A Novel Histologic Scoring System to Evaluate Mucosal Biopsies From Patients With Eosinophilic Esophagitis

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BACKGROUND & AIMS: Eosinophilic esophagitis (EoE) is characterized by medically/surgically-resistant gastroesophageal reflux symptoms and dense squamous eosinophilia. Studies suggest that histologic assessment of esophageal eosinophilia alone cannot reliably separate patients with EoE from those with gastroesophageal reflux disease (GERD). Our goal was to develop an assay to identify EoE patients and perhaps differentiate EoE from other causes of esophageal eosinophilia. **METHODS:** A monoclonal antibody specific for an eosinophil secondary granule protein (eosinophil peroxidase [EPX]) was developed and shown to specifically identify intact eosinophils and detect eosinophil degranulation in formalin-fixed specimens. A histopathologic scoring algorithm was developed to analyze data from patient evaluations; the utility of this algorithm was assessed by using archived esophageal tissues from patients with known diagnoses of EoE and GERD as well as controls from 2 tertiary care centers. **RESULTS:** Intraobserver/interobserver blinded evaluations demonstrated a significant difference ($P < .001$) between scores of samples taken from control subjects, from patients with esophageal eosinophilia who had a diagnosis of EoE, and from patients with GERD ($P < .001$). This algorithm also was able to identify patients whose clinical course was suggestive of a diagnosis of EoE, but that nonetheless failed to reach the critical threshold number of ≥15 eosinophils in a high-power (40×) microscopy field. **CONCLUSIONS: A novel immunohistochemical scoring system was developed to address an unmet medical need to differentiate histologic specimens from patients with EoE relative to those with GERD. The availability of a unique anti-EPX–specific monoclonal antibody, combined with the ease/rapidity of this staining method and scoring system, will provide a valuable strategy for the assessment of esophageal eosinophilia.**

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Eosinophilic esophagitis (EoE) is one of the leading causes of dysphagia and food impaction in adults and is an important cause of vague symptoms previously associated with gastroesophageal reflux disease (GERD) in children.[1] Despite increasing recognition of EoE, only recently have diagnostic criteria been published.[1] Although these criteria set new standards for clinical care, the established diagnostic histologic features focus solely on the numbers of intact eosinophils present in esophageal biopsies.[2] In addition, the diagnosis of EoE hinges on the fact that GERD has been excluded, thus requiring either pretreatment with a proton pump inhibitor or investigation with pH monitoring of the distal esophagus.[1,3,4]

The underlying mechanisms associated with the pathogenesis in EoE remain largely unresolved. However, eosinophil granule proteins are thought to play a functional role in the gastrointestinal tract by increasing permeability and colonic inflammation.[5,6] In this regard, it is important to realize that extracellular deposition of eosinophil granule proteins might be the only evidence of eosinophil activation in esophagitis. Earlier studies of EoE patients suggested that a subset of patients have extensive eosinophilic degranulation with few intact eosinophils.[7] However, because no studies have fully characterized degranulation patterns associated with EoE, the histologic diagnosis of EoE is often solely based on the numbers of eosinophils infiltrating the epithelium. Thus, the aim of our study was to improve current abilities to identify EoE patients on the basis of additional features of eosinophilic inflammation, including the use of eosinophil degranulation as an important diagnostic criterion of EoE.

This retrospective study used a novel monoclonal antibody specific for the secondary granule protein eosinophil peroxidase (EPX) as part of immunohistochemical assessments of formalin-fixed and paraffin-embedded esophageal biopsies from patients with EoE, GERD, or controls. The results from these assessments demonstrated the utility of anti-eosinophil peroxidase monoclonal antibody (EPX-mAb)–based immunohistochemistry to support clinical findings. Specifically, we report a quantitative scoring strategy based on the identification of infiltrating eosinophils and distinct patterns of degranulation. More importantly, this study showed that a diagnostic strategy that used EPX-mAb led to more expedient assessments of patients with esophageal diseases. We anticipate that future clinical studies will validate EPX-mAb–based evaluations, allowing

Abbreviations used in this paper: ANOVA, analysis of variance; EDN, eosinophil-derived neurotoxin; ELISA, enzyme-linked immunosorbent assay; EoE, eosinophilic esophagitis; EPX-mAb, anti-eosinophil peroxidase monoclonal antibody; ESGP, eosinophil secondary granule protein; GERD, gastroesophageal reflux disease; HPF, high-power field; MBP, major basic protein; SEM, standard error of the mean.

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distinctions between EoE, GERD, and other esophageal diseases. In turn, this will lead to more rapid diagnosis and treatment and thus provide a cost-effective and accurate means of achieving a definitive diagnosis.

Materials and Methods

Development of an Anti-Eosinophil Peroxidase Monoclonal Antibody and Diagnostic Scoring Algorithm to Evaluate Esophageal Patients

For a complete explanation of this process, see Supplementary Materials and Methods.

Human Subjects

Adult esophageal patients were identified retrospectively by gastroenterologists at Mayo Clinic Arizona. These patients were divided into 4 groups. Group I were patients diagnosed with EoE by virtue of (1) clinical symptoms at presentation (eg, dysphagia, vomiting, and/or food impaction); (2) GERD was ruled out with pretreatment with proton pump inhibition, a normal pH/impedance monitor, and/or response to topical or systemic steroids; (3) endoscopic findings (ie, corrugations and/or furrows) were characteristic of EoE; (4) histopathologic assessments of mid/proximal-esophageal biopsies demonstrating sclerosis of the lamina propria stroma, basal hyperplasia of the squamous epithelium, and/or intercellular epithelia edema; and, most important, (5) at least a single focus of ≥15 eosinophils/40× high-power field (HPF) from mucosal biopsies. Group II patients were diagnosed with GERD on the basis of symptoms that included response to proton pump inhibitors and epithelial biopsies that revealed <15 eosinophils/40× HPF with no evidence of intercellular edema, stromal fibrosis, or eosinophilic microabscesses. Control subjects (group III) were obtained from autopsy and clinical specimens from patients whose esophageal epithelium was unremarkable, and their medical records did not reveal history of IBD, reflux, Barrett's esophagus, adenocarcinoma of the esophagus, or eosinophilic disorders (eg, hypereosinophilic syndrome). Group IV subjects were suspected EoE patients by virtue of displaying a clinical presentation and/or endoscopic observations compatible with EoE. Moreover, the middle or proximal esophageal biopsies of these patients revealed at most only 2 ancillary histopathologies associated with EoE: sclerosis of the lamina propria stroma, basal hyperplasia of the squamous epithelium, intercellular epithelial edema, and/or the presence of eosinophilic microabscesses. Significantly, despite extensive reviews at high power (40×) of all sections of all biopsies taken from these patients, no foci of ≥15 eosinophils/40× HPF were identified, preventing an unambiguous diagnosis of EoE. Thus, these subsets of patients were specifically chosen on the basis of their clinical, endoscopic, and histopathologic outcomes as a preliminary means of developing and then using our EPX-mAb–based algorithm.

We also performed a retrospective analysis of the esophageal tissues from children who received care at The Children's Hospital, Denver, and underwent upper endoscopy during 2006. Well-documented clinical features, and in some cases the existence of follow-up assessments, allowed us to initially identify 48 children for potential study, of whom 14 were selected for analysis by using EPX-mAb–based immunohistochemistry on the basis of the availability of an unambiguous diagnosis (group I [EoE], 7; group II [GERD], 3; group III [control], 4). Children with EoE (group I) had symptoms referable to their esophagus, received at least 2 months of proton pump inhibition, ≥15 eosinophils/40× HPF in the esophageal epithelium with normal gastric and duodenal biopsies, and exhibited a clinical response to EoE treatment(s). GERD (group II) was documented by an abnormal pH/impedance monitoring of the distal esophagus or responsiveness to proton pump inhibitors, as well as a pathology report of <15 eosinophils/40× HPF in the esophageal epithelium. Control patients (group III) exhibited no evidence of esophageal inflammation. In addition, 8 indeterminate pediatric patients (group IV) were identified within this cohort of children and subjected to EPX-mAb–based immunohistochemistry.

Clinical descriptions of all patients included in this study, such as age, medical history/symptoms, and follow-up assessments (if available), are summarized in the Results section and/or in Supplementary Table 1. This study was reviewed and performed in accordance with Institutional Review Board approval at Mayo Clinic Arizona (IRB protocol number: 06-009236) and Colorado Multiple IRB (approval number 07-0888).

Anti-Eosinophil Peroxidase Monoclonal Antibody–Based Histopathologic Scoring

Slides with biopsies (n ≥ 4 per patient) from the mid-proximal esophagus (ie, >7 cm from the esophageal-gastric junction) were coded by clinical histopathology laboratory personnel and subsequently stained by research lab–based personnel by using EPX-mAb–based immunohistochemistry.

Our assessments of patients by using EPX-mAb–based staining led to the identification of 4 independent diagnostic markers on the basis of their presence versus absence in EoE relative to control subjects: (1) presence or absence of infiltrating tissue eosinophils, (2) evidence of eosinophil degranulation, (3) the extent of eosinophil infiltration and/or eosinophil degranulation in the maximally affected biopsy, and (4) the extent of eosinophil infiltration and/or eosinophil degranulation among the available patient biopsies. Detailed descriptions of each marker, including representative photomicrographs, are presented in the Supplementary Materials and Methods.

Statistical Analyses

Data were analyzed and graphed by using GraphPad Prism statistics program (GraphPad Prism Software, San Diego, CA). Results are presented as means ± standard error of the mean (SEM). Statistical analysis was performed by using analysis of variance (ANOVA) with Tukey. Differences between means were considered significant when $P < .01$.

Results

Anti-Eosinophil Peroxidase Monoclonal Antibody–Based Immunohistochemistry: Pathology Assessments of Eosinophilic Esophagitis Patients, Including the Detection of Eosinophil Activation (the Release of Granule Proteins [Degranulation])

EPX-mAb–based immunohistochemistry provided a strategy for the rapid detection of infiltrating eosinophils in esophageal biopsies. More important, this strategy also pro-

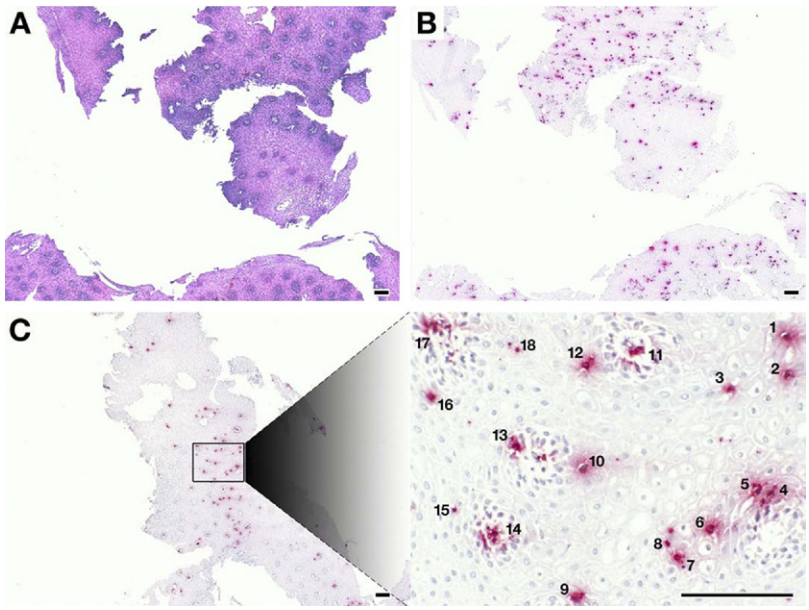

Figure 1. EPX-mAb–based immunohistochemistry provides an efficient and rapid strategy to identify intact eosinophils infiltrating biopsies from EoE patients. A comparison of low (5×, 20 mm^2 field of view) power microscopy of (*A*) hematoxylin-eosin–stained sections and (*B*) serial sections of the same patient after EPX-mAb–based immunohistochemistry demonstrated that this immunohistochemical strategy easily permits the identification of infiltrating eosinophils in multiple biopsies within the field. (*C*) EPX-mAb–based immunohistochemistry permits a rapid evaluation for the presence of intact infiltrating eosinophils of entire esophageal biopsies and the location of focal areas of eosinophil accumulation. The *insert photograph* in this panel is a high-power (40×, 0.29 mm^2 field of view) field that was quickly/efficiently identified as a focal area of eosinophil accumulation (identified eosinophils are numbered *1–18*) without the need of laborious time-consuming cell differential analyses. Scale bar = 100 μm.

vided a method with which to identify quickly areas of biopsies that are likely to contain the focus of ≥15 eosinophils/40× HPF needed to meet the histologic guidelines for a diagnosis of EoE. The photomicrographs of Figure 1 demonstrated the ease of identifying eosinophils within tissue sections by using EPX-mAb–based immunohistochemistry compared with hematoxylin-eosin–stained esophagus sections at low (5×, 20 mm^2 field of view) power (Figure 1*A, B*). Immunohistochemistry with EPX-mAb also allowed rapid identification of areas within the sections with a density of infiltrating eosinophils likely to achieve the ≥15 eosinophil/40× HPF needed for a guideline-driven EoE diagnosis (Figure 1*C*)**.** More important, these photomicrographs highlighted the ease and utility of locating focal accumulation of eosinophils at low power before more detailed assessments at a higher magnification.

A unique observation from our studies of EoE patients with EPX-mAb–based immunohistochemistry was that degranulation (ie, extracellular matrix deposition of EPX) was common (Figure 2). The importance of this observation is hard to overestimate because EPX-mAb–based immunohistochemistry detected not only intact eosinophils but also eosinophil degranulation in areas with nominal numbers of intact eosinophils. Moreover, our examination of both adult and pediatric patients showed that extensive degranulation in the biopsies was nearly always associated with EoE, whereas GERD patients displayed lower levels of degranulation.

Anti-Eosinophil Peroxidase Monoclonal Antibody–Based Immunohistochemistry and the Development of a Strategy to Evaluate Eosinophilic Esophagitis Versus Gastroesophageal Reflux Disease Patients

EPX-mAb–based immunohistochemistry of mid-proximal biopsies (>7 cm from the esophageal-gastric junction) from EoE patients (group I), GERD patients (group II), and control subjects (group III) allowed for the identification of several EPX-mAb–based histopathologic markers that correlated with disease pathologies (see Methods and Supplementary Materials and Methods). No observer-to-observer variations were observed in 3 of the 4 identified EPX-mAb–based histopathologic markers, with only minor variations in the counting of intact eosinophils (<5%) observed among the 4 evalua-

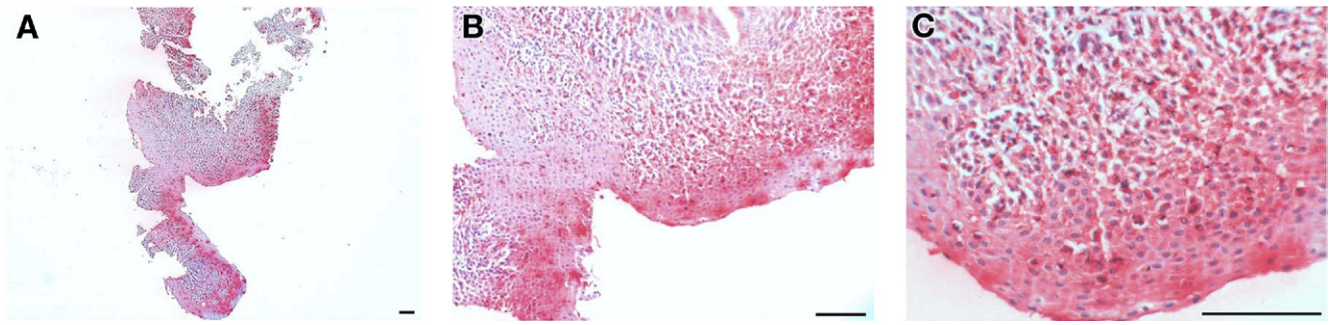

Figure 2. EPX-mAb–based immunohistochemistry represents a novel strategy to detect eosinophil degranulation and the presence of released EPX bound to tissue extracellular matrix. (*A*) Low (5×, 20 mm^2 field of view), (*B*) medium (16×, 1.8 mm^2 field of view), and (*C*) high (40×, 0.29 mm^2 field of view) microscopic fields of a proximal esophageal biopsy from an EoE patient demonstrate the utility of EPX-mAb–based immunohistochemistry to detect eosinophil degranulation (ie, EPX bound to extracellular matrix) in these biopsies. The results with this biopsy are representative of EoE patients who often display significant eosinophil degranulation. Scale bar = 100 μm.

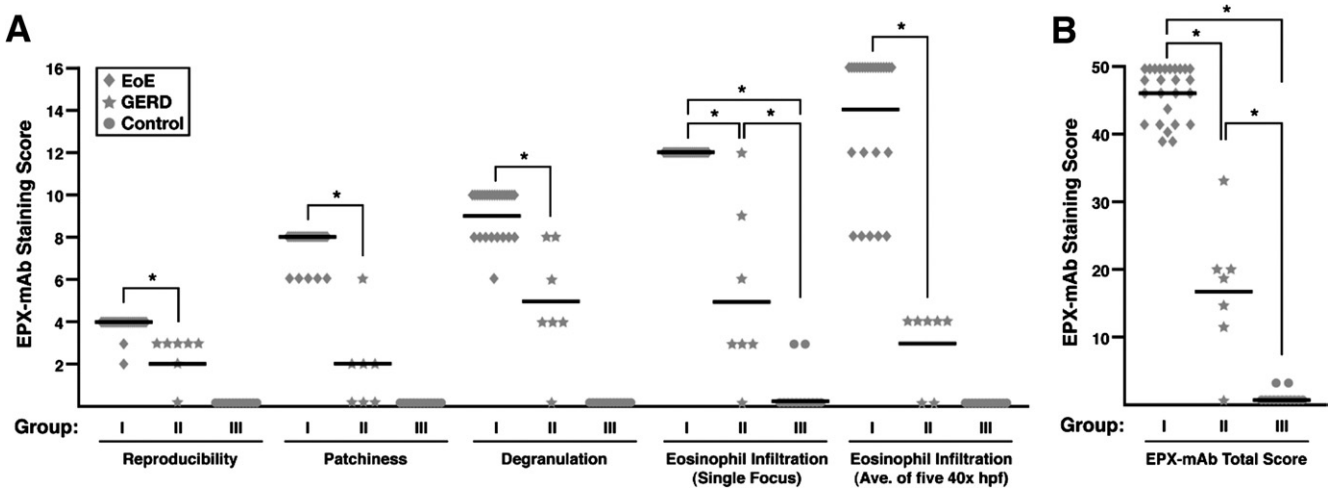

Figure 3. EPX-mAb–based immunohistochemistry provides a quantitatively significant strategy (Supplemental Table 3) to distinguish EoE vs GERD patients. (*A*) Examination of the scores for individual EPX-mAb–based parameters associated with the EoE (group I), GERD (group II), and control patients (group III) found in Supplemental Table 2 demonstrated statistical differences (*$P < .001$) for each of the parameters comprising the EPX-mAb–based algorithm. (*B*) Statistical assessments (ANOVA with Tukey) of the average total EPX-mAb–based staining scores (means ± SEM) for the EoE, GERD, and control patients from Supplemental Table 2 demonstrated the utility of this algorithm to distinguish between these patient populations (*$P < .001$).

tors; in no case did these variations lead to different EPX-mAb–based scores for given patient. However, the significantly increased sensitivity afforded by EPX-mAb–based staining is noteworthy and represents a significant improvement over existing abilities. Specifically, 2 board-certified pathologists each counted eosinophils from serial sections of biopsies, one of which was stained with hematoxylin-eosin to determine an average eosinophil count/40× HPF (average of 10–20 HPF). The paired serial section was stained via EPX-mAb–based immunohistochemistry, and in a blinded fashion, the same pathologists determined an average eosinophil count/40× HPF (average of 10–20 HPF). These assessments showed that EPX-mAb–based immunohistochemistry was able to detect >4-fold more eosinophils relative to inspection of hematoxylin-eosin–stained slides. The EPX-mAb–based assessments of adult and pediatric patients are shown in Supplementary Table 2, and summations of these scores are presented in Figure 3. These results showed that with one exception, the EPX-mAb–based assessments were consistent with the previously established clinicopathologic diagnosis based on tissue histopathology. In addition, statistically different EPX-mAb–based scores ($P < .001$) were observed between EoE, GERD, and control patients for each of the markers comprising the numeric algorithm (Figure 3*A, B*). EPX-mAb scores showed that most control subjects displayed total scores of 0, with no control patients exceeding a score of 4. In contrast, EoE patients were uniformly identified ($P < .001$) on the basis of scores at the other end of the scale within the range of 36–50. The EPX-mAb–based scores also permitted identification of GERD patients ($P < .001$) relative to either control or EoE patients, with each of these GERD subjects scoring in the range of 5–35 (Supplementary Table 2 and Figure 3*B*). Thus, EPX-mAb–based histopathologic scoring not only identified EoE patients but also provided qualitative measures and a quantitatively significant ($P < .001$) means to distinguish EoE patients from patients with GERD. Significantly, our assessments of a well-characterized cohort of children with histologic evidence of EoE, GERD, and otherwise unremarkable control tissues demonstrated that EPX-mAb–based immunohistochemical staining patterns replicated those observed in adults.

Anti-Eosinophil Peroxidase Monoclonal Antibody–Based Immunohistochemistry Provides a Unique Ability to Identify Indeterminate Patients Who Fail to Achieve an Unambiguous Diagnosis With Current Clinicopathologic Guidelines

The utility of these quantitative assessments with difficult-to-diagnose patients was demonstrated in subjects with a likely diagnosis of EoE but who nonetheless failed to achieve the prerequisite guideline of at least a single focus of ≥15 eosinophils/40× HPF (group IV). Histologic assessment of these patients' epithelium by using EPX-mAb–based immunohistochemistry in some cases revealed scores within the range of subjects with EoE as identified in group I (Supplementary Tables 1 and 2) or scores less than 36 (but ≥5) and were thus within the diagnostic range of GERD patients (group II [Supplementary Tables 1 and 2]). In particular, assessments with EPX-mAb–based scoring of a cohort of indeterminate pediatric patients (Supplementary Tables 1 and 2) demonstrated that most of these patients (75%) achieved scores supporting a diagnosis of EoE (ie, an EPX score of 36–50). However, perhaps as many as 25% of these children had an EPX-mAb–based score consistent with a GERD diagnosis (ie, EPX-mAb score of 5–35). Unfortunately, clinical follow-up assessments were not available, preventing us from correlating final clinical outcome of these patients with their initial EPX-mAb–based diagnosis.

The availability of clinical follow-up assessments of many of our adult indeterminate patients demonstrated the difficult-to-diagnose character of these esophageal patients exclusively on the basis of existing clinicopathologic criteria. Figure 4 represents serial slides (hematoxylin-eosin vs EPX-mAb–based immunohistochemistry stained slides) from 2 problematic adult patients (ie, patients no. 44 and no. 46 from Supplementary

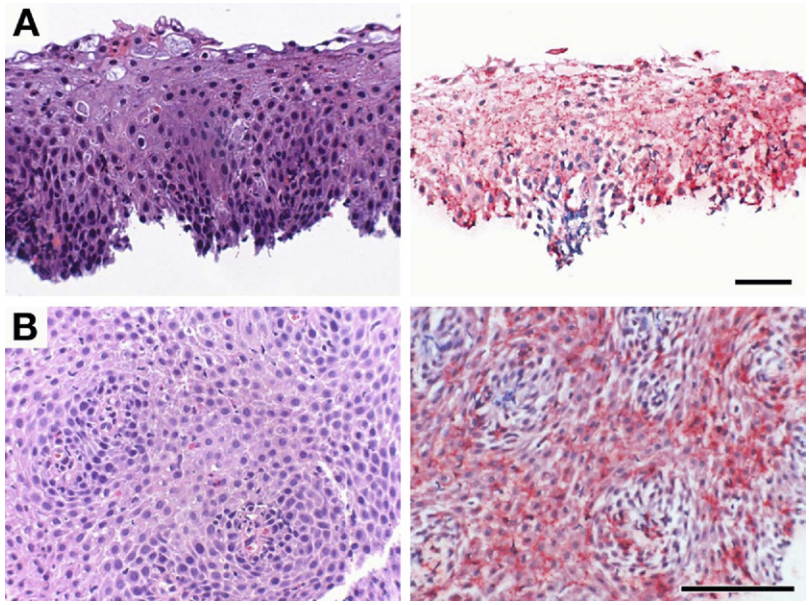

Figure 4. EPX-mAb–based immunohistochemistry permits a diagnosis of EoE among patients with appropriate clinical symptoms and borderline endoscopic/histologic results but who fail to achieve the current guideline recommendations of at least a single focus of ≥15 eosinophils/40× HPF among the available biopsies. Serial sections of proximal esophageal biopsies from (*A*) patient no. 44 and (*B*) patient no. 46 (Supplemental Tables 1 and 2) were either stained with hematoxylin-eosin (*left panels*) or subjected to EPX-mAb–based immunohistochemistry (*right panels*) and photographed at high (40×, 0.29 mm^2 field of view) power. Although both patients had failed to meet traditional pathology guidelines for a diagnosis of EoE, EPX-mAb–based immunohistochemistry detected the presence of extensive eosinophil degranulation in the absence of ≥15 intact eosinophils/40× HPF (0.29 mm^2 field of view), elevating their EPX-mAb–based total score within the range indicating an EoE diagnosis. Scale bar = 50 μm.

Tables 1 and 2). These data demonstrate that although both traditional pathologic assessment and EPX-mAb–based immunohistochemistry each failed to detect the guideline required focus of ≥15 eosinophils/40× HPF, EPX-mAb–based immunohistochemistry revealed significant areas of eosinophil degranulation. Thus, as noted earlier, the EPX-mAb scoring provided additional histologic support for the diagnosis of EoE (ie, an EPX-mAb score of 36–50). Indeed, follow-up assessments of these patients revealed that patient no. 44 responded to corticosteroid treatment, whereas patient no. 46 has thus far failed to display symptomatic improvement during an extended period of treatment with proton pump inhibitors. In contrast, another indeterminate adult patient (patient no. 45) was identified as a GERD patient on the basis of this subject's EPX-mAb–based score, and follow-up assessments demonstrated that this patient became symptom-free after treatment with a proton pump inhibitor. Moreover, follow-up EPX-mAb–based immunohistochemistry on biopsies on several other GERD patients responding to proton pump inhibitor–based therapies demonstrated that the EPX-mAb scores of these patients were reduced to the control range of <5 (data not shown). These data highlight both the difficulties associated with identifying some esophageal patients solely on the basis of existing diagnostic guidelines and the utility of EPX-mAb–based immunohistochemistry as a strategy to resolve difficult and/or ambiguous cases.

Discussion

The EPX-mAb–based immunohistochemical assay described in this report represents a novel tool and systematic method to assess esophageal tissues for evidence of eosinophilic inflammation in both children and adults. The previously limited availability of eosinophil-specific antibody staining options for immunohistochemical assays that are sensitive, reproducible, and useful in the most commonly available format (ie, archived formalin-fixed paraffin-embedded tissues) had prevented the development and use of this strategy to evaluate patients. However, development of an eosinophil granule protein–specific monoclonal antibody, together with a novel histologic scoring system, allowed for not only the specific detection of eosinophils and eosinophil degranulation but also provided a sensitive and cost-effective method that specifically identified patients with EoE versus GERD on the basis of a single histologic evaluation. In addition, it allowed for the differentiation of indeterminate patients in whom the diagnosis of EoE was not certain.

This study assessed children and adults with previously established diagnoses and not consecutive patients. In turn, this might have led to a selection bias, ultimately leading to either higher EPX-mAb–based scores for patients with EoE or lower EPX-mAb–based scores for GERD and/or control subjects. However, in this rapidly evolving field, the utility of our retrospective analysis of very well-defined patients has proved to be quite valuable, especially in light of the fact that a number of challenges have led to difficulty in obtaining a true diagnosis of EoE.[4,8] As a consequence, we propose the current scoring system as an initial effort to evaluate tissues that can now be used to prospectively study consecutive patients with esophageal diseases.

These findings address several critical and timely unmet needs in the care of patients who display eosinophilic esophageal inflammation. (1) The use of this EPX-mAb–based scoring system allowed for the rapid identification of patients with EoE. (2) Esophageal eosinophilia as a pure numeric value of intact eosinophils was improved by the additional sensitivity of EPX-mAb–based immunohistochemical detection of tissue infiltrating eosinophils. This issue was highlighted in our assessments of indeterminate pediatric patients, in which we found that although traditional hematoxylin-eosin histopathologic assessments failed to reveal a focus of ≥15 eosinophils/40× HPF, 75% of these cases displayed this guideline prerequisite as determined by EPX-mAb–based immunohistochemistry. The sensitivity of this strategy was further enhanced by expanding the term *eosinophilia* to include assessment and quantification of eosinophil degranulation. (3) Because an increasing body of evidence suggests that tissues from patients with GERD might have a similar number of esophageal eosinophils as with EoE, this

system allowed for differentiation between these 2 diseases.[1,3] The inclusion of multiple EPX-mAb–based histologic scoring parameters provided not only a statistically significant quantitative means by which to identify EoE patients but also qualitative measures that appear to reliably differentiate these subjects from patients with GERD. In particular, a subset of EPX-mAb–based parameters might be sufficient to achieve a quick, but accurate, differential diagnosis between these esophageal diseases. For example, 24 of 26 EoE patients displayed extensive degranulation in multiple biopsies compared with only 2 of 7 biopsies from GERD patients showing a similar level of degranulation. In addition, low-power assessments of the fraction of patient biopsies displaying eosinophil degranulation (reproducibility) and the fractional area of the maximally affected tissue fragment that displayed evidence of degranulation (patchiness) together were alone sufficient to accurately identify EoE patients relative to subjects with GERD. (4) Indeterminate diagnoses were clarified with EPX-mAb–based immunohistochemistry. This strategy allowed for a diagnosis of EoE and GERD in patients who achieved many, but not all, of the clinical, endoscopic, and histopathologic features of these diseases. Indeed, our study provides examples of indeterminate EoE patients who nonetheless failed to achieve the prerequisite ≥15 eosinophils/40× HPF.

It is interesting that some EoE and GERD patients had biopsies with many intact eosinophils but minimal levels of degranulation (eg, patients no. 6 and no. 10 and patients no. 27 vs no. 29 [Supplementary Table 2], respectively), whereas other patients displayed widespread degranulation in the presence of very few intact eosinophils (eg, patients no. 44, no. 46, and no. 47). These observations suggest that the degranulation detected in the tissue sections of this study was specific/unique to individual patient biopsies and unlikely to be a consequence of artifactual events associated with tissue processing and/or handling.[9] Thus, although current guidelines propose that identification of ≥15 eosinophils/40× HPF (in the proper clinical/endoscopic context) is required for the diagnosis of EoE, the EPX-mAb–based scoring system's utilization of multiple parameters such as eosinophil degranulation provides an alternative diagnostic strategy for pathologists/clinicians.

EPX-mAb–based immunohistochemical evaluations address long-held technical issues related to assessment of eosinophil degranulation. Previous studies of a limited number of subjects identified that the mucosa affected by EoE displayed enhanced eosinophil degranulation (ie, deposition of eosinophil-derived neurotoxin[10] [EDN] or major basic protein[9] [MBP]) relative to GERD patients. However, issues arise with the use of antibodies reactive to each of these granule protein constituents. For example, although EDN is a prominent eosinophil secondary granule protein (ESGP), several studies have demonstrated that unlike EPX, EDN is not eosinophil-specific and is expressed in both other leukocytes (eg, neutrophils[11]) and tissue/organs (e.g, liver[12]). In addition, the cationic character of MBP, together with its propensity to "stick" to virtually any substratum as well as its near insolubility in environments at neutral pH, has been problematic issues that might have biased earlier studies by artifactually limiting the extent of observable areas of degranulation. Moreover, these intensely staining local aggregates might also have been responsible for the perception that tissue handling/processing in earlier studies led to eosinophil degranulation.[9] In contrast, the nominal cationic character of EPX (isoelectric point, ~8.9[13]) together with its greater solubility at neutral pH would prevent aggregation and allow this granule protein to disperse to a greater extent.

The practical utility of this method lies in its simplicity, durability, and cost-saving features. Immunohistochemical staining with EPX-mAb is straightforward, can be performed on archived formalin-fixed paraffin-embedded tissues, and requires no unique technology. The assessment and scoring evaluations of the EPX-mAb–based staining patterns in children and adults were reproducible, as evidenced by negligible interobserver variability. The added time and cost for this assessment will be minimal when taken in the context of achieving the correct diagnosis in a rapid time frame. For example, current diagnostic guidelines support the strategy that a diagnosis of GERD must be ruled out as a cause for esophageal eosinophilia before assigning a diagnosis of EoE. In this light, a patient would need to undergo either 2 months of proton pump inhibition or a pH/impedance monitoring of the distal esophagus, both of which can be time-consuming, costly, and potentially uncomfortable. However, in the context of a preponderance of clinical symptoms, endoscopic results, and evaluations of histopathology, the EPX-mAb–based scoring algorithm could provide a quantitatively definitive evaluation of initial biopsies permitting an immediate diagnosis of EoE, including potentially the differentiation between difficult-to-diagnose EoE versus GERD patients (ie, indeterminate cases). Despite guideline-driven diagnoses that had been unclear, EPX-mAb–based immunohistochemistry appears capable of identifying subsets of patients as either EoE or GERD. These observations suggest either an overlap between these 2 types of esophageal patients on the basis of clinicopathologic findings, or that a unique subset of EoE patients are instead simply difficult-to-diagnose GERD patients. In summary, we anticipate that validation and future use of this system will improve care of children and adults and allow for greater understanding of esophageal inflammatory diseases.

Supplementary Data

Note: to access the supplementary materials accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at doi:10.1016/j.cgh.2009.03.022.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Mice

IL-5 transgenic mice (NJ.1638[1]) were used as the source of peripheral blood eosinophils and, in turn, eosinophil secondary granules from which EPX was purified. In addition, gene knockout mice deficient of EPX were used in the production of anti-EPX monoclonal antibodies. The mice were maintained in ventilated micro-isolator cages housed in the specific pathogen-free animal facility at Mayo Clinic Arizona. Protocols and studies involving animals were carried out in accordance with National Institutes of Health and Mayo Foundation institutional guidelines.

Isolation of Mouse Eosinophils and Eosinophil Secondary Granules

Eosinophils and, in turn, eosinophil secondary granules were purified from peripheral blood and spleens harvested from 4-month-old IL-5 transgenic mice. Specifically, 1–1.2 mL of peripheral blood (>100,000 eosinophils/mm^3, representing 50% of total white blood cells) was collected (20 U/mL heparin was added to prevent clotting) from mice via cardiac puncture and stored on ice until use. After resection, spleens were diced into small sections and sheared through 18-, 20-, and 22-gauge needles in PBS containing 20 U/mL heparin and filtered through a 40-μm nylon mesh (Cat. no. 35-2340; Becton Dickinson, Franklin Lakes, NJ).

Peripheral blood and disassociated splenocytes were pooled, and the red blood cells were lysed in ice-cold distilled water for 20 seconds before low-speed centrifugation (1000*g*, 10 minutes at 4°C) to collect intact cells. This lysis cycle was repeated as necessary to remove all evidence of red blood cell contamination (usually 2–3 times). The final preparation of leukocytes was resuspended in the RPMI 1640/5% fetal calf serum, and total cell counts were acquired by using a hemacytometer; cell differentials were performed from cytospin preparations counting >300 cells. The recovered leukocytes were overlaid onto Percoll E as described previously, and the buffy coats from these discontinuous gradients were harvested.[2] These buffy coat cells were washed with PBS/2% bovine serum albumin, incubated with antibodies recognizing CD90 and CD45R for 15 minutes at 4°C (10 μL antibody/10^7 cells; Miltenyi Biotec, Inc, Auburn, CA), and eosinophils were purified to >95% homogeneity by using deletion chromatography on an MACS immunomagnetic separation column as per the manufacturer's instructions (Miltenyi Biotec, Inc). Purified eosinophils recovered after MACS were lysed at 37°C for 30 minutes by incubation in 0.5 mL of 0.25 mol/L sucrose containing heparin (10,000 U/10^8 cells) and DNase I (100 U/10^8 cells). Eosinophil secondary granules were subsequently harvested by high-speed centrifugation of these lysed leukocytes (12,000*g* at 4°C for 20 minutes).

Purification of Eosinophil Peroxidase

Granules derived from ~10^9 purified eosinophils were resuspended in 10^{-4} mol/L HCl, and the granules were disrupted by probe sonication with a Branson 450 sonifier (Branson Ultrasonics, Danbury, CT) at a constant duty cycle setting with a pulse of 30 seconds. The pH of the resulting sonicate was assessed with pH paper, and 5–10 μL of 10^{-2} mol/L HCl was added before repeating the sonication of the granules. If necessary, this process was repeated a third time or until the pH of the granule lysate reached 3.5. This acidified suspension was centrifuged at 8000*g* (20 minutes at 4°C) to remove any acid insoluble proteins/debris. The supernatant from this centrifugation represents the collective sum of the cationic ESGPs from which EPX was purified.

Protein concentration of the ESGPs was determined by BCA assay (Pierce Endogen, Inc, Rockford, IL), and an aliquot of the ESGPs (20 mg) was salt-precipitated (4°C for 1 hour) by the addition of ammonium sulfate ($(NH_4)_2SO_4$) to a concentration of 10%. EPX and other precipitated proteins were recovered by centrifugation (5000*g* for 20 minutes at 4°C). These ammonium sulfate–precipitated proteins were resuspended in 0.025 mol/L sodium acetate (NaOAc) pH 5.0 in preparation for size selection with Sepharose G-50 chromatography. EPX and several contaminating proteins within the void volume of the G-50 column were subsequently subjected to high-performance liquid chromatography by using a CM preparative column. EPX (~70 kd) was uniquely recovered (as judged by silver-stained PAGE) by elution from this column with an increasing gradient of NaCl in 0.025 mol/L NaOAc pH 5.0.

Monoclonal Antibody Production and Screening

Monoclonal antibodies recognizing mouse EPX were generated by repeated sensitization of EPX knockout mice ($EPX^{-/-}$)[3] by using established methods and/or protocols.[4] Briefly, $EPX^{-/-}$ mice (C57BL/6J background) were immunized (by intraperitoneal injection) a total of 4 times at 2- to 4-week intervals with 25 μg of purified mouse EPX (injection emulsified in RIBI adjuvant; RIBI ImmunoChem Research Inc, Hamilton, MT). Antibody titers were assessed from blood recovered from the tail vasculature. Immunized mice received a final intravenous injection of 25 μg of purified EPX 3–4 days before recovery of spleens and generation of antibody secreting hybridomas (Myeloma Fusion Partner, P3X63-Ag8.653; ATCC, Manassas, VA).

Potential hybridomas resulting from the immunization of $EPX^{-/-}$ mice with purified mouse EPX were screened by using a stepwise strategy (~2000 total hybridomas). (1) An initial enzyme-linked immunosorbent assay (ELISA)–based screen selected for IgG-secreting cells (470 IgG^+ hybridomas). (2) By using purified EPX and purified eosinophil MBP, all IgG-positive hybridomas were subsequently screened by ELISA for reactivity to EPX and the lack of a response to MBP (130 $IgG^+/EPX^+/MBP^-$ hybridomas). (3) Randomly selected hybridomas were cloned by limiting serial dilution (20 hybrid-

omas). (4) These mouse monoclonal anti-mouse EPX antibodies were finally screened on the basis of their abilities to function in mouse sample-based assays such as immunohistochemistry, Western blots, and ELISA and then on their abilities to detect human EPX in both immunohistochemical and ELISA formats (7 total hybridomas achieved these criteria). Hybridoma MM25-82.2.1 was identified on the basis of its robust responses in the assays outlined above and was selected for the studies presented.

Tissue Preparation and Slide Production

Esophageal biopsies were fixed in 10% formalin and embedded in paraffin. Four-micrometer-thick sections were obtained for traditional histopathology (eg, staining with hematoxylin-eosin) and immunohistochemistry by using EPX-mAb. Hematoxylin-eosin sections were analyzed for numbers of inflammatory cells including eosinophils and neutrophils in up to 10 HPFs, eosinophilic microabscesses (aggregates of $\geq$4 eosinophils), presence or absence of intercellular edema, basal zone hyperplasia $>$20% of the epithelial thickness, lamina propria papillae elongation to $>$2/3 of the epithelial height, lamina propria eosinophils, and lamina propria fibrosis. Eosinophils were counted in the areas in which they appeared most numerous. For all cases of histologic EoE, accompanying clinical data and gastric and duodenal biopsies when available were reviewed to exclude eosinophilic gastroenteritis and other inflammatory bowel diseases.

Immunohistochemistry Protocol

Infiltrating intact eosinophils and evidence of eosinophil degranulation (ie, the presence of free cytoplasmic granules and/or extracellular matrix deposition of ESGPs) were assessed by immunohistochemistry by using the mouse monoclonal EPX-mAb noted above (MM25-82.2.1). Immunocytochemical staining was performed with Dako detection/visualization reagents purchased from Dako Cytomation (Carpinteria, CA). Positive control slides (eosinophil-containing sections from patients identified by traditional pathologic assessments) and negative control slides (both antibody isotype controls and negative tissue control sections) were included as part of the processing of each group of slides examined. It is noteworthy that this staining protocol was designed specifically for use in typical academic/hospital histology units, requiring no specialized equipment or technical insights beyond those currently available in these settings. Tissue sections were dried in an oven at 65°C for 60 minutes before deparaffinization, rehydration, and target retrieval. This extended drying time is necessary to ensure maximum adherence of esophageal tissue to the slides. Subsequent to this drying step, the tissue sections were deparaffinized in xylene (3 changes in fresh xylene for 5 minutes each) before rehydration in a descending series of ethanol/H_2O slide baths. Antigen retrieval[5] was performed on the rehydrated sections at 125°C for 30–60 seconds at a pressure of 17–23 psi by using a Decloaking Chamber (as per the manufacturer's instructions; Biocare Medical, Concord, CA; Cat No. DC2002) and 1× Dako Target Retrieval Solution (Dako; Cat. no. S1699). After antigen retrieval, the slides were rinsed with deionized water and incubated at room temperature in Dako Cytomation Proteinase K (Dako; Cat no. S3020) for 5 minutes (~200 μL/slide). Proteinase K digested slides were subsequently rinsed (3 times for 5 minutes each) with 1× Dako Wash Buffer (Dako Cytomation; Cat. no. S3006) before blocking the slides in preparation of antibody staining with a 10-minute incubation (~200 μL/slide) in Dako Dual Endogenous Blocking Solution (Dako Cytomation; Cat. no. S2003). The blocked slides were rinsed (1 time for 5 minutes) with 1× Dako Wash Buffer. These slides were then incubated (40 minutes) with EPX-mAb at a concentration of 10 μg/mL. Specifically, EPX-mAb (1 mg/mL) was diluted 1:100 (~200 μL/slide) with Dako antibody diluent with background reducing components (Dako Cytomation; Cat. no. S3022). Negative control slides were stained with IgG2a mouse antibodies (10 μg/mL) derived from antigen-naive wild-type mice (Dako Cytomation; Cat. no. X0943) diluted again with Dako antibody diluent with background reducing components. The antibody-stained slides were rinsed (3 times for 5 minutes each) with 1× Dako Wash Buffer before application of a secondary visualizing biotinylated antibody/streptavidin–alkaline phosphatase conjugate (Dako Cytomation LSAB 2 System, Cat. no. K0674) as per the manufacturer's instructions. Specific EPX-mAb–based staining was visualized with a 10-minute incubation with Permanent Red substrate-chromogen (Dako Cytomation; Cat. no. K0695) followed by a single rinse with distilled water. Permanent Red visualized slides were counterstained (approximately 1 minute) with methyl green (Dako Cytomation Ready-to-use Methyl Green; Cat. no. S1962), rinsed in free-flowing deionized water, and air-dried. Stained slides were dipped once quickly in xylene and coverslipped with Consul mount/xylene mounting media (Shandon Cat. no. 9990441; Thermo Scientific, Pittsburgh, PA) before photomicroscopy with a Zeiss Axiophot microscope and an AxioCam MRc5 digital camera.

Eosinophil Peroxidase Monoclonal Antibody Production

Monoclonal antibodies reactive to mouse EPX were generated as part of ongoing studies evaluating the role(s) of eosinophils in various disease settings (eg, asthma and other allergic disorders) as well as assessments of mouse models of human diseases. EPX was purified from peripheral blood and spleen-derived eosinophils of IL-5 transgenic mice (NJ.1638[1]) as described in the Materials and Methods and outlined in Supplementary Figure 1. Typically, several hundred micrograms of purified EPX was recovered from the blood/spleen of 10 transgenic animals.

Previous attempts to generate mouse monoclonal and/or rabbit polyclonal antibodies with a high degree of reactivity/specificity to either human or mouse EPX that are also useful in a variety of detection platforms (eg, immunohistochemistry, Western blots) have been problematic (our unpublished observations), most likely, in part, because of the extraordinary sequence identity that these proteins display among mamma-

lian species (eg, >94% amino acid identity between mouse and human EPX).[6] We used the novel experimental strategy of sensitizing knockout mice devoid of EPX with purified EPX as a means of increasing the immunogenicity of this protein and maximizing the number of available epitopes. This strategy resulted in several hundred antibody-producing hybridomas that met 2 basic selection criteria: they secreted monoclonal antibodies that displayed reactivity to mouse EPX in a single antibody ELISA format, and the monoclonal antibodies were of the IgG subtype. These antibodies were further screened in a stepwise strategy for their utility in detection formats with samples derived from mouse models of disease and, subsequently, their applicability in assays with clinical samples derived from patients. Thus, the several hundred hybridomas that were initially generated and preliminarily characterized were further selected to a group of 7 monoclonal antibodies on the basis of their utility in multiple detection formats in the mouse including immunohistochemistry, Western blots, and ELISA and their ability to display cross-reactivity with human EPX in similar assays. A single mouse monoclonal antibody from this selected group (EPX-mAb; MM25-82.2.1) was chosen for further study and used for the histologic studies of this report.

Validation of Eosinophil Specificity in Clinical Biopsies

Although our screening strategy generated mouse anti-mouse EPX monoclonal antibodies with cross-reactivity to human EPX, this strategy did not address questions of eosinophil specificity in clinical samples or the sensitivity of immunohistochemical assays with this monoclonal antibody. The specificity and sensitivity of an EPX-mAb–based immunohistochemical assay for human tissue samples were determined through the assessment of bone marrow biopsies derived from patients (processed via several different fixatives and postbiopsy methods [eg, plus or minus decalcification]) who presented with unique marrow disorders (eg, lymphoma, idiopathic eosinophilic syndrome, mastocytosis, and myeloproliferative disease [data not shown]). Supplementary Figure 2 presents a representative assessment of the marrow from patients with yet another of these marrow disorders, myelodysplastic syndrome. Marrow clot biopsies from myelodysplastic syndrome patients were fixed with formalin before paraffin embedding and the generation of 4-μm sections. Marrow sections were stained for histologic assessments followed serially by EPX-mAb–based immunohistochemistry. A representative hematoxylin-eosin–stained section is presented in Supplementary Figure 2*A*. This section displays the multitude of leukocyte subtypes characteristic of these biopsies, including megakaryocytes, neutrophils, eosinophils (*arrowheads*), mast cells, and mononuclear leukocytes such as lymphocytes and monocytes. Subsequent to the staining and photographing of this hematoxylin-eosin section, the coverslip was removed after a brief immersion in xylene, and the hematoxylin-eosin was removed through the decloaking process associated with antigen retrieval. The destained slide was then subjected to EPX-mAb–based immunohistochemistry, and the region of the slide previously examined by hematoxylin-eosin staining was once again photographed (Supplementary Figure 2*B*). This strategy revealed the following: (1) Eosinophils identified by hematoxylin-eosin staining also stained positive after EPX-mAb–based immunohistochemistry (eg, compare the leukocytes with *arrowheads* in *panels A* vs *B*). (2) Positive staining by EPX-mAb immunohistochemistry was limited only to eosinophils, with no detectable signal derived from any of the other cell types present in the section. (3) The EPX-mAb–based immunohistochemical assay displayed a significant level of sensitivity, with the identification of eosinophils possible with a 1:100 dilution of a 1 mg/mL EPX-mAb stock (ie, final working concentration of 10 μg/mL).

Development of a Numeric Algorithm for the Diagnosis of Eosinophilic Esophagitis

EPX-mAb–based immunohistochemistry of mid-proximal biopsies (>7 cm from the esophageal-gastric junction) from EoE patients (group I), GERD patients (group II), and normal control subjects (group III) allowed for the identification of several EPX-mAb–based histopathologic markers that correlated with disease pathologies. Beyond the simple appearance of these markers in EoE patients (and to some extent GERD), quantitative differences were also observed, suggesting a hierarchy whereby some markers were more likely to be associated with disease than others. In an attempt to stratify esophageal patients, a scoring system was developed with which to identify subjects with EoE (Supplementary Table 3). The resolution of the scoring strategy developed was designed low enough to negate/minimize observer-to-observer variability. Specifically, the observed magnitude of the diagnostic markers examined was scored on a scale of 1–4; each marker was further assigned a 1–4 priority factor (see detailed descriptions in Supplementary Materials and Methods) on the basis of the frequency by which they were observed in EoE patients diagnosed by traditional pathologic assessments (ie, previously established diagnostic guidelines). These assessments have been incorporated into a quantitative algorithm that permits a histologic scoring strategy for patient diagnosis. The scoring system developed is based on a scale of 0–50, the extremes of which are representative of the esophagi of control subjects and severe EoE patients, respectively. The histopathologic scoring of the slides was performed independently by 2 research lab–based staff and 2 hospital/clinic-based colleagues, including a senior pathologist with a specialty in gastrointestinal diseases.

Anti-Eosinophil Peroxidase Monoclonal Antibody Diagnostic Marker 1: Presence or Absence of Infiltrating Tissue Eosinophils

Similar to traditional histopathologic assessments, evidence of eosinophils infiltrating the epithelial areas of esophageal biopsies by using EPX-mAb–based immunohistochem-

istry (Figure 1) was consistently observed among the EoE patients we have examined. Our quantitative assessments of EoE patients identified 2 parameters that correlated with disease as determined by the current guidelines: the maximal number of eosinophils in a single focus within the available biopsies from a patient and the average number of eosinophils/40× HPF as determined from 5 selected areas within the available biopsies.

Maximum in a single focus. Patient biopsies were scanned at low (5×, 20 mm^2 field of view) power to identify foci associated with elevated numbers of accumulating eosinophils. Selected areas were chosen, and the number of intact eosinophils/40× HPF was determined. (An intact eosinophil is either an EPX^+ leukocyte or an EPX^+ cellular fragment associated with a morphologically identifiable eosinophilic nucleus.) The sample was assigned a maximum in a single focus score on the basis of the number of intact eosinophils present as follows: 0, <2; 1, 2–5; 2, 6–10; 3, 11–14; 4, ≥15. Similar to traditional histopathologic assessments, any patient who displayed ≥15 eosinophils/40× HPF by using EPX-mAb–based immunohistochemistry was in fact an EoE patient. However, GERD patients also potentially displayed this marker, prompting us to differentially weight the importance of this parameter by assigning a priority factor of 3 and thus create an EPX intact eosinophils single focus score range of 0–12.

Average of five 40× (HPF) fields. A total of 5 designated 40× fields were examined, and the number of intact eosinophils present was determined. (Designated fields are defined as microscopic fields at the center of the 5 largest biopsies available from a given patient. In cases with less than 5 available biopsies, designated fields are defined as microscopic fields at the center of the available biopsies, with additional fields included as needed to a total of 5. These additional fields are taken from the largest available biopsy, starting first in the upper left quadrant and successively moving in a clockwise fashion to the other quadrants of the biopsy as needed. It is noteworthy that these designations of fields are independent of identifying areas of localized concentrations of infiltrating eosinophils and thus are likely to yield average scores that, if anything, might underestimate the density of eosinophils in affected areas.) The average number of intact eosinophils present in 5 fields was determined, and an average of five designated fields score based on these counts was determined: 0, <2; 1, 2–5; 2, 6–10; 3, 11–14; 4, ≥15. This parameter displayed a one-to-one correlation with an EoE diagnosis, prompting us to assign a priority factor of 4, the highest among the parameters examined. This yielded an EPX average intact eosinophils score range of 0–16.

Anti-Eosinophil Peroxidase Monoclonal Antibody Diagnostic Marker 2: Evidence of Eosinophil Degranulation

A unique observation from our studies of EoE patients by using EPX-mAb–based immunohistochemistry was that degranulation was common (Figure 2). The importance of this observation is hard to overestimate because this parameter was independent of identifying intact infiltrating tissue eosinophils. EPX-mAb–based immunohistochemistry detects not only intact eosinophils but also eosinophil degranulation in areas with nominal numbers of intact eosinophils. Moreover, our examination of EoE versus GERD patients showed that extensive degranulation in the biopsies was always associated with EoE, whereas GERD patients displayed lower levels of degranulation. This has led to the identification of 2 parameters that were used as part of our histopathologic scoring algorithm.

Level of degranulation in the maximally affected biopsy. (The maximally affected biopsy is subjectively defined by scanning the available patient biopsies at low [5×, 20 mm^2 field of view] power, identifying the one with the greatest percent area displaying a significant eosinophil infiltration and/or degranulation.) The level of degranulation observed within the maximally affected patient biopsy was determined by scanning 40× HPF (Supplementary Figure 3) by using the following scale: 0 = no identifiable eosinophil infiltration or degranulation observed at high (40×, 0.29 mm^2 field of view) power in any of the available biopsies (identifiable eosinophil infiltration or degranulation is defined as >3 intact eosinophils in at least a single 40× HPF and/or >10% of any single 40× HPF displaying evidence of extracellular deposition of EPX); 1 = the presence of >3 intact eosinophils (ie, eosinophilic microabscesses) in at least a single 40× HPF with no evidence of extracellular release of EPX; 2 = EPX extracellular release is evident in at least a single 40× HPF but is limited to areas surrounding >3 otherwise intact eosinophils; 3 = the presence of enucleated eosinophils (ie, cytoplasmic fragments), the presence of free granules (ie, EPX-containing secondary granules not associated with fragmented eosinophils) and/or nominal evidence of EPX extracellular matrix deposition in at least a single 40× HPF; 4 = biopsies containing at least a single 40× HPF that displayed robust eosinophil degranulation (ie, release of EPX) characterized by the presence of free secondary granules and extensive (>50% of the field) extracellular matrix deposition of EPX. The correlation of this parameter with EoE was not absolute because not all patients with a diagnosis via traditional pathologic assessments displayed the maximal level (ie, level 4), and some GERD patients also displayed the maximal level of this marker.

This prompted us to differentially weight the importance of this parameter by assigning a priority factor of 2 and thus create an EPX degranulation in the maximally affected biopsy score range of 0–8.

Eosinophil degranulation in multiple biopsies. Eosinophil degranulation associated with a given patient was further evaluated on the basis of whether multiple (ie, ≥2) biopsies display at least a single 40× HPF with eosinophil degranulation characterized as level 3 or 4. If yes, the degranulation EPX score was increased by 2 points. If the answer was no, additional degranulation points were not assigned. The inclusion of these additional degranulation points as part of our biopsy evaluations derives from our assessments of EoE patients showing that eosinophil degranulation within multiple biopsies occurred more frequently in EoE (>90% of all cases examined) and thus provided a slightly greater predictive value of this disease relative to degranulation occurring in a single biopsy.

Anti-Eosinophil Peroxidase Monoclonal Antibody Diagnostic Marker 3: Extent of Eosinophil Infiltration and/or Eosinophil Degranulation in the Maximally Affected Biopsy

EPX-mAb immunohistochemistry allowed for the assessments at low (5×, 20 mm^2 field of view) power of entire sets of biopsies (Supplementary Figure 4). This ability facilitated the identification and preliminary characterization of the maximally affected biopsy among the available tissue fragments. Significantly, our preliminary assessments of the maximally affected biopsies from EoE patients demonstrated that the extent to which this biopsy displayed significant eosinophil infiltration and/or degranulation was nominally predictive of disease. This parameter (patchiness) was incorporated in the histopathologic scoring algorithm on the basis of the percent area of the maximally affected biopsy displaying significant eosinophil infiltration and/or degranulation by using the following scale: 0 = <10%; 1 = 10%–24%; 2 = 25%–49%; 3 = 50%–74%; 4 = 75%–100%. Because the maximal level of patchiness (ie, level 4) was common in EoE (ie, >85% of all cases examined) and was never observed in GERD patients, this parameter was predictive of disease. This prompted us to assign a priority factor of 2, yielding an EPX patchiness score range of 0–8.

Anti-Eosinophil Peroxidase Monoclonal Antibody Diagnostic Marker 4: Extent of Eosinophil Infiltration and/or Eosinophil Degranulation Among the Available Patient Biopsies

The ability to scan and identify quickly/efficiently eosinophils and/or degranulation among multiple available biopsies from individual EoE patients provided yet another parameter that appeared to correlate with disease. Specifically, examination at low (5×, 20 mm^2 field of view) power of the available biopsies from patients after EPX-mAb immunohistochemistry (Supplementary Figure 4) often showed that only a fraction of the available biopsies displayed significant eosinophil infiltration and/or eosinophil degranulation, thus representing yet another parameter that correlated with the probability of an EoE diagnosis. This parameter (reproducibility) was incorporated in the histopathologic scoring algorithm as percentage of the available patient biopsies that display significant eosinophil infiltration and/or degranulation by using the following scale: 0 = <1%; 1 = 1%–24%; 2 = 25%–49%; 3 = 50%–74%; 4 = 75%–100%. Similar to the limited extent by which the parameter patchiness was associated with a differential diagnosis of EoE, the parameter reproducibility was common (ie, >80% of all cases examined) in both EoE and GERD. Accordingly, this parameter was assigned a minimal priority factor of 1, yielding an EPX reproducibility score range of 0–4.

This EPX-mAb–based immunohistochemistry scoring system was applied to EoE, GERD, and control subjects. The results from application of this scoring system are summarized in Figure 3 and Supplementary Table 2.

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Supplementary Table 1. Clinical and Endoscopic Assessments as well as Clinicopathologic Diagnoses of Esophageal Study Patients

Patient group (cohort)		Patient number	Age (*y*)	Clinical symptoms	Endoscopic observations	Clinicopathologic diagnosis
I	Adult	1	53	Dys, Imp, Ref	Ery	EoE
		2	37	Imp, Ref	HH, Ery	EoE
		3	72	Dys, Imp, Vom	HH, Ring	EoE
		4	38	Imp, Ref	Unremarkable	EoE
		5	63	Dys, Imp, Ref	Ery	EoE
		6	50	Dys, Imp, Ref	Ery	EoE
		7	38	Dys	Unremarkable	EoE
		8	25	Dys, Ref, Vom, CP	Unremarkable	EoE
		9	26	Dys, Imp, Ref, AP	Ring, HH	EoE
		10	53	Dys, Imp, Ref, Vom	Ring, Fur, Ery	EoE
		11	54	Dys	Ring, Ery	EoE
		12	28	Dys	Str, HH, Ery	EoE
		13	53	Dys, Imp, Vom	Ring, Fur, HH, Ery	EoE
		14	47	Dys, Imp, Ref	Ring, Fur, Ery	EoE
		15	75	Dys, Imp, Ref, CP	Ring, HH, Ery	EoE
		16	32	Dys, Imp, Ref	Unremarkable	EoE
		17	72	Dys, Imp, Ref	Ery	EoE
		18	47	Dys, Imp, Ref, CP	Ery	EoE
		19	54	Dys, Ref, AP	Ring, HH, Ery	EoE
	Pediatric	20	2	Ref, Vom	Fur, Plq	EoE
		21	2	Vom, Feed	Fur	EoE
		22	10	Dys, Ref	Fur, Plq	EoE
		23	9	Vom	Ring, Plq	EoE
		24	2	AP, Vom, Feed	Unremarkable	EoE
		25	2	Feed, Vom, Dys	Unremarkable	EoE
		26	2	FTT	Plq	EoE
II	Adult	27	68	Ref	HH, Ery	GERD
		28	45	Dys, Ref	HH, Ery	GERD
		29	58	Ref, Vom	HH, Ery	GERD
		30	80	Ref	HH	GERD
	Pediatric	31	3	Ref, Vom	Unremarkable	GERD
		32	18	AP, Ref	Ery	GERD
		33	1	Vom	Unremarkable	GERD
III	Adult	34	47	Ref	Ring, HH	Control
		35	52	Dys, Ref, Vom	Unremarkable	Control
		36	57	AP	Unremarkable	Control
		37	51	AP	Unremarkable	Control
		38	64	Dys, Imp, Vom	Unremarkable	Control
		39	78	Dys, Vom	Unremarkable	Control
	Pediatric	40	0.67	Vom	Unremarkable	Control
		41	2	Feed	Unremarkable	Control
		42	9	Ref, AP	Unremarkable	Control
		43	16	AP	Unremarkable	Control
IV	Adult	44	44	Dys, Imp, Vom	Ring, Fur, Ery	Indeterminate
		45	38	Dys, Imp, Ref	Ring, Str, HH	Indeterminate
		46	40	Dys, Ref	Ery	Indeterminate
		47	29	Imp	Ring, Ery	Indeterminate
	Pediatric	48	17	AP	Unremarkable	Indeterminate
		49	4	Feed	Unremarkable	Indeterminate
		50	3	Vom, Ref	Unremarkable	Indeterminate
		51	15	Vom	Unremarkable	Indeterminate
		52	7	AP	Unremarkable	Indeterminate
		53	13	Dys, Imp	Ery	Indeterminate
		54	3	AP	Unremarkable	Indeterminate
		55	13	AP, FTT	Fur	Indeterminate

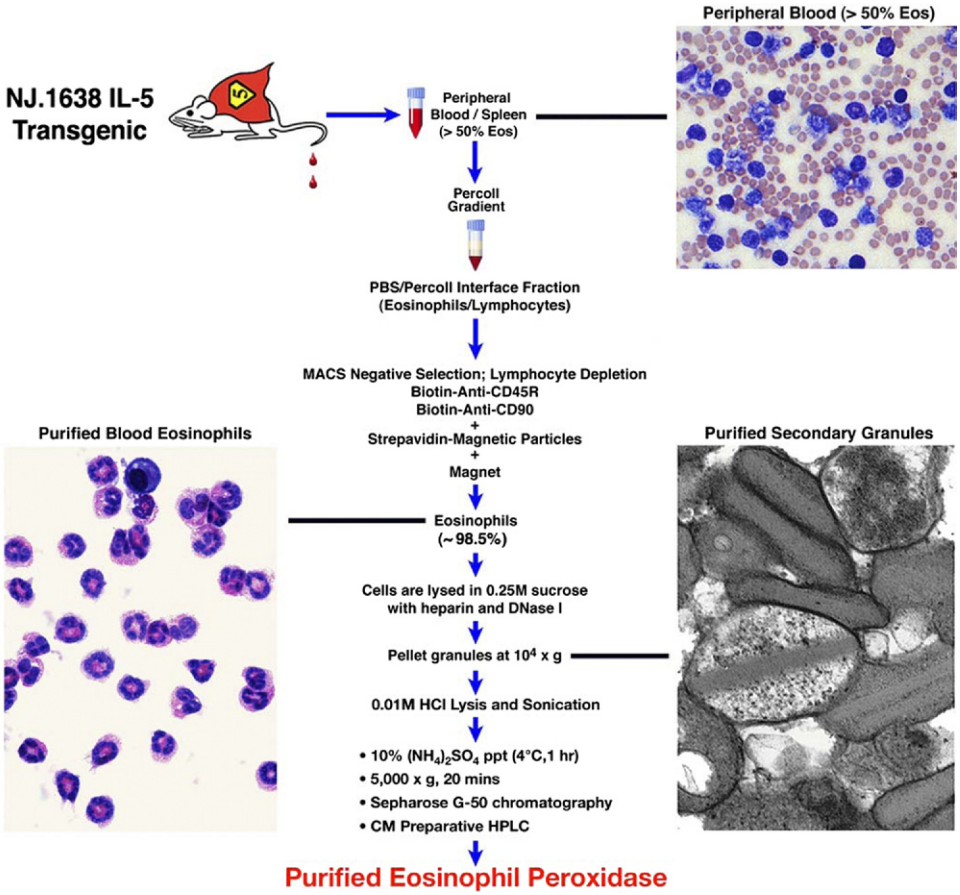
AP, abdominal pain; CP, chest pain; Dys, dysphagia; Ery, erythema; Feed, feeding intolerance; Fur, furrowing; FTT, failure to thrive; HH, hiatal hernia; Imp, impaction; Ring, ring structures; Ref, reflux; Str, strictures; Vom, vomiting; Plq, plaque.

Supplementary Table 2. Intraobserver/Interobserver-Blinded Assessments of Patients by Using EPX-mAb–Based Immunohistochemistry

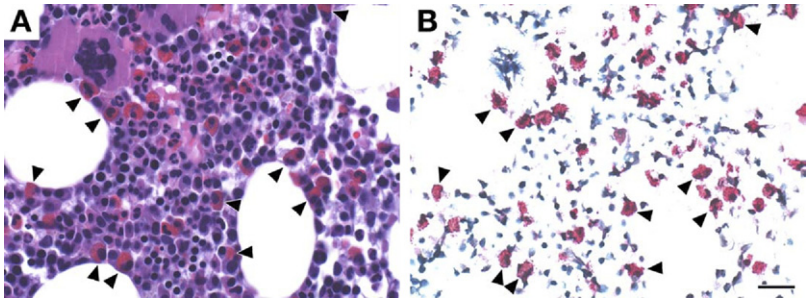
				EPX-mAb staining scores							
						Degranulation (C)					
Patient group (cohort)		Patient number	Traditional pathology diagnosis	Reproducibility (A)	Patchiness (B)	Part 1	Part 2	Eosinophil infiltrate: maximum single focus (D)	Eosinophil infiltrate: average of five designated foci (E)	Total EPX score (A+B+C+D+E)	EPX-based diagnosis
I	Adult	1	EoE	4	8	8	2	12	8	42	EoE
		2	EoE	4	8	8	2	12	8	42	EoE
		3	EoE	4	8	8	2	12	12	46	EoE
		4	EoE	4	6	6	2	12	16	46	EoE
		5	EoE	4	8	8	2	12	16	50	EoE
		6	EoE	3	6	6	0	12	12	39	EoE
		7	EoE	4	8	6	2	12	16	48	EoE
		8	EoE	4	8	8	2	12	12	46	EoE
		9	EoE	4	8	6	2	12	12	44	EoE
		10	EoE	2	6	6	0	12	16	42	EoE
		11	EoE	4	8	8	2	12	16	50	EoE
		12	EoE	4	6	8	2	12	16	48	EoE
		13	EoE	4	8	8	2	12	8	42	EoE
		14	EoE	4	8	6	2	12	16	48	EoE
		15	EoE	4	8	8	2	12	16	50	EoE
		16	EoE	4	8	8	2	12	16	50	EoE
		17	EoE	4	8	8	2	12	16	50	EoE
		18	EoE	4	8	8	2	12	16	50	EoE
		19	EoE	4	8	6	2	12	16	48	EoE
	Pediatric	20	EoE	4	8	8	2	12	16	50	EoE
		21	EoE	4	8	8	2	12	16	50	EoE
		22	EoE	4	8	6	2	12	8	40	EoE
		23	EoE	4	8	8	2	12	16	50	EoE
		24	EoE	4	6	6	2	12	8	38	EoE
		25	EoE	4	8	8	2	12	16	46	EoE
		26	EoE	4	8	6	2	12	16	50	EoE
II	Adult	27	GERD	3	0	4	0	9	4	20	GERD
		28	GERD	2	2	4	0	3	4	15	GERD
		29	GERD	3	6	6	2	12	4	33	GERD
		30	GERD	3	2	6	2	3	4	20	GERD
	Pediatric	31	GERD	3	2	4	0	6	4	19	GERD
		32	GERD	0	0	0	0	0	0	0	Control
		33	GERD	3	0	6	0	3	0	12	GERD
III	Adult	34	Control	0	0	0	0	0	0	0	Control
		35	Control	0	0	0	0	0	0	0	Control
		36	Control	0	0	0	0	0	0	0	Control
		37	Control	0	0	0	0	0	0	0	Control
		38	Control	0	0	0	0	0	0	0	Control
		39	Control	0	0	0	0	0	0	0	Control
	Pediatric	40	Control	0	0	0	0	0	0	0	Control
		41	Control	0	0	0	0	0	0	0	Control
		42	Control	0	0	0	0	0	0	0	Control
		43	Control	0	0	0	0	0	0	0	Control
IV	Adult	44	Indeterminate	4	8	8	2	9	8	39	EoE
		45	Indeterminate	3	4	6	2	6	4	25	GERD
		46	Indeterminate	4	8	8	2	9	8	39	EoE
		47	Indeterminate	4	8	8	2	9	8	39	EoE
	Pediatric	48	Indeterminate	4	6	6	2	12	12	42	EoE
		49	Indeterminate	4	4	4	0	6	4	22	GERD
		50	Indeterminate	4	8	6	2	12	16	48	EoE
		51	Indeterminate	4	8	8	2	12	16	50	EoE
		52	Indeterminate	4	8	8	2	12	16	50	EoE
		53	Indeterminate	4	4	8	2	12	16	46	EoE
		54	Indeterminate	2	0	4	0	6	4	16	GERD
		55	Indeterminate	4	6	8	2	12	12	44	EoE

Supplementary Table 3. Worksheet for Calculation of EPX-mAb–Based Immunohistochemistry Diagnostic Scoring

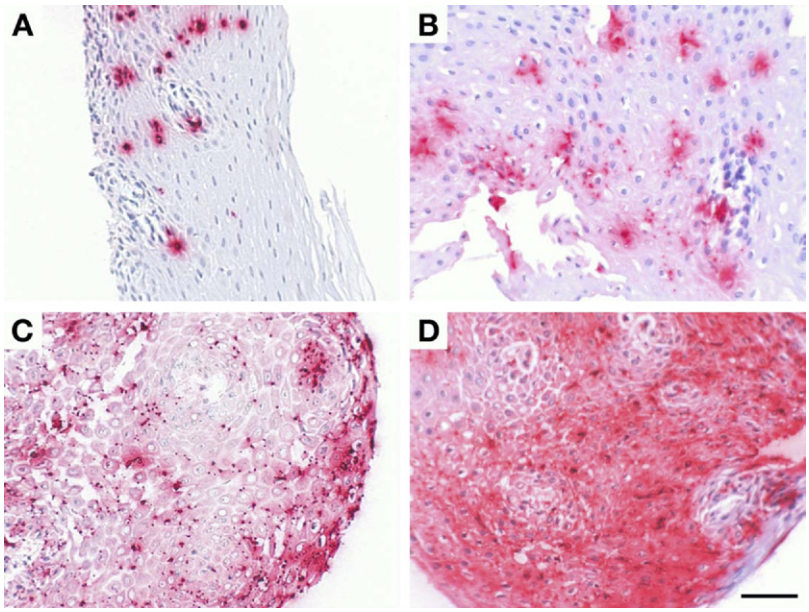
EPX-mAb staining parameter	Numeric score	Priority factor	EPX-mAb staining parameter score
Reproducibility	Percent of all biopsies with significant eosinophil infiltration and/or degranulation 0 (<1%) 1 (1–24%) 2 (25–49%) 3 (50–74%) 4 (75–100%)	1	
Patchiness	Percent area of the maximumly affected biopsy showing significant eosinophil infiltration and/or degranulation 0 (<10%) 1 (10–24%) 2 (25–49%) 3 (50–74%) 4 (75–100%)	2	
Degranulation	Part 1: Level of degranulation observed in maximumly affected biopsy 0 1 2 3 4	2	
	Part 2: Extent of level 3 or 4 degranulation either ≤ 1 or ≥ 2 biopsies 0 (≤1 biopsy) 2 (≥2 biopsies)	1	
Eosinophil infiltrate: maximum single focus	Number of intact eosinophils - peak value in a single 40× HPF 0 (<2) 1 (2–5) 2 (6–10) 3 (11–14) 4 (≥15)	3	
Eosinophil infiltrate: average of five designated foci	Number of intact eosinophils - peak value in an average of 5 40× HPFs 0 (<2) 1 (2–5) 2 (6–10) 3 (11–14) 4 (≥15)	4	
Total EPX-mAb–based immunohistochemistry score			
Scoring scale: 0–50 <5 = Control	5–35 = GERD		36–50 = EoE



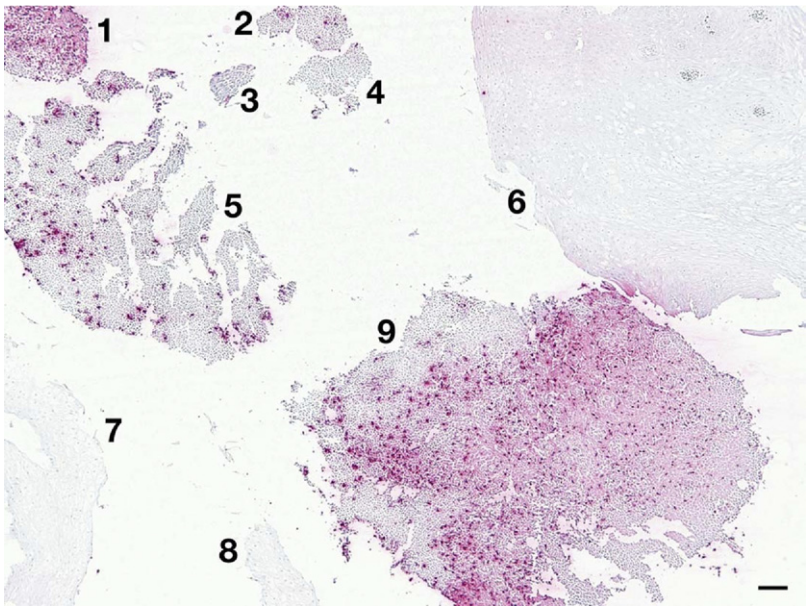
Supplementary Figure 1. A schematic review of the protein purification strategy of mouse EPX. Blood/spleen eosinophils from the IL-5 transgenic line of mice NJ.1638 were the source material for the purification of EPX. The strategy uses both physical means of eosinophil isolation (discontinuous Percoll E gradient centrifugation) as well as cell surface marker selection (MACS) to achieve the isolation of sufficient numbers of eosinophils that also displayed a purity of >95% (contaminating leukocytes include neutrophils and monocytes). The subsequent purification of EPX occurred in a 3-step approach: (1) cell lysis, physical separation/isolation of secondary granules, and the disruption/sonication of granules to isolate acid soluble proteins; (2) separation of acid-soluble proteins by size selection chromatography; and (3) purification of EPX by preparative high-performance liquid chromatography. The photomicrographs provided include a representative peripheral blood film from NJ.1638 mice and a cytospin of the resulting purified eosinophil population used to isolate EPX. In both cases, the slides were stained with Wright-Giemsa (Diff-Quik, Fisher; Dade Behring Inc, Newark, DE) and coverslipped before photo documentation. Purified secondary granules were fixed and subjected to electron microscopy as previously described.[7] The electron photomicrograph (original magnification, 512,500×) is representative of the isolated granule fraction containing predominantly membrane-bound secondary granules with a distinct electron microscopic morphology: MBP-derived electron-dense cores surrounded by an electron-translucent matrix area that contains the remaining abundant acid-soluble granule proteins (ie, EPX and the eosinophil associated ribonucleases).



Supplementary Figure 2. Immunohistochemistry of human bone marrow biopsies shows that the mouse anti-mouse EPX monoclonal antibody MM25-82.2.1 is eosinophil-specific, displaying no reactivity toward other human leukocytes. A bone marrow biopsy from a patient with myelodysplastic syndrome was initially subjected to hematoxylin-eosin staining, and a selected field was photographed, allowing for a cell differential. After photography, the coverslip was removed (brief incubation in xylene), and the hematologic stains were removed as part of the antigen retrieval process. EPX-mAb–based immunohistochemistry was performed on the destained slide, and the exact location of the previously photographed hematoxylin-eosin–stained field was determined and photographed for comparison. This strategy demonstrated that among all the marrow-derived leukocytes, only eosinophils were identified by staining with EPX-mAb (corresponding *arrowheads* in both photographs identify a subset of these eosinophils). Scale bar = 20 μm.



Supplementary Figure 3. EoE patients display quantitatively different levels of eosinophil degranulation that correlate with disease severity. Representative photomicrographs at high (40×, 0.29 mm^2 field of view) power of the 4 described levels of eosinophil degranulation within esophageal biopsies. (*A*) Degranulation level 1: the presence of >3 intact eosinophils with no extracellular release of EPX; (*B*) degranulation level 2: release of EPX is evident (ie, degranulation is observed) but limited to areas surrounding >3 intact eosinophils; (C) degranulation level 3: detection of eosinophil cytoplasmic fragments and free granules, including limited areas of extracellular matrix deposition; (*D*) degranulation level 4: extensive EPX extracellular matrix deposition and detection of free granules in >50% of at least a single microscopic field. Scale bar = 50 μm.



Supplementary Figure 4. EPX-mAb–based immunohistochemistry demonstrated that often only a fraction of available biopsies displayed either focal areas of eosinophil accumulation and/or areas of eosinophil degranulation. A representative low (5×, 20 mm^2 field of view) power photomicrograph is shown that contains 9 available biopsies from an EoE patient, highlighting the often observed heterogeneity encountered with these biopsies. Scale bar = 100 μm.