

Activated Mucosal Mast Cells Differentiate Eosinophilic (Allergic) Esophagitis From Gastroesophageal Reflux Disease

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ABSTRACT

Objectives: To evaluate the utility of activated mucosal mast cells (MC) in the differential diagnosis of eosinophilic esophagitis (EE) and gastroesophageal reflux disease (GERD).

Methods: Intraepithelial eosinophils and MC were quantified in esophageal biopsies from 25 children with EE, 22 children with GERD and 22 controls. MCs were identified by immunohistochemistry for MC tryptase, whereas MC activation status was evaluated by immunohistochemistry for immunoglobulin E (IgE) and by electron microscopy.

Results: Esophageal biopsies from patients with EE showed higher intraepithelial eosinophil counts (55 ± 27.5 vs 6.9 ± 9.7 , $P < 0.0001$) and MC counts (26.3 ± 12.7 vs 7.8 ± 8.9 , $P < 0.0001$) than those from patients with GERD. Almost all EE biopsies (24 of 25 patients; 96%) contained IgE-bearing cells compared with 9 of 22 (41%) GERD biopsies ($P < 0.001$). GERD biopsies with intraepithelial eosinophil counts >7 /high-

power field (suggesting an allergic component) contained IgE-bearing cells in 6 of 7 (86%) cases compared to 3 of 15 (20%) cases with eosinophil counts <7 /h.p.f ($P < 0.01$). No intraepithelial eosinophils, MC or IgE-positive cells were present in controls. Electron microscopy confirmed the presence of intraepithelial MC and changes in cytoplasmic granules indicative of MC and eosinophil activation.

Conclusions: Intraepithelial MC counts and IgE-bearing cells may help to differentiate EE and GERD and to define a subset of GERD patients in which an allergic component is present. The findings support a role for a MC-mediated hypersensitivity reaction in the pathogenesis of EE. *JPGN* 44:20–26, 2007.

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INTRODUCTION

Intraepithelial eosinophils are frequently encountered in esophageal biopsy specimens from pediatric patients, most commonly in the setting of gastroesophageal reflux disease (GERD) (1). Intraepithelial eosinophils may also be seen in a variety of other conditions including eosinophilic esophagitis (EE), eosinophilic gastroenteritis, idiopathic hypereosinophilic syndrome, inflammatory bowel disease, drug reactions and fungal and parasitic infections (2,3). Of the above-mentioned conditions, EE is the most frequently encountered in pediatric practice and its prevalence appears to be increasing, mainly as a consequence of its increased recognition by both

gastroenterologists and pathologists (4). Because there is considerable overlap in the clinical and histological features of EE and GERD, differentiating between these conditions may prove challenging (5). At each end of the spectrum, patients with EE and GERD are easily recognized. EE tends to present at an older age than GERD, shows a stronger male predilection, is classically associated with dysphagia for solids (6) and is usually accompanied by other allergic conditions (7). EE is associated with normal or near-normal acid pH probe studies and a poor response to antireflux therapy (7,8). EE characteristically involves both the proximal and distal esophagus, whereas involvement in GERD is limited to the distal esophagus (5). Endoscopic findings such as esophageal rings, vertical furrows, white papules and strictures, when present, are suggestive of EE, although not entirely specific (4). Histologically, EE is characterized by high intraepithelial eosinophil counts, superficially located eosinophils and eosinophilic microabscesses (2,5,9,10). In a proportion of patients with EE, the full array of clinical and histological features is not

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present and the distinction of EE from GERD remains challenging. There have been numerous attempts to define the density of eosinophils that will allow for a diagnosis of EE (2,8–11). Intraepithelial eosinophil counts exceeding 15 to 20/high-power field (400 $\times$ magnification) have been widely used in the literature for the diagnosis of EE (2,5,10,12), and it has been suggested that counts between 7 and 24/high-power field may represent a combination of GERD and food allergy (2,13). Some have cautioned against the use of eosinophil counts as the sole criterion for the diagnosis of EE and emphasize the need to interpret these in the clinical context (5).

There have been few attempts to identify additional histological or immunohistochemical markers of EE that may help to differentiate it from GERD. Although there is strong evidence implicating mast cells (MC) and immunoglobulin E (IgE)-mediated hypersensitivity in the pathogenesis of EE (14–18), the utility of immunostaining for IgE in differentiating EE from GERD has not been evaluated in large series of cases. Justinich et al. demonstrated increased numbers of MC in a small number of patients with EE compared to GERD but without assessing the status of IgE immunostaining (19). Because MC may infiltrate tissues in response to both allergic and nonallergic stimuli (20,21), the presence of IgE-bearing cells is a more specific indicator of an allergic process than MC per se. We therefore hypothesized that activated MC identified by IgE positivity coupled with cytoplasmic degranulation by electron microscopy should be present in esophageal biopsies from patients with EE but not in those with GERD, allowing for differentiation of these 2 conditions.

PATIENTS AND METHODS

This retrospective study was approved by the Research Ethics Board of the Hospital for Sick Children, Toronto, Canada. Cases were retrieved from the clinical and pathology files at the Hospital for Sick Children over a 7-year period (1999–2006) and included clinically and histologically confirmed cases of EE ($n=25$; 21 male, 4 female; mean age 12 years, range 3–17 years), GERD ($n=22$; 13 male, 9 female, mean age 10.8 years, range 3–17 years) and normal controls ($n=22$; 18 male and 4 female, mean age 10.1 years, range 3–17 years). Distal esophageal biopsies were evaluated in all subjects. Hematoxylin-and-eosin-stained sections from formalin-fixed, paraffin-embedded tissue were reviewed by 3 pathologists (R.B., E.C. and R.K.) who, where possible, were blinded to the clinical information and previous pathological diagnoses. The number of intraepithelial eosinophils was counted in 10 high-power field and the mean values were calculated. For immunohistochemical staining, 5- μ m formalin-fixed, paraffin-embedded sections were mounted on positively charged slides, baked overnight at 60°C, dewaxed in xylene and hydrated to distilled water through decreasing concentrations of alcohol. MCs were identified using monoclonal antibodies directed against MC tryptase (DakoCytomation, Carpinteria, CA, dilution 1:50), and IgE-bearing cells were identified

using polyclonal antibodies directed against IgE (Behring, Marburg, Germany, 1:200 dilution). Immunohistochemical procedures were performed using the NEXES autoimmunostainer (Ventana Medical Systems, Tucson, AZ) using the Ventana iView DAB (3-3'-diaminobenzidine) ABC detection kit. All tissue sections were treated with Ventana Protease I for 16 min, and endogenous biotin blocked (Ventana Avidin/Biotin Block). As a positive control for MC tryptase and IgE, we used sections of lungs that contained numerous IgE bearing cells from patients with bronchial asthma who died in status asthmaticus. As a negative control, the primary antibody was omitted. The number of tryptase-positive cells was counted in 10 high-power field as described above and the mean values were calculated. Immunohistochemical staining for IgE was evaluated as either positive (when unequivocal, strong membrane staining was observed) or negative. Data were subjected to statistical analysis using the Student *t* test for comparison of means and the χ^2 test for evaluation of differences in the frequency of IgE positivity in the 2 patient groups.

Transmission electron microscopy (TEM), using standard methods, was performed initially on biopsies reprocessed from paraffin blocks ($n=3$ /group) to correlate ultrastructural changes with IgE immunoreactivity status. To obtain better ultrastructural preservation and to compare with reprocessed material, some samples were processed directly for TEM ($n=3$ /group). Tissue was fixed in universal fixative, postfixed in 1% osmium tetroxide, dehydrated in graded alcohols and propylene oxide and embedded in Epon resin. Ultrathin sections were mounted on copper grids and stained with uranyl acetate and lead citrate. Ultrastructural investigation was performed using a JEOL 1230 transmission electron microscope (JEOL, Tokyo, Japan).

RESULTS

Eosinophil and Mast Cell Counts

Esophageal biopsies from patients with EE showed significantly higher mean intraepithelial eosinophil counts (55 ± 27.5 vs 6.9 ± 9.7 , $P < 0.0001$) and MC counts (26.3 ± 12.7 vs 7.8 ± 8.9 , $P < 0.0001$) than did

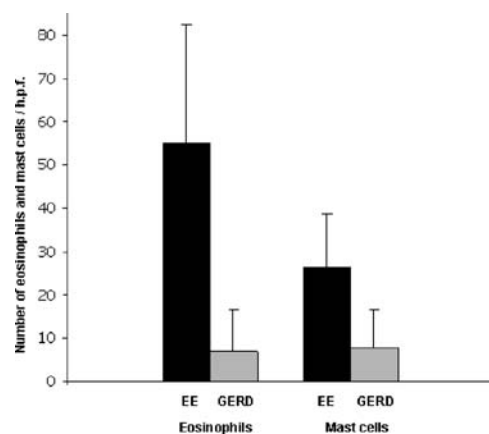


Fig. 1. Intraepithelial eosinophil and MC counts in distal esophageal biopsies from patients with EE and GERD (mean $\pm$ SD). (EE vs GERD, $P < 0.0001$.)

biopsies from patients with GERD (Fig. 1). Neither intraepithelial eosinophils nor MC were identified in control biopsies although occasional MCs were noted in the lamina propria. Figures 2–4 are representative micrographs of control, GERD and EE biopsies, respectively. Within the GERD group, a subset of patients ($n=7$) had eosinophil counts exceeding 7/high-power field, a level considered to indicate an allergic component (“GERD plus allergy” [GERD+A], or so-called overlap syndrome) (2,13). Review of the clinical files of this

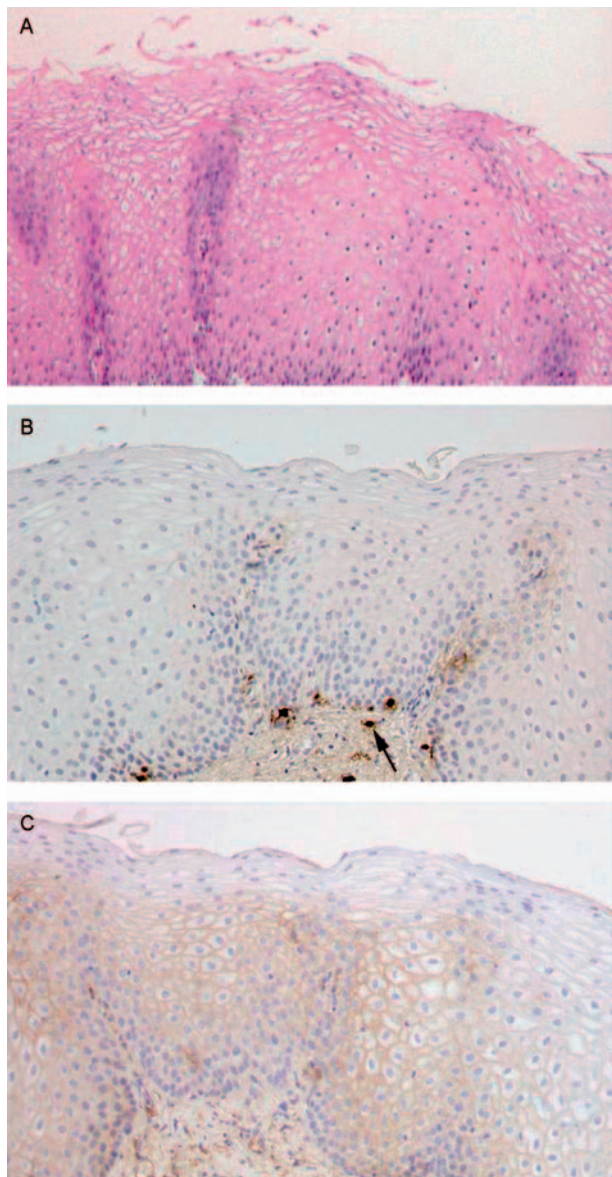


Fig. 2. Distal esophageal biopsy from a control subject. a, Normal esophageal mucosa, HE objective 10 $\times$. b, Immunohistochemistry for MC tryptase showing a few MC in the superficial lamina propria (arrow) but none within the epithelium, objective 10 $\times$. c, Immunohistochemistry for IgE is negative, objective 10 $\times$.

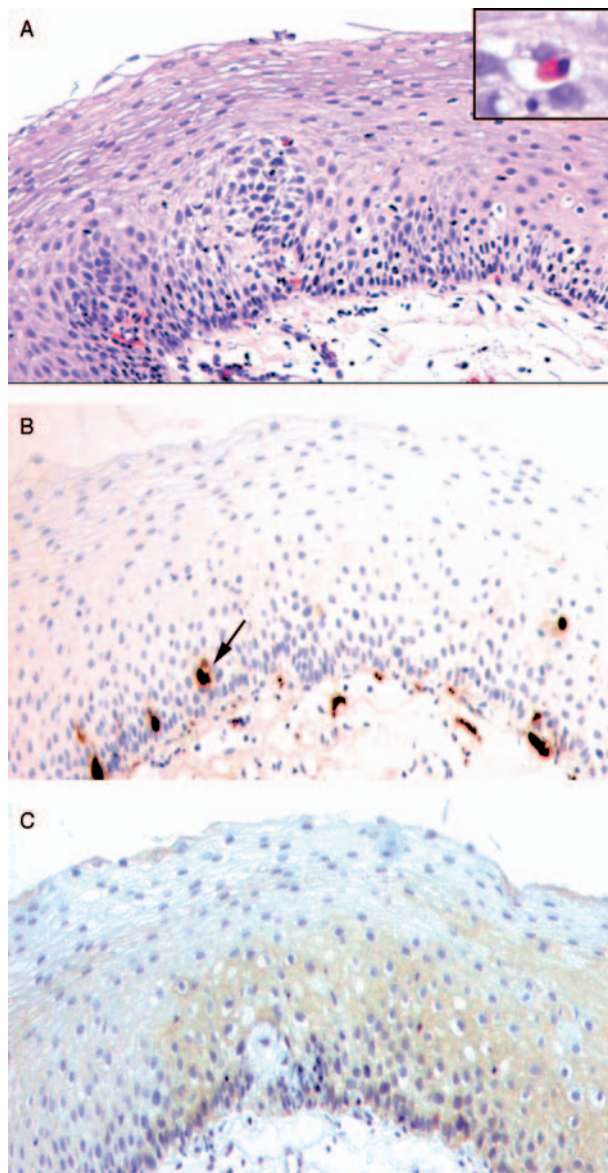


Fig. 3. Distal esophageal biopsy from a patient with GERD. a, Esophageal mucosa showing basal cell hyperplasia, intercellular edema and a mild eosinophil infiltrate, HE objective 10 $\times$ (inset: intraepithelial eosinophil). b, Immunohistochemistry for MC tryptase showing occasional intraepithelial MC, mostly basally located (arrow), objective 10 $\times$. c, Immunohistochemistry for IgE is negative, objective 10 $\times$.

subset of patients revealed an allergic phenotype in at least 4 of these patients (food allergy in 2 patients, asthma in 1, food allergy and asthma in 1). Subset analysis of the GERD group revealed significantly higher mean eosinophil and MC counts in the GERD+A group (defined as those with eosinophils >7/high-power field; $n=7$) than in the remaining conventional GERD subset (defined as those with eosinophils <7/high-power field; $n=15$)

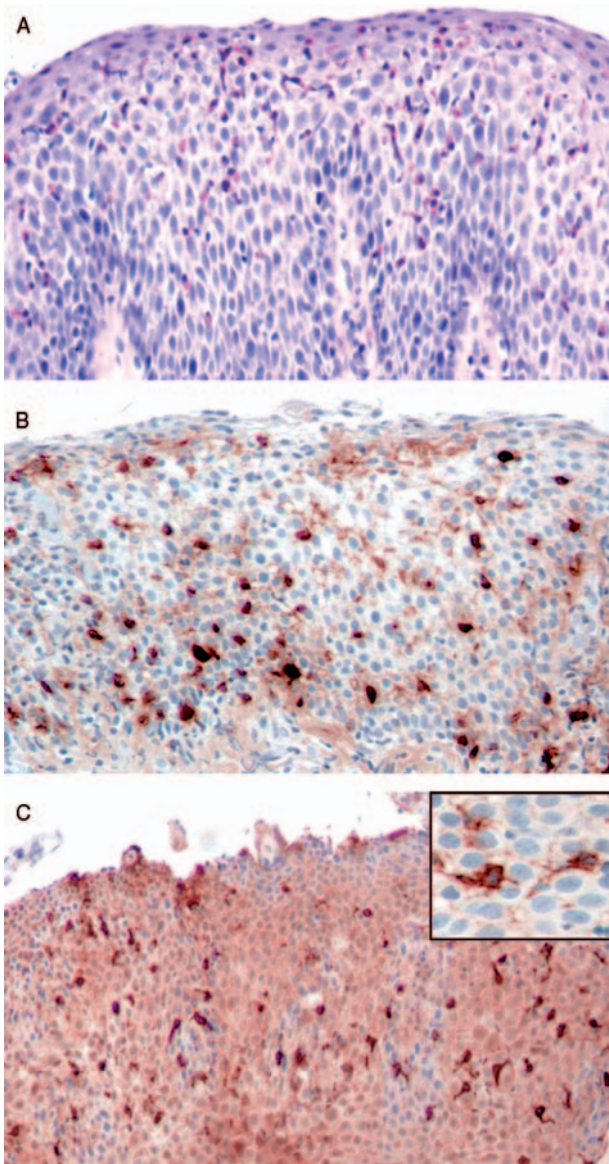


Fig. 4. Distal esophageal biopsy from a patient with EE. a, Esophageal mucosa showing marked basal cell hyperplasia, intercellular edema and large numbers of intraepithelial eosinophils, many of which are superficially located, HE objective 10 $\times$. b, Immunohistochemistry for MC tryptase showing large numbers of intraepithelial MC present throughout all layers of the epithelium, objective 10 $\times$. c, Immunohistochemical stain for IgE showing large numbers of IgE-positive cells present throughout all layers of the epithelium, objective 10 $\times$. (Inset: Higher magnification showing IgE-positive membrane staining of MC.)

(18.7 ± 9.2 vs 1.4 ± 1.4 , $P < 0.0001$ and 18 ± 9.7 vs 3.1 ± 2 , $P < 0.0001$, respectively) (Fig. 5).

IgE-bearing Cells

Within the EE group, 24 of 25 biopsies (96%) contained intraepithelial MC showing extensive membrane

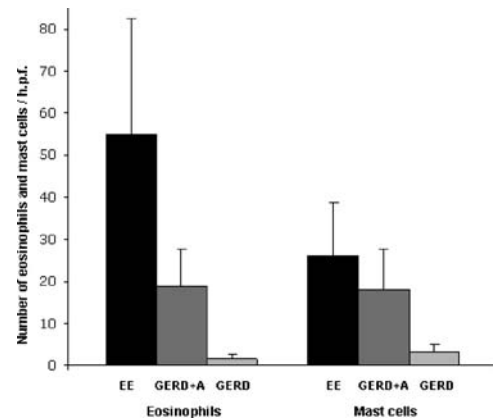


Fig. 5. Intraepithelial eosinophil and MC counts in distal esophageal biopsies from patients with EE, GERD+A (defined by intraepithelial eosinophil counts >7 /high-power field) and conventional GERD (defined by intraepithelial eosinophil counts <7 /high-power field) (mean $\pm$ SD). (GERD+A vs GERD, $P < 0.0001$).

immunostaining for IgE. In most of these EE biopsies, IgE-bearing cells were numerous and diffusely distributed throughout the epithelium (Fig. 4). Within the GERD group only 9 of 22 GERD biopsies (41%) contained IgE-bearing cells (EE vs GERD, $P < 0.001$). Intraepithelial IgE-bearing cells were not identified in control biopsies.

Subset analysis of the GERD group revealed the majority of GERD+A patients (6 of 7, 86%) as having IgE-positive biopsies compared with only 3 of 15 (20%) of the conventional GERD patients ($P < 0.01$). In the latter 3 patients, IgE-bearing cells were sparse and basally located.

Ultrastructural Studies

Transmission electron microscopy confirmed the presence of intraepithelial MC and eosinophils in cases of EE and GERD. In control cases only occasional MCs and eosinophils were identified in the subepithelial lamina propria. As expected, in cases of EE, IgE-bearing cells showed extensive changes of cytoplasmic degranulation with loss of electron-dense granule matrix, particularly near the plasma membrane (Fig. 6a). This TEM appearance, characteristic of activated MC, contrasted with relatively well-preserved electron density of MC granule matrix in control cases (Fig. 6b). The eosinophils also showed ultrastructural changes of activation characterized by alterations in cytoplasmic granules, including fragmentation and formation of intragranular, vesiculotubular structures (Fig. 6c). These features contrasted with well-preserved electron density and crystalloid-like inclusions of the matrix in eosinophils from control samples (Fig. 6d). In cases of GERD the TEM changes in both MC and eosinophils were variable, and because of the small sample size, they were not considered useful in

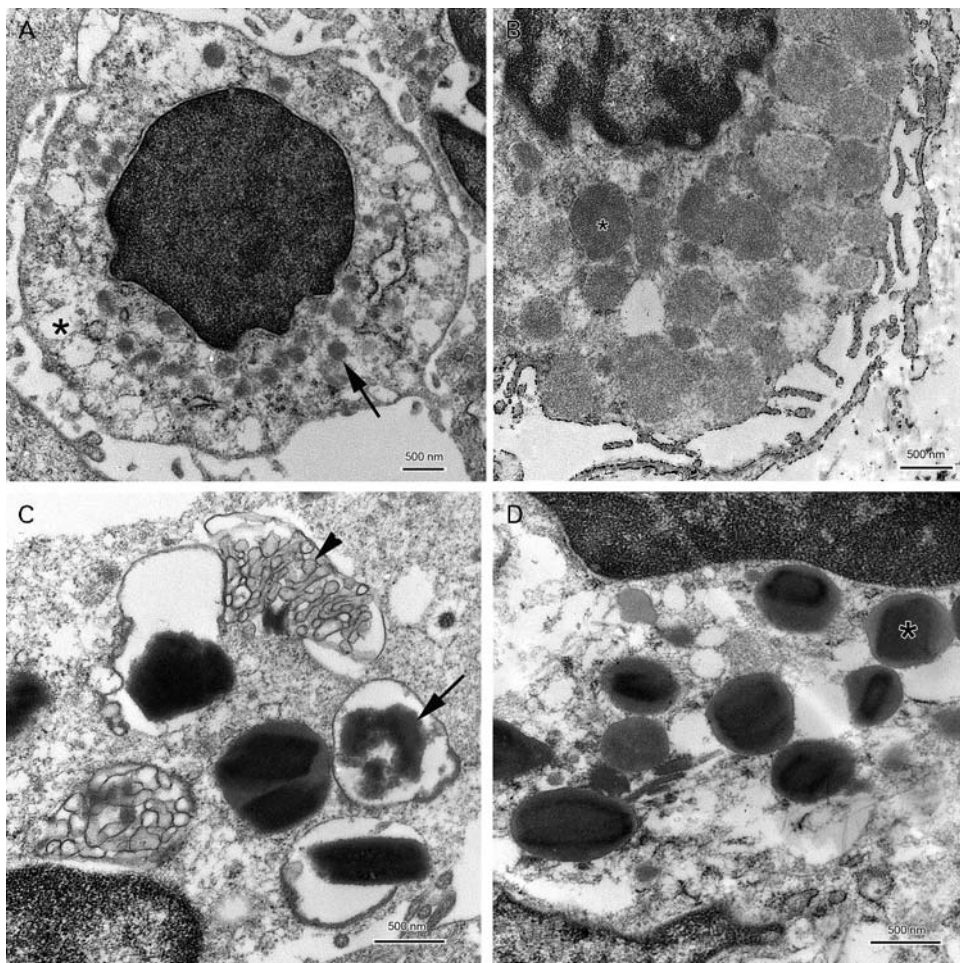


Fig. 6. MC and eosinophils in esophageal biopsies from patients with EE and controls, reprocessed from paraffin blocks. a, Intraepithelial IgE-bearing cells in a case of EE with cytoplasmic granules, particularly those close to plasma membrane, showing loss of electron density because of degranulation (*). A few granules near nucleus (Nu) show partial preservation of granule matrix (arrows), ruling out processing artifact. b, In comparison with Fig. 6a, this MC from a control case shows preservation of most cytoplasmic granules, which contain moderately electron-dense granule matrix (*). c, Close-up of eosinophil granules in a case of EE with evidence of activation characterized by fragmentation of cytoplasmic granules (arrow) and formation of vesiculotubular structures (arrowhead). d, Typical eosinophil cytoplasmic granules in a control case contain oval or rectangular electron-dense crystalloid-like inclusions (*).

the differential diagnosis. There were no significant differences in the TEM appearance of cytoplasmic granules in either MC or eosinophils between samples reprocessed from paraffin blocks and those processed directly for TEM (data not shown).

DISCUSSION

Consistent with previous studies (2), we found intraepithelial eosinophil and MC counts to be significantly higher in esophageal biopsies from patients with EE than with GERD. In addition, 96% of biopsies from patients with EE were found to contain intraepithelial IgE-bearing cells compared with 41% of GERD biopsies. The finding of IgE-bearing cells in a proportion of GERD biopsies was unexpected given that GERD is etiologically

unrelated to allergy. This prompted further evaluation of the GERD group to determine whether it may include a subset of patients with allergic features. Rothenberg et al. have proposed that patients with eosinophil counts between 7 and 24 eosinophils/high-power field may have an intermediate phenotype with features of both GERD and EE and used GERD+A to describe this group (2,13). Patients with eosinophil counts >7 eosinophils/high-power field have been reported to have a suboptimal response to antireflux therapy and to benefit from control of allergen exposure and/or therapy directed against allergy (9,10). On this basis, we stratified the GERD group into those with eosinophils <7/high-power field (conventional GERD) and those with eosinophils >7/high-power field (GERD+A). Analysis of these 2 GERD subsets revealed marked differences in mean eosinophil

and MC counts ($P < 0.0001$) and in the prevalence of intraepithelial IgE-bearing cells ($P < 0.01$). In all, 86% (6 of 7) of GERD+A biopsies contained IgE-bearing cells compared with only 20% (3 of 15) of the conventional GERD biopsies. Interestingly, review of the clinical files of the GERD+A patients revealed evidence of an allergic phenotype in 4 of these patients (food allergy in 2, asthma in 1 and both food allergy and asthma in 1). The presence of IgE-bearing cells in 3 of 15 conventional GERD biopsies is less easy to explain. In these patients, the IgE-bearing cells were sparse and tended to be basally located compared with a more diffuse infiltrate seen in most cases of EE. Although the significance of these cells is uncertain, the possibility of a mild, subclinical allergic reaction to ingested or inhaled allergen cannot be excluded. In 1 patient, a subsequent biopsy showed intraepithelial eosinophil counts exceeding 20/high-power field, suggesting the presence of an allergic component in that patient.

Our findings suggest that immunohistochemical stains for IgE and MC may have a role to play in the differential diagnosis of EE and GERD. In the setting of a heavy intraepithelial eosinophil infiltrate (>20 /high-power field), the presence of intraepithelial IgE-bearing cells (particularly when numerous and diffusely distributed) is strongly supportive of EE. Although a small minority of conventional GERD biopsies may contain isolated IgE-bearing cells, their paucity, basal location and the lack of an associated heavy eosinophil or MC infiltrate facilitates their distinction from EE.

Our findings are consistent with the proposal that a subset of GERD patients with eosinophil counts exceeding 7/high-power field may have an allergic component to their esophagitis (2,13) because this subset was associated with both intraepithelial IgE-bearing cells (in 6 of 7 cases) and allergy (4 of 7 cases) (Fig. 7). Interpretation of our findings is limited, however, by small patient numbers and the retrospective nature of this study, which was not specifically designed to address this issue. It is

possible that IgE immunohistochemistry has a role to play as an adjunct to eosinophil counts in confirming an allergic component in a subset of GERD patients who are poorly responsive to standard therapy. Such a role would need to be evaluated in prospective studies designed for a more detailed characterization of the GERD+A subgroup, with special attention paid to esophageal pH studies, skin testing, allergic history and response to antireflux therapy. Immunohistochemistry for IgE does not have application in the differential diagnosis of EE and GERD+A because the majority of patients in both groups will have intraepithelial IgE-bearing cells. Transmission electron microscopy studies on esophageal biopsies from cases of EE confirmed the presence of intraepithelial MC and eosinophils with ultrastructural evidence of activation that indicates release of preformed mediators from these cells. Our ultrastructural findings in MC from patients with EE are similar to TEM studies in well-documented allergic reactions such as asthma and allergic rhinitis (22). A previous TEM study described activated eosinophils in esophageal biopsies from children with GERD but also other gastrointestinal disorders, indicating a relative nonspecificity of this finding (23).

Our finding of numerous intraepithelial IgE-bearing cells in esophageal biopsies from children with EE confirm the findings of Straumann et al. in adults. As in that study, we assume that the vast majority of IgE-bearing cells in these biopsies are MC (18). High-affinity FcεRI IgE receptors are present in large numbers on the membranes of tissue MCs (and blood basophils) compared with much lower levels in Langerhans' cells, monocytes platelets and eosinophils (24). Given this and the presence of large numbers of tryptase-positive MC in these biopsies, it seems reasonable to assume that a high proportion of IgE-bearing cells identified in this study are likely to be MC. Our findings are consistent with accumulating evidence suggesting that IgE-mediated hypersensitivity may play a role in the pathogenesis of EE. Most patients with EE have a history of atopy (7,10) and demonstrate hypersensitivity to dietary and/or inhaled allergens on skin prick tests and/or radioallergen sorbent test (13,25,26). Removal of the offending allergen from the diet is reported to result in both resolution of symptoms and a marked reduction in intraepithelial eosinophil counts in most children with EE (26). The reported seasonal variation in the symptoms of EE and its relationship to pollen (16,17,25) appears to further support a role for environmental allergens in the pathogenesis of EE, although the response of the vast majority of patients with EE to an elemental diet without any change in their environment brings into question the exact role of these agents. Finally, studies evaluating cytokine profiles in both human EE and an animal experimental model of EE provide evidence to suggest an IL-5-, T_H2 cell-, and MC-driven allergic inflammatory process (14,15,18,27,28).

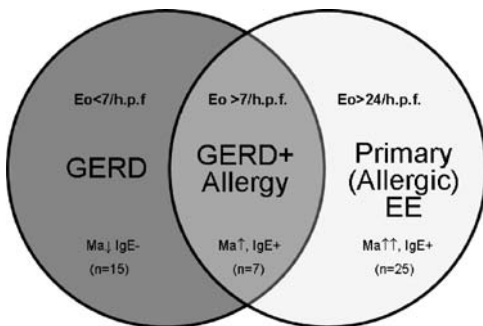


Fig. 7. The spectrum of features in GERD and primary (allergic) EE, including overlapping features of GERD and allergy. The predominant findings in each group are summarized. Eo = eosinophils, MC = mast cells. Figure modified from Rothenberg et al. (2).

The role of MC in the pathogenesis of EE has recently been questioned because of a study that found that mRNA for IL-4 and IL-13 (T_H2 cytokines that induce IgE production by B cells) was not increased in esophageal biopsies from patients with EE (28). In that study cytokine mRNA expression, but not corresponding cytokine levels, was measured. This may be important because preformed IL-4 is known to be stored within MC secretory granules and is rapidly released during degranulation without preceding upregulation of mRNA transcription (29). Interestingly, it has been suggested that many T_H2 -type cytokines may in fact be generated in largest quantities by activated MC and eosinophils (30). Thus, once initiated, MC and eosinophils may be able to sustain the T_H2 polarized environment in EE. Although the precise cellular sources of T_H2 cytokines in EE and their roles in initiating and perpetuating the hypersensitivity reaction require further study, the identification of intraepithelial, activated IgE-bearing cells in EE implies the presence of a T_H2 -type response sufficient to induce B cell IgE production.

In summary, our findings suggest a role for IgE immunohistochemistry in the differential diagnosis of EE and GERD and in better defining a subset of GERD patients with overlapping features of acid reflux and allergy. In addition, our findings support a role for MC-mediated hypersensitivity in the pathogenesis of EE, although the precise nature of this role requires further study.

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