eosinophilic inflammation also occurs in patients in allergic rhinitis (AR), suggesting different pathophysiologic roles

for the eosinophilic leukocyte because its presence is associated with both of these clinically very distinct entities.

Remarkably, analyses of eosinophil-associated inflammation in CRS have shown accumulation and cluster formation of eosinophils in the mucus, indicating migration through the submucosa and epithelium into the mucus.^{7,8,10} Within those clusters, eosinophils can be observed to degranulate in a specific fashion, namely to diffusely release large amounts of major basic protein (MBP).¹⁰ Altogether, those observations suggest that the eosinophils' final target for degranulation in CRS is within the clusters in the mucus.

This degranulation observed in the mucus of CRS patients is distinctively different from the presence of occasional free but intact granules in the tissue (Fig. 1B).¹⁰ This finding suggests a different degranulation pattern between mucus and tissue, where massive amounts of the highly toxic eosinophil MBP are being released into the mucus, in contrast to the absence of diffuse MBP release in the tissue and epithelium.^{10,11} This deposition of MBP, with its known toxicity to respiratory epithelium, is directly associated with the mucosal damage found in CRS and likely predisposes patients to frequent secondary bacterial infections.⁹ The lack of detectable MBP in the mucus of AR patients might also explain why epithelial damage and subsequent bacterial infections are absent in AR.¹²

MBP comprises the crystalloid core of the eosinophil secondary granule, whereas other eosinophil granular proteins are located in the matrix. MBP is uniquely specific to the eosinophil and does not exist in other human cells (except in very small amounts in basophils).^{13,14} Pathophysiologic effects, besides erosion and direct destruction of the surfaces it is released upon, include its important role in bronchial asthma.¹⁵ In asthma MBP is known to mediate epithelial erosion, directly causing bronchoconstriction and inducing hyperresponsiveness of the lower airway to external triggers.^{16,17} In fact, the majority of physiologic and pathologic changes of the lower airway in asthma can be explained by the presence of MBP in the bronchial lumen.^{16,17}

Specific biomarkers for CRS, which could be used as a diagnostic test for CRS with both a high sensitivity and specificity, are presently lacking. Other modalities currently used for diagnosis, such as a CT scan or an endoscopic examination, are impacted by radiation exposure, high costs, or the lack of availability of a trained specialist for nasal endoscopy. A simple, noninvasive screening test specific for CRS would help identify these patients, facilitating direct referral to the specialist offering a specific tailored treatment.

The fact that MBP is present in the airway mucus of CRS patients and absent in both AR and healthy controls makes the detection of released MBP, so called "free MBP," a viable candidate for a screening test. The purpose of this study was to extract MBP from human mucus, develop a reliable enzyme-linked immunosorbent assay (ELISA) assay to detect extracted MBP, and establish the sensitivity, specificity and reliability of intranasal (free) MBP levels in

the mucus as a diagnostic marker of CRS, including the comparison with appropriate controls.

Patients and methods

Patient selection criteria

The Mayo Clinic and the University at Buffalo institutional review boards approved the study. Eighty-five randomly selected patients seen by otolaryngologists with confirmed CRS having symptoms for at least 3 months, inflammatory mucosal thickening on endoscopy, and a coronal CT scan demonstrating mucosal thickening of greater than 3 mm in 2 or more sinuses were recruited for the study. Samples from healthy controls had the absence of all these criteria. Disease control samples were taken from 5 patients with AR who had seasonal symptoms during the allergy season. Because systemic corticosteroids significantly reduce the number of eosinophils in the systemic circulation and in the diseased tissue, and consequently the levels of MBP in the nasal mucus,¹⁸ both patients and controls who used systemic steroids within 3 months were excluded. Patients having used topical corticosteroids were permitted to participate.

Specimen collection and handling

Approximately 0.1 to 0.2 mL of mucus was suctioned under direct vision, using a nasal speculum, from the area of the middle meatus into a specific trap (Immco Diagnostics, Amherst, NY). No liquids were applied to the patients' noses prior to sampling (such as decongestants or anesthetics) in order to avoid dilution of the sample. The extraction involved adding a 3-fold excess (by weight) of normal saline (0.15 M NaCl) to the specimen, vigorous vortexing for 10 seconds ($\times 3$), and centrifugation. The supernatant fluid was transferred to a polypropylene tube and stored at -70°C .

Description of the assay

The MBP ELISA is a so-called "sandwich" assay, which has high sensitivities. The analyte is bound between 2 antibodies: (1) the 173-14E10-1D1 capture antibody (a monoclonal antibody against mature 14 kDa MBP); and (2) a non-interfering biotinylated monoclonal detection antibody against mature MBP (171-8B2-A10).

All human-derived components used were tested for hepatitis B surface antigen (HBsAg), hepatitis C virus (HCV), human immunodeficiency virus (HIV)-1 and 2, and human T-lymphotropic virus (HTLV)-1 and found negative by U.S. Food and Drug Administration (FDA)-required tests. Good laboratory practices in storing, dispensing, and disposing were followed.¹⁹

All samples, including MBP standard, must undergo reduction and alkylation prior to use in the MBP ELISA.

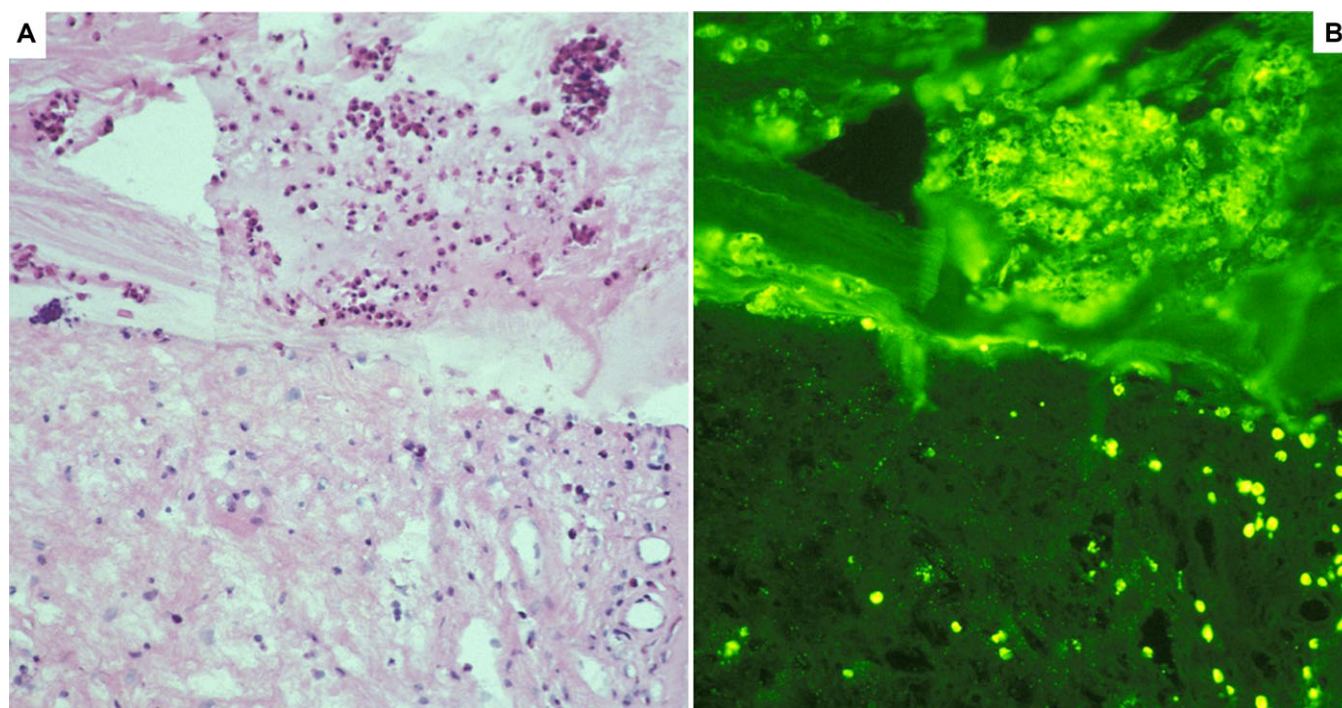


FIGURE 1. Histologic section visualizing the transition between tissue, epithelium, and mucus in CRS. Special care in the collection and handling was applied to ensure that the mucus remained attached to the tissue. (A) Typical finding of an eosinophilic inflammation in the tissue, severely eroded epithelium (damaged from the outside), and heavier concentrations of eosinophils in clusters in the mucus (hematoxylin and eosin, original magnification $\times 400$). (B) Serial section of (A) stained with an antibody to MBP. Note that the MBP is contained within intact eosinophils in the tissue; however, massive amounts of toxic MBP are released within the clusters in the mucus (anti-MBP immunofluorescence, original magnification $\times 400$). CRS = chronic rhinosinusitis; MBP = major basic protein.

Preparation of stabilized ELISA plates

Microtiter plates (Thermo Scientific, Rochester, NY) were coated with 100 μL /well of 1 $\mu\text{g}/\text{mL}$ 173-14E10-1D1 antibody prepared in 0.05 M carbonate buffer, pH 9.4 for 4 hours at room temperature. Plates were washed once with 300 μL /well phosphate-buffered saline (PBS, Life Technologies, Buffalo, NY) (0.0027M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ /0.0015M KH_2PO_4 /0.155M NaCl, pH 7.2), and blocked with 250 μL /well 0.5% bovine serum albumin (BSA, SeraCare Life Sciences, Milford, MA)-PBS for 1 hour at room temperature. Plates were washed 3 times with PBS (300 μL /well) and stabilized with a Trehalose solution (0.1% BSA/5% Trehalose dihydrate [MP Biomedicals, Santa Ana, CA]/0.09% NaAz [Sodium Azide, Sigma, St. Louis, MO]/PBS, pH 7.2) for 2 hours, and aspirated and stored overnight (both at room temperature). After drying, plates were placed in heat-sealable Mylar bags with desiccant and stored refrigerated at 4°C.

Preparation of the patient samples

First, a 30-mM tris(2-carboxyethyl)phosphine (TCEP, Sigma, St. Louis, MO) working solution was made using 700 μL Reaction Buffer (0.25M Tris, pH 7.5) and 300 μL of 100 mM TCEP stock. Each sample was reduced by combining 110 μL Reaction Buffer, 20 μL of 30 mM TCEP, and 50 μL saline-extracted mucus test sample in a 10-mL borosilicate glass tube for 30 minutes. A 35-mM

iodoacetamide working solution was prepared (650 μL reaction buffer plus 350 μL of 100 mM iodoacetamide stock [Sigma, St. Louis, MO]) and 20 μL of the prepared 35-mM iodoacetamide working solution was added to each reduced sample, and then incubated for an additional 30 minutes at room temperature. Finally, 100 μL assay diluent (0.1% Proclin-300 [Sigma, St. Louis, MO]/0.5% BSA/PBS) was added for a 6 $\times$ dilution factor. Each sample was run in duplicate.

Preparation of the MBP standard

Human MBP was isolated from peripheral blood eosinophils from patients with eosinophilia.²⁰ Treatment of MBP standard mimicked that of the patient samples. A 30 mM TCEP working solution was made using 700 μL Reaction Buffer (0.25M Tris, pH 7.5) and 300 μL of 100 mM TCEP stock. MBP standard was reduced in a 10-mL borosilicate glass tube by combining reagents in the following order: 800 μL Reaction Buffer, 100 μL of 30 mM TCEP, and 0.72 μL MBP Standard (1.4 mg/mL). Treated standard was incubated for 30 minutes prior to addition of 100 μL of the prepared 35-mM iodoacetamide working solution and incubation for an additional 30 minutes at room temperature. Finally, 4.0 mL assay diluent (0.1% Proclin-300/0.5% BSA/PBS) was added to the reduced and alkylated standard and mixed, for a final concentration of

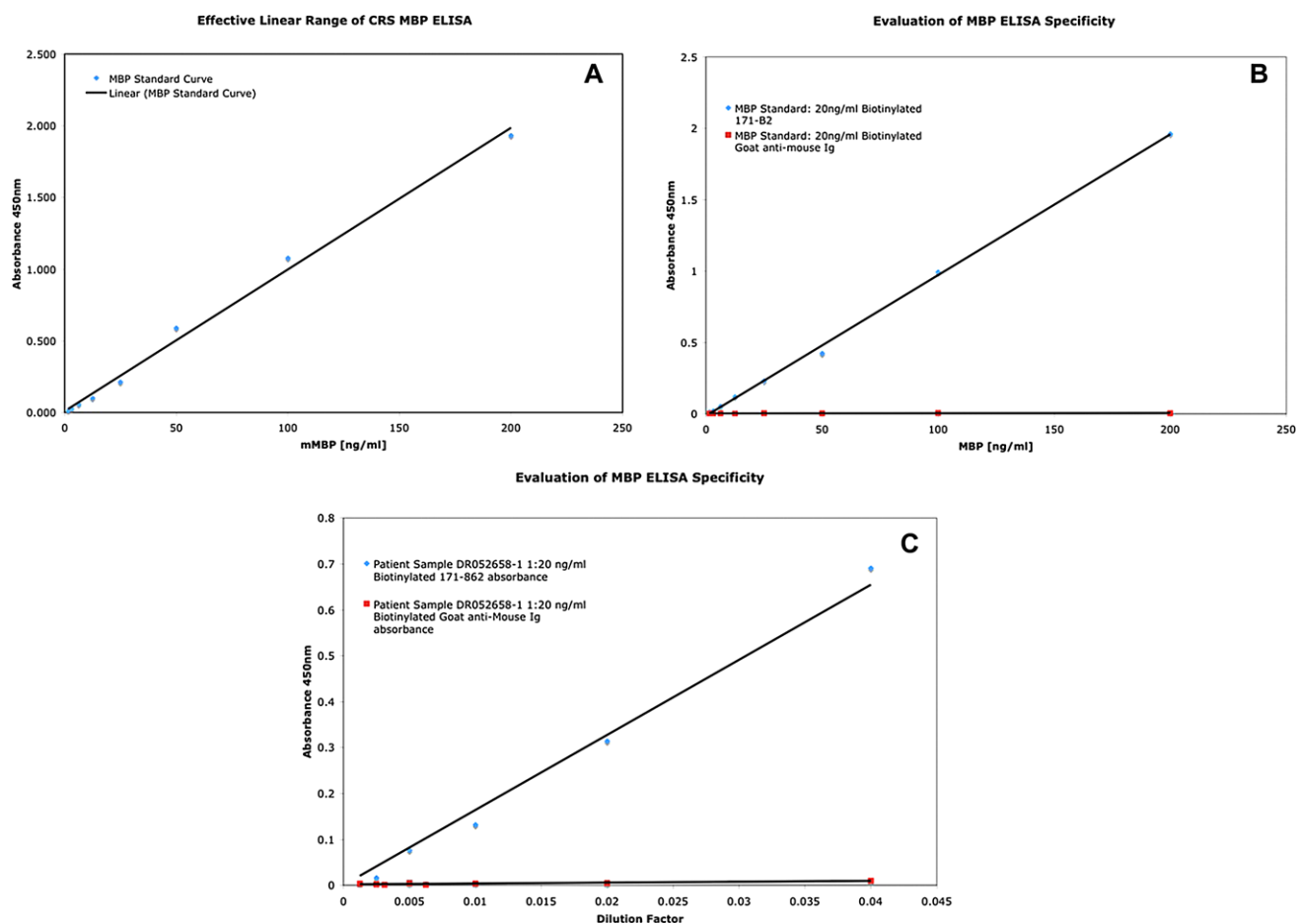


FIGURE 2. (A) Evaluation of effective linear range of the CRS MBP ELISA. The low and high limits of detection for the assay are 1.56 ng/mL and 200 ng/mL, respectively. The effective linear range for the assay is 1.56 to 200 ng/mL. (B, C) Evaluation of MBP ELISA specificity/background employing a nonspecific antibody. CRS = chronic rhinosinusitis; ELISA = enzyme-linked immunosorbent assay; Ig = immunoglobulin; MBP = major basic protein.

200 ng/mL. Two-fold serial dilutions were made to generate a standard curve ranging from 3.13 to 200 ng/mL.

MBP ELISA

All materials and reagents were warmed to room temperature. First, 150- μ L duplicates of reduced and alkylated test samples were transferred into wells of a 96-well polypropylene dilution plate (Corning Life Sciences, Lowell, MA), together with the duplicate, and 100 μ L of all samples transferred to the 173-14E10-1D1-coated capture plate. The plate was incubated at room temperature for 30 minutes, and then washed 4 times with 300 μ L/well of PBS. All wells were treated with 100 μ L of 20 ng/mL biotinylated 171-8B2-A10 antibody (prepared in assay diluent) and incubated at room temperature for 30 minutes. The plate was then washed 4 $\times$ with 300 μ L/well of PBS, and 100 μ L/well of a 0.05 μ g/mL solution of streptavidin-horseradish peroxidase (HRP, Life Technologies, Buffalo, NY) prepared in assay buffer was added, incubated for 30 minutes at room temperature, washed again with 300 μ L/well of wash buffer, then 100 μ L/well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (BioFxx, Surmodics, Eden Prairie, MN) was added. The plate was

incubated for 15 minutes at room temperature and 100 μ L/well of stop solution (0.5M H₂SO₄, Sigma, St. Louis, MO) was added before absorbance was measured at 450 nm (A450).

Assay limitations

Mucus specimens had to meet minimum volume requirements (0.1 to 0.2 mL) and processed within 2 days of harvest in order to ensure that eosinophils did not disintegrate, releasing intracellular MBP into the mucus and potentially yielding false-positive results. Repeated freezing and thawing of samples must be avoided. No interference of the assay was associated with tested mucus specimens; however, all specimens showing visible signs of contamination were rejected.

Validation of the assay's quality

The MBP assay was first established as a radioimmunoassay (RIA) in the Allergic Disease Research Laboratory of the Mayo Clinic, Rochester, MN. This assay was revalidated in ELISA format using known MBP-positive clinical specimens, CRS specimens, disease controls, and normal

human sinus mucus samples. The FDA's Quality System Regulation for assay design control and validation were followed. The validations included verification of positive specimens using a monoclonal antibody, purified MBP standards, evaluation of specificity of the system by employing nonspecific antibodies, analysis of matrix stability, durability of assay components, runs of 103 specimens, and precision studies with multiple runs of specimens.

Evaluation of MBP ELISA specificity/background employing a nonspecific secondary antibody

The validity of the test could be called into question if MBP was detected after a nonspecific antibody was used in place of the biotinylated secondary antibody to MBP. To ensure the validity, the MBP ELISA procedure was run several times (CRS patient samples as well as MBP standard) to test for recovery of MBP after using 20 ng/mL of the specific biotinylated secondary antibody to MBP (171-8B2-A10) or 20 ng/mL of nonspecific biotinylated goat anti-mouse immunoglobulin (Ig, Southern Biotech, Birmingham, AL).

Validity of the matrix stability

Specimens containing MBP have been validated stable at room temperature and in overnight courier shipping conditions for a period of 48 hours. Specimens may be viable for longer periods; however, longer stability studies have not yet been completed.

Validity and durability of materials in the assay after a prolonged storage

Each of the components used in the assay was assessed for durability over an extended period of time. Stability for each reagent was evaluated side-by-side with its fresh counterpart, using MBP standard reactivity as a benchmark, and concordance correlation coefficient analysis (CCC) was used to determine equivalence.²¹

Evaluation of negative control patients and samples

Of paramount concern was that other endogenous and exogenous elements in the mucus may cross react and yield false-positive results. Nasal mucus is a heterogeneous mixture of peptidoglycans, deceased epithelial cells, leukocytes, and inhaled exogenous materials. Thus mucus specimen from healthy controls and patients with confirmed AR were analyzed via the MBP ELISA.

In addition, endogenous and exogenous airway mucus proteins may mask MBP and lead to false-negative results. Thus, negative control samples were spiked with mature MBP (mMBP). Two separate trials were done on 3 negative controls; 2 sets were spiked with 100 ng/mL and 200 ng/mL mMBP. The recovered MBP concentrations were measured and the percentages of the spikes recovered calculated.

Accuracy

Purified MBP from the Allergic Disease Research Laboratory of the Mayo Clinic (Rochester, MN) was used for quality control and to establish reference standards for the assay. The nature of this material has been confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoassay sequence analysis.^{13,22} Reactivity was tested by coating a monoclonal antibody against mature MBP (173-14E10-1D1) onto microtiter plates and evaluating a titration series using the protocol previously documented in the Description of the Assay section. Mucus specimens from positive and negative CRS patients, which included disease (AR) controls, were tested to calculate the sensitivity and specificity of the MBP assay.

Precision and reproducibility

In order to assure integrity and reproducibility, duplicate specimens from 59 confirmed CRS-positive patients were analyzed over several runs in the MBP ELISA, including testing within and across coated capture plates of the same run to establish intraassay and interassay variance. Following industry standards the level of acceptance was set at <15% Clinical Variance (CV).

Effectiveness of different incubation periods and MBP extraction methodologies on patient mucus, including saline, gelsolin, and deoxyribonuclease (DNase) treatment, was also evaluated using the same samples.

MBP RIA and ELISA comparison

A comparison of the MBP ELISA with the previous method of detecting MBP by RIA was done, where mildly reduced and alkylated MBP was iodinated with radioactive iodine-125.^{19,22-24} Analysis of the MBP via RIA is cumbersome and expensive, and handling radioactive substances requires specific precautions and procedure, which would make the assay impractical for routine use as a diagnostic tool.

Results

Analysis of effective linear range

The absorbance value of the 200 ng/mL standard was generally between 1.5 and 2.5 at A₄₅₀. Any value below an A₄₅₀ of 1.2 invalidated the assay run, with the assay being within linear parameters above an A₄₅₀ of 1.2.

Evaluation of MBP ELISA specificity/background employing a nonspecific secondary antibody

The results shown in Figure 2A-C confirm the specificity of the biotinylated detection antibody for MBP. Although the biotinylated 171-8B2-A10 detection antibody demonstrated reactivity to both purified MBP standard (Fig. 2B) and MBP within a CRS-positive patient mucus sample (Fig. 2C), a nonspecific biotinylated goat anti-mouse Ig evidenced no reactivity under the same conditions.

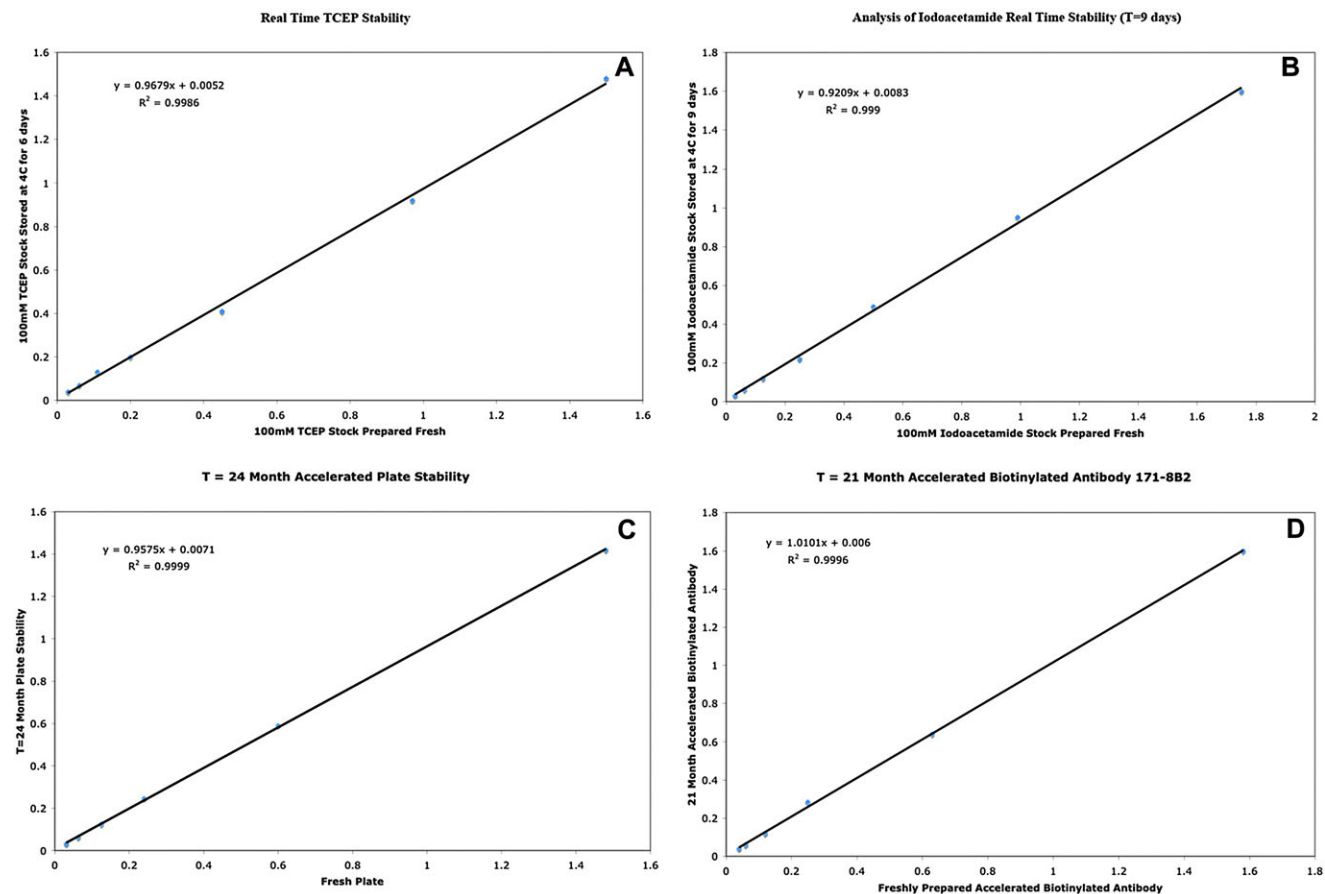


FIGURE 3. (A) Analysis of TCEP real-time stability (T = 6 days). (B) Analysis of iodoacetamide real-time stability (T = 9 days). (C) Concordance correlation coefficient analysis of plate stability (T = 24-month accelerated). (D) Concordance correlation coefficient analysis of biotinylated 171-8B2-A10 stability (T = 21-month accelerated). TCEP = tris(2-carboxyethyl)phosphine.

Validity and durability of materials in the assay after a prolonged storage

Many of the components used in the assay were evaluated for durability over an extended period of time. These components included TCEP real-time stability after 6 days, iodoacetamide real-time stability after 9 days, plate stability after 24 months, and biotinylated (171-8B2-A10) antibody stability after 21 months. Reactivity of the MBP standard was evaluated side-by-side using the time-elapse test reagent vs their fresh counterparts. All of the reagents were shown to be durable up to the elapsed time (Fig. 3A-D).

Evaluation of negative control patients and samples

All 18 non-CRS control patients (13 healthy controls, 5 AR controls) had MBP mucus levels of less than 7.8 ng/mL, which was below the low limit of detection for the system. The percentage of recovered mMBP in the 3 negative samples spiked with mMBP was found to be adequate and not inhibited by exogenous proteins (Table 1).

TABLE 1. Spike recovery evaluation results			
Sample code	Spiked MBP (ng/mL)	Recovered MBP (ng/mL)	Spike recovery (%)
GM102952	100	90.7	90.7
TN112661	100	97.7	97.7
VR021266	100	159.2	159.2
			Average % recovery 115.9
GM102952	200	159.8	79.9
TN112661	200	167.8	83.9
VR212266	200	229.0	114.5
			Average % recovery 92.8

MBP = major basic protein.

Accuracy

The mucus of 78 of 85 patients tested positive for MBP, whereas none of the healthy controls (0/13) and none of

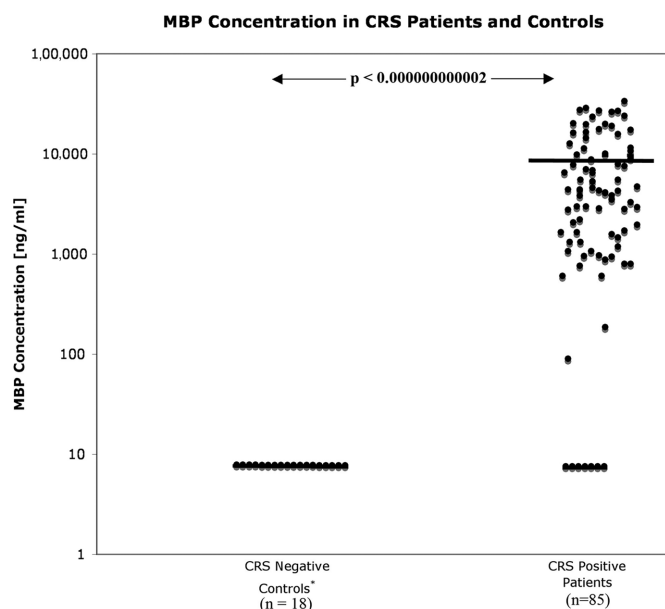


FIGURE 4. Evaluation of CRS positive and negative patients via the MBP ELISA. The difference in concentration of MBP found in the mucus of these patients is striking. Horizontal bars indicate mean values for each group. *Values were detected below the lower limit of <7.8 ng/mL. CRS = chronic rhinosinusitis; ELISA = enzyme-linked immunosorbent assay; MBP = major basic protein.

the AR controls (0/5) had detectable MBP in their nasal mucus (Fig. 4). The mean (MBP) for all confirmed CRS-positive patients was found to be 7722 ng/mL, which is a striking difference from the <7.8 ng/mL found in CRS-negative patients ($p < 0.000000000002$, Wilcoxon rank-sum test).

The data indicate that the MBP ELISA is a highly sensitive (92%) and specific (100%) method for identification of patients with CRS and allows for differentiation between AR and CRS patients.

Precision and reproducibility

Duplicate specimens from 59 of 85 confirmed CRS positive patients were analyzed with several runs through the MBP ELISA in order to establish intraassay and interassay clinical variance. Average CV within each study ranged from 7.8% to 13.1%; all MBP values remained strongly positive. Duplicate specimens from 16 of 17 healthy and AR controls were also analyzed and remained negative.

Effect of extraction method on interassay reproducibility

Test samples were processed using different extraction methods (including saline, gelsolin, and DNase) to co-evaluate the effectiveness to extract MBP from patient mucus. The results (not shown) indicated that the MBP ELISA assay is reproducible using a variety of extraction methods and incubation times. Furthermore, all of the positive controls remained strongly positive and none of the

negative controls were positive in either trial, confirming the sensitivity and specificity of the assay system for CRS.

Similar sensitivity of MBP ELISA to RIA

When compared to analysis with MBP RIA, the samples had a sensitivity of 91% (77/85) for detecting MBP in CRS mucus samples, confirming the precision of the MBP ELISA assay (78/85).

Discussion

Previous studies have shown that the eosinophilic degranulation, specifically the release of the toxic MBP, is responsible for the mucosal damage in CRS.^{8–10} These observations further strengthens the argument that there are different degranulation patterns between AR and CRS, because toxic MBP is released in the nasal mucus in CRS but not in AR. In addition, in AR there is a lack of tissue remodeling and lack of epithelial damage typically seen in CRS, potentially explaining some of the clinical differences between those 2 distinct diseases. Logically, testing for MBP in the airway mucus would differentiate between these 2 clinical entities, and identify those patients who may benefit from specific anti-inflammatory treatments using topical or systemic steroids.

Immunofluorescence was the original immunohistochemical method used to help visually identify MBP in the airway mucus.^{25–27} In order to quantify the MBP in mucus specimens, digital analysis is required.¹⁰ Furthermore, careful sampling and preparation of the mucus and nasal epithelium prior to formalin fixation and paraffin embedding. The degree of skill and resources required makes MBP immunofluorescence an impractical screening test for CRS. With the advent of the ELISA, there is now an expedient, highly sensitive, and specific test for MBP in the mucus, and consequently for CRS.

The development of the MBP ELISA allowed for a rigorous analysis and validation of every step and component of the assay. The lower limit of detection of MBP concentration in the assay was naturally a concern, because too high of a cutoff could potentially have resulted in numerous false negatives, thereby missing actual CRS patients. With the lowest limit for CRS mucus set at 28 ng/mL, all CRS positive patients were identified, whereas mucus from healthy and diseased controls had MBP concentrations of less than 7.8 ng/mL and thus were below the low limit of detection of the MBP ELISA.

It is important to understand that the concentration of extracted MBP is significantly less (by a magnitude of more than 1000) than the focal concentration in the CRS mucus indicated by immunofluorescence, which is in the milligram per milliliter (mg/mL) range.¹⁰ This was due to the careful extraction process to not damage eosinophils, releasing intracellular MBP. This resulted that only released (“free”) MBP was detected, which was crucial for AR specimen to remain negative.

Although other MBP ELISAs exist, their antibodies detect 43-kDa and 64-kDa recombinant MBP. However, mature MBP released in humans is 14 kDa, which is detected by the antibodies used in this assay. In addition, this is the only ELISA validated in mucus samples, which makes it unique given the specific processing required (eg, extraction, reduction, and alkylation).

We can only speculate why 8% of the tested CRS specimens fulfilling the clinical criteria for CRS were not MBP-positive. Were patients not reporting the use of corticosteroids? Did they have symptoms of CRS and CT findings

associated with CRS, but not CRS itself? Or does CRS exist in some cases without an eosinophilic inflammation?

Conclusion

The severe morbidity and increasing prevalence of CRS clearly point to the great need to rapidly and accurately identify CRS patients. The introduction of this novel MBP ELISA represents a first immunologic test specific for CRS and has the potential to be used on a large scale and direct CRS patients to the appropriate care. 

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