

Electron microscopy elucidates eosinophil degranulation patterns in patients with eosinophilic esophagitis

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Background: In patients with eosinophilic esophagitis (EoE), eosinophils accumulate and release granule proteins onto esophageal epithelium. However, little is understood about the mechanism of eosinophil degranulation.

Objective: To determine and quantify eosinophil degranulation patterns, we studied esophageal biopsy specimens from both the proximal and distal esophagi of 9 randomly selected patients with EoE.

Methods: The specimens were fixed in glutaraldehyde, embedded, sectioned, and imaged by means of transmission electron microscopy. Eosinophils and their granules were identified by their distinctive morphology, and all eosinophils and granules were imaged. A total of 1672 images from 18 esophageal specimens were evaluated and graded. Eosinophils were categorized based on membrane integrity and by cytoplasmic vesiculation as evidence of piecemeal degranulation. Granules were categorized based on reversal of staining (eosinophil granule core lightening) and localization within and outside the cells.

Results: The results revealed that greater than 98% of eosinophils infiltrating the esophagus in patients with EoE demonstrate morphologic abnormalities ranging from granule changes with reversal of staining to marked cytoplasmic vesiculation to loss of cellular membrane integrity with cytolytic disruption and release of intact membrane-bound granules into the tissues. Approximately 81% of eosinophils showed membrane disruption. Extracellular granules were abundant in at least 70% of the images, and approximately 50% of these granules showed reversal of staining. On the basis of the prominence of tubulovesicular development, piecemeal degranulation appears closely related to the other morphologic changes seen in patients with EoE.

Conclusion: These findings reveal that eosinophils in esophageal biopsy specimens from patients with EoE are abnormal, with greater than 80% showing cytolysis, and therefore that evaluation by means of light microscopy after hematoxylin and eosin staining might not accurately reflect eosinophil involvement. (*J Allergy Clin Immunol* 2014;■■■:■■■-■■■.)

Key words: *Eosinophilic esophagitis, eosinophils, activation, piecemeal degranulation, electron microscopy, cytolysis*

Eosinophilic esophagitis (EoE) is an increasingly common disease characterized by eosinophilic invasion of the esophageal mucosa in which eosinophil granulocytes accumulate and are activated. Activated eosinophils release granule proteins into both the epithelial and submucosal layers.¹⁻⁴ Evidence of eosinophil activation includes expression of membrane markers, formation of cytoplasmic vesicles, and changes in eosinophil granules with loss of electron density of the granule cores.⁵ In patients with EoE, eosinophil granule proteins, including major basic protein 1, eosinophil-derived neurotoxin (also known as ribonuclease 2), eosinophil peroxidase, and eosinophil cationic protein (also known as ribonuclease 3), are localized on esophageal epithelial cells.^{4,6} Remarkably, eosinophil-derived neurotoxin is extensively deposited on the diseased esophagus, implying eosinophil activation and release of eosinophil-derived neurotoxin from its storage site in the granule matrix.⁴ However, the mechanisms responsible for eosinophil activation and the process by which granule proteins are deposited in the disease-affected esophagus remain incompletely described.

Secretion of eosinophil granule proteins occurs through eosinophil cytolysis and release of intact granules, which then liberate granule proteins, or through piecemeal degranulation, a unique secretory process characterized by transport of vesicles to the cell surface.^{7,8} Tubulovesicular carriers, which are referred to as sombrero vesicles because they resemble the well-known Mexican hat, have been identified as playing a key role in transporting eosinophil proteins from granules to the plasma membrane for extracellular release.⁸ Therefore, loss of membrane integrity is associated with cytolysis, and the presence of cytoplasmic vesiculation, including sombrero vesicles, is taken as evidence of piecemeal degranulation.

Here we identified and quantified eosinophil degranulation patterns by means of transmission electron microscopy in esophageal biopsy specimens from patients with EoE. The patterns were classified and graded according to cell membrane integrity, cytoplasmic vesiculation, granule morphology with respect to core staining and granule membrane integrity, and the occurrence of extracellular granules.

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Abbreviations used

EoE: Eosinophilic esophagitis

H&E: Hematoxylin and eosin

METHODS**Patients**

Nine patients (3 female and 6 male patients) were randomly selected from 20 patients participating in a study of omalizumab treatment in the setting of EoE (institutional review board 0013623, "A pilot study of the treatment of eosinophilic esophagitis with omalizumab," [ClinicalTrials.gov](#) identifier: NCT00123630); the results of this study will be reported separately. All of these patients satisfied the diagnostic criteria for EoE.³ Esophageal biopsy specimens were obtained during endoscopy concurrently for histology and for transmission electron microscopy at baseline before entering the study. Peak esophageal eosinophil counts derived from examination after staining formalin-fixed biopsy specimens with hematoxylin and eosin (H&E) for individual patients are included in [Table 1](#) (referred to in the Results section below). All patients had at least 15 eosinophils per high-power field at $\times 400$ magnification in at least 1 biopsy specimen after high-dose acid suppression therapy (twice-daily proton pump inhibitor) for 8 weeks and before commencing treatment with omalizumab (or placebo control).

Electron microscopy

Tissue specimens were fixed in 2.5% glutaraldehyde, postfixed in 4% osmium tetroxide, dehydrated in graded ethanol, and stained with uranyl acetate and Reynolds lead citrate. A total of 18 tissue blocks, 1 biopsy specimen each from the proximal and distal esophagi of each of the 9 patients, were sectioned. Thin sections (90 μm thick) from each block were mounted on separate electron microscopy grids and then examined with transmission electron microscopy (Hitachi H7100, Gatan digital camera; Gatan, Pleasanton, Calif). For each section, the operator systematically scanned across the grid from left to right and top to bottom at $\times 2000$ magnification and photographed every eosinophil and all granules, both intracellular and extracellular, identified based on the distinctive morphology of the cells and granules, particularly the characteristic cytoplasmic granule cores. The tissue sections from the distal and proximal esophageal specimens were evaluated for each patient.

Eosinophil classification

A total of 1672 images were acquired during the grid scans, and all were examined and graded. Three graders (H.S., L.H.H., and G.J.G.) individually and independently inspected each image. Averages of the scores assigned by the 3 graders are reported. Eosinophils and their granules in the images were evaluated based on cell membrane integrity, occurrence of cytoplasmic vesiculation, recording the specific presence of sombrero vesicles, granule morphology, and occurrence of extracellular granules. Eosinophils that were only partially captured within an image were not graded.

[Figs 1-5](#) show a gallery of findings in the images analyzed that were defined and inventoried as follows: (1) normal eosinophils (with no granule changes, no cytoplasmic vesiculation, and no cytoplasmic membrane disruption; [Fig 1](#)); (2) intracellular eosinophil granules displaying reversal of staining (core relatively radiolucent compared with the matrix, [Fig 2](#)); (3) prominent cytoplasmic vesiculation, including piecemeal degranulation (tubulovesicular development with sombrero vesicles, [Figs 2-4](#)); and (4) occurrence of extracellular eosinophil granules (individual eosinophil granules or clusters of <5 eosinophil granules with loss of cell membrane, surrounding cytoplasm, or both; [Fig 5](#)). Cytoplasmic membrane integrity was classified as intact (no membrane loss), mainly intact ($\leq 20\%$ membrane loss), partially disrupted ($>20\%$ membrane loss), or fully disrupted (no recognizable membrane). Each category was tabulated only once per image, although multiple categories could be marked for that image. For example, if 10 free granules were present,

6 displaying reversal of staining and 4 without granule changes, in 1 electron micrograph, each category (reversal of core staining and no granule changes) was counted once. For detailed averages and SDs of 1672 electron photomicrographs, see [Table E1](#) in this article's Online Repository at www.jacionline.org.

RESULTS

We examined the ultrastructural appearance of eosinophils and their granules in esophageal biopsy specimens from 9 patients with EoE using transmission electron microscopy. A total of 1672 electron microscopic images obtained from systematically scanning grids of tissue sections from the specimens were examined. We observed a spectrum of morphologies ranging from rare intact eosinophils with appearances comparable with eosinophils in peripheral blood of healthy persons ([Fig 1](#)),⁹ to many cells showing disruption and loss of cytoplasmic membranes ([Fig 4](#)), to prominent cytoplasmic changes ([Figs 2 and 3](#)), including cytoplasmic vesiculation, especially with sombrero vesicles, and granules showing reversal of core staining ([Fig 2](#)), and to individual extracellular eosinophil granules scattered in the intercellular spaces among esophageal epithelial cells ([Fig 5](#)). We scored each image for the presence of eosinophils and extracellular granules; 809 of the images showed 1 or more eosinophils, which was defined as at least 5 eosinophil granules in a cluster and in close proximity regardless of the membrane integrity, and 1185 images showed extracellular granules defined by characteristic core morphology with fewer than 5 clustered in an area of the image. We further categorized eosinophils as to their specific features, including whether they had intact or disrupted cell membranes; by the presence of cytoplasmic vesicles, including sombrero vesicles, because these have been identified in association with eosinophil piecemeal degranulation⁸; and, for the intracellular granules (similar to the extracellular granules), by using granule core morphology, particularly whether the granules showed reversal of core staining.

Nine hundred eosinophils were recognized in the 1672 electron micrographic images; the overall distribution of findings in these 900 eosinophils is represented in the Venn diagram in [Fig 6](#), including overlap when more than 1 finding was observed. Of the 900 eosinophils, 80.6% showed loss of membrane integrity, varying from $<20\%$ to total loss of cytoplasmic membrane. Of the 80.6% eosinophils with loss of membrane integrity, 43% had fully disrupted cytoplasmic membranes. Considering cytoplasmic changes within eosinophils, we found that most of the eosinophils (91.6%) displayed increased cytoplasmic vesiculation, with the presence of sombrero vesicles in greater than 50%; sombrero vesicles could not always be identified definitively in the eosinophils with vesiculation, likely in part because the sections were thin and might not have included identifying features and likely because the extent of the vesiculation in some of the cells obfuscated the defining appearance of sombrero vesicles. Most of the 900 eosinophils (88%) demonstrated granule changes, especially reversal of core staining. Most of the eosinophils examined in the images displayed more than 1 of the categorized features. As shown in [Fig 6](#), 70.3% of the eosinophils showed all 3 features. Considering eosinophils with 2 features, 76.9% (70.3% plus 6.6%) showed loss of membrane integrity and cytoplasmic vesiculation, 73.1% (70.3% plus 2.8%) showed loss of membrane integrity and reversal of granule core staining, and

TABLE I. Patients' characteristics and study values

Patient no.	Age/sex	IgE Level*	Maximum eosinophils per hpf (H&E)	No. of images/no. of eosinophils	Percentage of free granules†	Percentage of eosinophils showing PMD and reversal of staining‡	Percentage of normal eosinophils‡
1	50/M	116	28	253/120	76.4	84.5	0.0
2	28/M	82	33	263/188	67.4	73.2	0.2
3	29/M	554	28	226/92	74.3	79.3	4.0
4	44/F	32	27	114/78	62.0	91.5	0.9
5	36/F	171	30	169/131	55.8	78.4	2.0
6	23/M	53	16	129/68	71.8	97.0	1.5
7	36/F	609	30	185/33	93.2	72.7	7.1
8	29/M	82	30	188/110	65.7	92.1	0.0
9	26/F	35	47	145/80	65.3	89.6	0.8

F, Female; hpf, high-power field ($\times 400$ magnification); M, male; PMD, piecemeal degranulation.

*International units per milliliter.

†Calculated based on number of images showing free granules/total number of images $\times 100$.

‡Calculated based on numbers of eosinophils showing these abnormalities/total number of eosinophils $\times 100$.

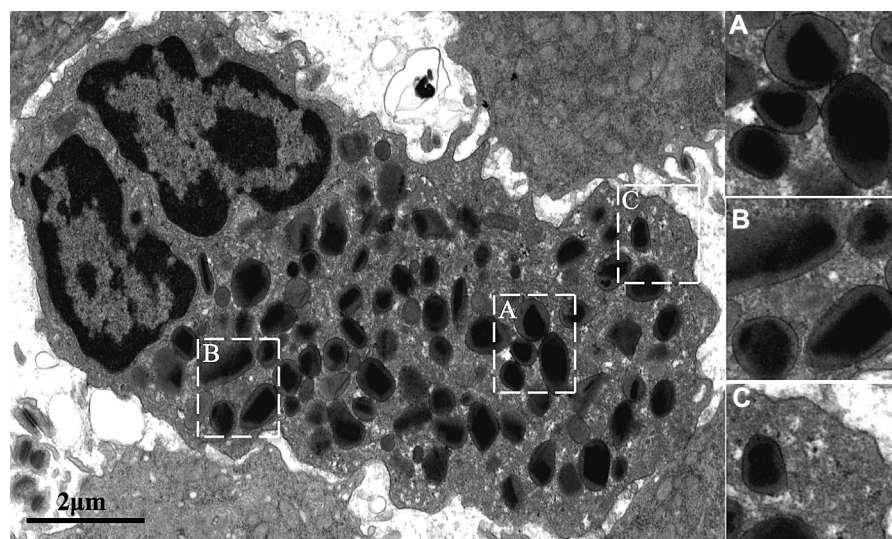


FIG 1. Eosinophil with normal granules; no evidence of cytoplasmic vesiculation, including sombrero vesicles; and fully intact cytoplasmic membrane. **A-C**, Individual, intact, membrane-bound cytoplasmic granules.

83.2% (70.3% plus 12.9%) showed cytoplasmic vesiculation and reversal of granule core staining. Relatively small proportions of the eosinophils demonstrated only 1 feature: 0.9% showed loss of membrane integrity alone, 1.7% showed cytoplasmic vesiculation alone, and 2% showed reversal of granule core staining alone. Very few of the eosinophils (1.3%) appeared normal (not on diagram) with intact membranes and no evidence of cytoplasmic vesiculation or granule changes.

We observed a surprising frequency of extracellular eosinophil granules in these esophageal specimens. As noted above, in 1672 electron photomicrographs, 1185 (71%) images showed eosinophil granules, which were recognizable by their characteristic morphology, clearly outside of cells (Fig 5). From the 1185 images of free granules, 48% showed abnormalities with loss of core density, granule membrane loss, or both. In many images a single granule was present without other eosinophil remnants. In other images isolated eosinophil granules were found with remnants of cell structures, such as nuclear fragments. Many

of the free granules were in fields largely devoid of other structures, suggesting the presence of tissue edema, likely in dilated intercellular spaces.

Table I summarizes information individually for each patient and presents observations, including the maximum eosinophil counts in esophageal biopsy specimens determined by using H&E histology and the average percentages of common findings in the images: extracellular eosinophil granules (71% [1185/1672] of electron photomicrographic images); eosinophils showing both reversal of granule core staining and cytoplasmic vesiculation, including sombrero vesicles (83.2% [749/900] of eosinophils); and proportions of normal eosinophils. The total number of images graded for each patient and the total number of eosinophils (excluding the free granules) are charted. Note that the data are presented in percentages of the total number of images graded per patient for the extracellular granules and in percentages of the total number of eosinophils identified per

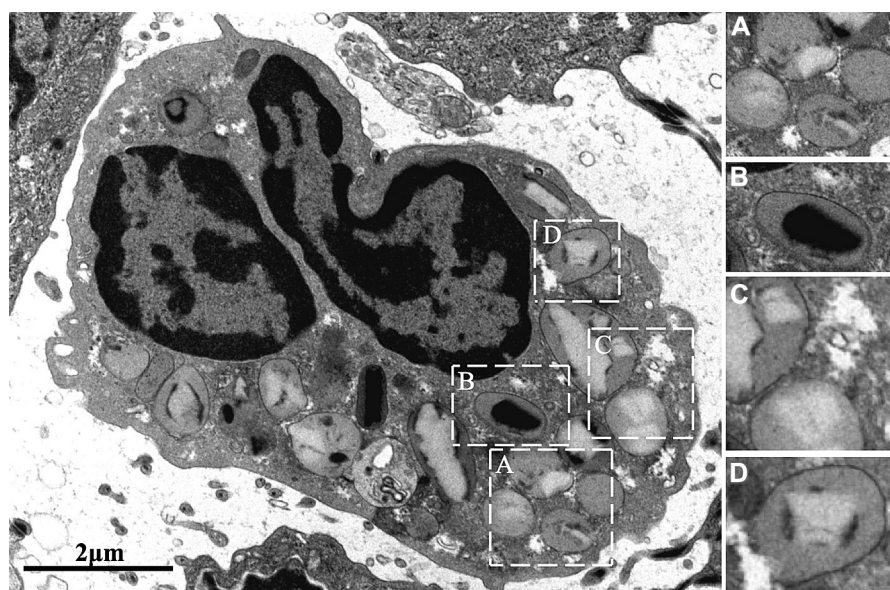


FIG 2. Eosinophil with intact cytoplasmic membrane showing reversal of granule core staining and cytoplasmic vesiculation. **A, C, and D,** Reversal of granule core staining with radiolucent core areas. **B,** Intact normal-appearing granule with an adjacent sombrero vesicle to the right.

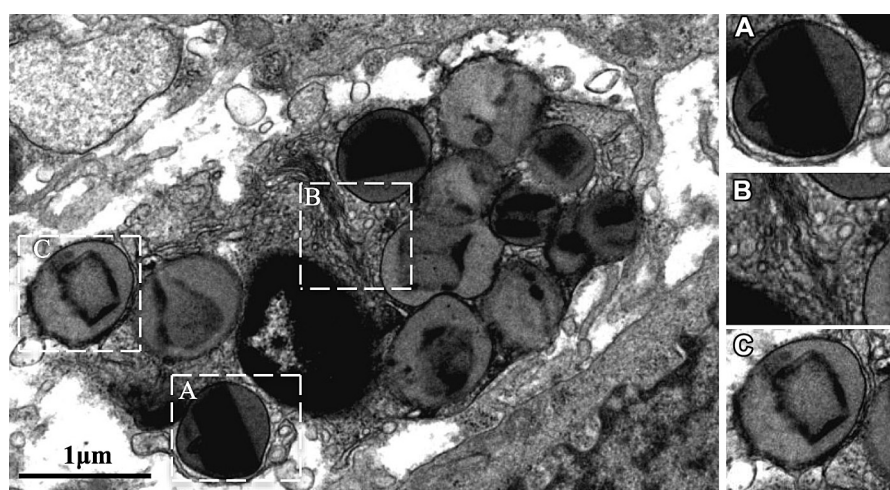


FIG 3. Eosinophil showing partial cytoplasmic membrane disruption (>20%), granules with reversal of core staining, and overall increased cytoplasmic vesiculation. **A,** Intact granule and cytoplasmic vesiculation. **B,** Cytoplasmic vesicles. **C,** Cytoplasmic granule with reversal of core staining.

patient for the combined features of cytoplasmic vesiculation and reversal of granule core staining and for normal eosinophils. The results show that the patients with the highest IgE levels (patients 3 and 7) also had high proportions of images with extracellular granules and normal eosinophil numbers, although the normal eosinophil numbers are small. Patient 7, with an IgE level of 609 IU/mL, had the highest percentage of free granules (93.2%).

DISCUSSION

The diseases in which eosinophil degranulation are prominent and the processes by which eosinophils release granule proteins

into tissues have been studied for almost 3 decades. Identification and isolation of eosinophil granule proteins permitted development of antibodies to recognize the granule proteins in tissues and investigate their participation in disease.¹⁰ One of the first studies was of tissues from patients dying of bronchial asthma. As expected, striking eosinophil infiltration was present, but unexpectedly, dramatic deposition of the eosinophil granule major basic protein 1 occurred on bronchial epithelium, in mucous plugs, and on respiratory epithelium.¹¹ Subsequent studies have largely confirmed the remarkable occurrence of eosinophil granule protein deposition in affected tissues from patients with a wide variety of diseases.¹²⁻²³ Perhaps this is most striking in patients with atopic dermatitis, in whom numbers

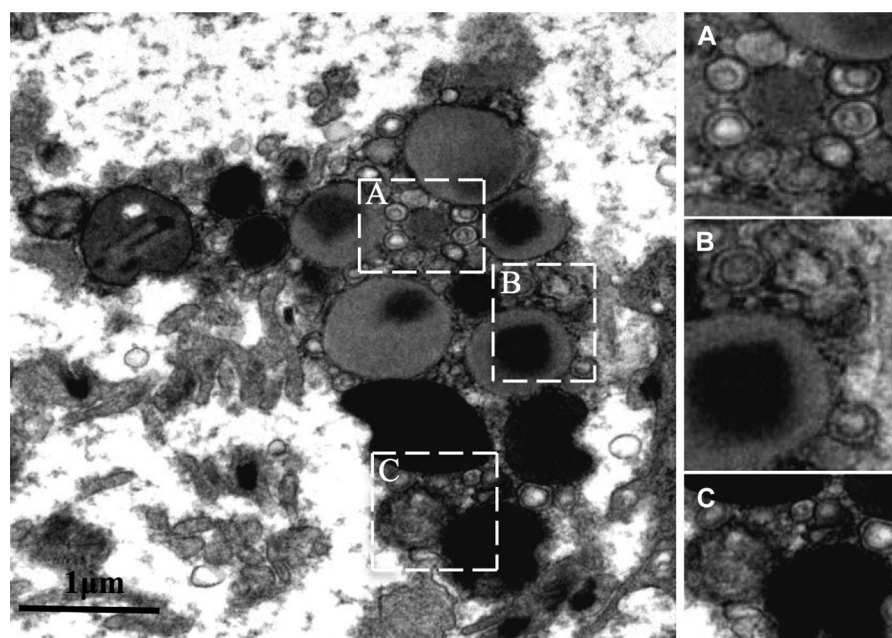


FIG 4. Eosinophil showing fully disrupted cell membrane and cytoplasmic vesiculation. **A-C**, Numerous sombrero vesicles as part of tubulovesicular development.

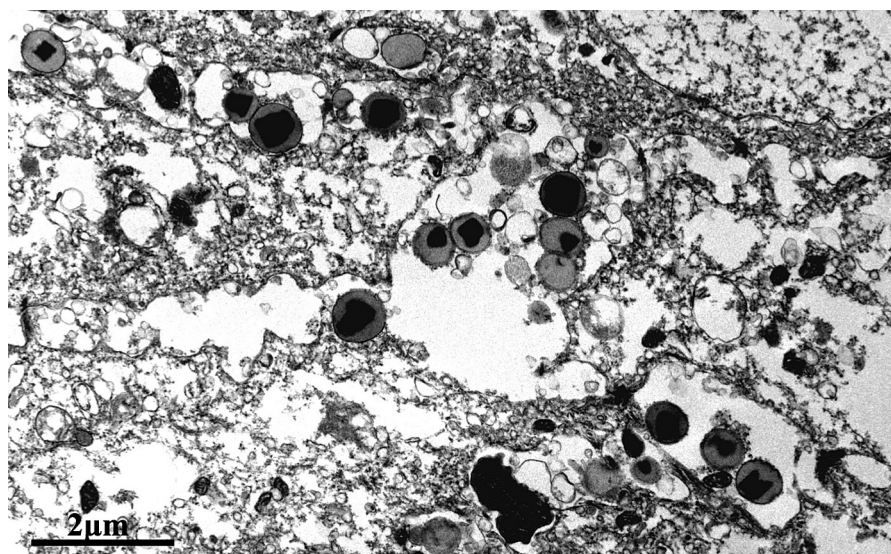


FIG 5. Eosinophil granules in epithelial intercellular spaces.

of intact eosinophils are not or are minimally increased, but affected skin shows striking deposition of eosinophil granule proteins.²⁴

Studies of the mechanisms of eosinophil degranulation in diseased tissues show 2 key modes of granule protein release. Electron microscopic evidence demonstrates that, with eosinophil activation, there is loss of the usual granule core electron density, indicating that the core protein major basic protein²⁵ has been released.²⁶ Electron microscopic evidence also demonstrates cytolytic changes in eosinophils and the accompanying presence of extracellular eosinophil

granules with intact cores, often with reversal of granule core staining.^{27,28} *In vitro* studies have shown secretion of eosinophil granule proteins by means of vesicular transport and, in particular, in association with tubulovesicular development, including sombrero vesicles.⁸ Thus 2 predominant mechanisms of eosinophil degranulation are known: cytolytic degranulation and piecemeal degranulation. Cytolytic degranulation is shown by disruption of the cell membrane and granules deposited outside of cells (typically associated with increased inflammatory actions of eosinophils), whereas piecemeal degranulation is associated with cytoplasmic

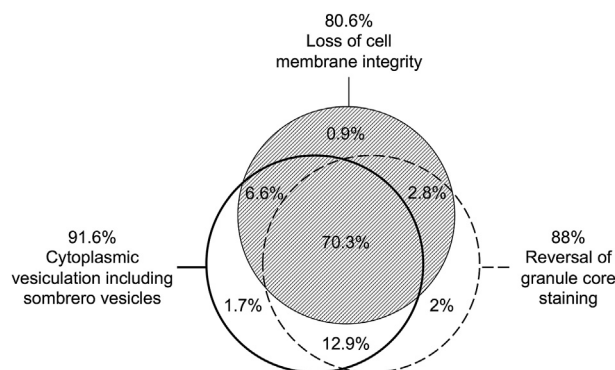


FIG 6. Venn diagram of the average percentage of findings in esophageal eosinophils from patients with EoE (n = 900 eosinophils) by means of transmission electron microscopy scored by 3 observers based on membrane integrity, cytoplasmic vesiculation, and reversal of granule core staining.

vesiculation.⁸ Analyses of tissues from patients with atopic dermatitis showed evidence of both cytolytic disruption of eosinophil membranes and extracellular granules.²⁸ A quantitative study of the mechanisms of eosinophil degranulation in nasal polyps found that approximately 33% of eosinophils had undergone cytolysis, whereas most eosinophils showed evidence of piecemeal degranulation.⁷

In this study we found that almost all of the eosinophils invading the esophagus in patients with EoE showed changes of activation and degranulation with marked cytoplasmic vesiculation and varying degrees of membrane disruption. These changes were associated with the release of intact, membrane-bound granules into the tissues. The proportion of eosinophils with no changes or only 1 change was remarkably small: 1.3% of eosinophils appeared normal without change, eosinophils with cytoplasmic vesiculation and no loss of membrane integrity or reversal of granule core staining comprised 1.7%, eosinophils with reversal of granule core staining and no loss of membrane integrity or cytoplasmic vesiculation comprised 2%, and eosinophils with disruption of membrane, no cytoplasmic vesiculation, or reversal of granule core staining comprised 0.9%. Therefore, fewer than 6% of the eosinophils showed only 1 or none of the scored features, and approximately 93% showed 2 or 3 of the features.

Fig 7 is a bar graph summarizing the distribution of features observed in the eosinophils in the biopsy specimens from the 9 patients with EoE based on the evidence that activated and degranulating eosinophils exhibit reversal of granule core staining and piecemeal degranulation. In comparison with the previous study showing the predominance of piecemeal degranulation over cytolysis,⁷ we observed that the majority of eosinophils (>80%) undergoing piecemeal degranulation and reversal of granule core staining also showed cytolysis, as evidenced by loss of cytoplasmic membrane integrity (70.3%, Fig 6). This finding suggests that cytoplasmic vesiculation leads to enhanced secretion and eosinophil cytolysis.

Another striking finding in these studies was the frequency of extracellular eosinophil granules in tissues from patients with EoE, indicating that cytolysis results in liberation of

granules into extracellular sites. Studies of isolated eosinophil granules have demonstrated that extracellular granules retain secretory activity and thus, have the ability to secrete individual granule proteins into tissues after stimulation.²⁹ Notably, prior studies demonstrated that striking eosinophil granule protein deposition occurs in patients with EoE.^{4,6} The compilation of findings in this electron microscopic study along with the existing understanding of eosinophil activity provides strong evidence of eosinophil activation with degranulation in EoE tissues, including deposition of cytotoxic granule proteins.

The findings reported in this study further indicate that a large proportion of eosinophils in biopsy specimens from patients EoE enumerated by means of light microscopy after H&E staining are undergoing cytolysis, leading to release of granules and granule proteins onto esophageal tissues. The finding that approximately 80% of eosinophils recognized by electron microscopy in biopsy specimens from patients with EoE are undergoing cytolysis suggests that most of the eosinophils counted by pathologists using light microscopy are, in fact, already showing membrane disruption and thus are classifiable as dying cells. Along with the observations that striking eosinophil granule protein deposition occurs in tissues from patients with EoE, these observations provide compelling evidence that the full gamut of proinflammatory activities of the granule proteins is unleashed onto the epithelial cells. Moreover, it seems likely that eosinophil participation in patients with EoE is only partly recognized by enumerating eosinophils. In some cases, the possibility exists that the process of eosinophil cytolysis and loss of morphology is sufficiently advanced that few, if any, intact cells are recognizable. In these cases one would overlook a diagnostic criterion for EoE when the effects of eosinophil inflammation are maximal. Overall, our findings imply that localization of eosinophil granule proteins by using immunostaining, such as for major basic protein 1 or cytochemical localization for eosinophil peroxidase, might be a better indicator of disease activity than counting of dying eosinophils.

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Key messages

- Degranulation patterns of esophageal biopsy specimens obtained at endoscopy from 9 randomly selected patients with EoE were studied.
- Eosinophils in esophageal biopsy specimens from patients with EoE are abnormal, with greater than 80% showing cytolysis.
- Evaluation by means of light microscopy after H&E staining might not accurately reflect eosinophil involvement.

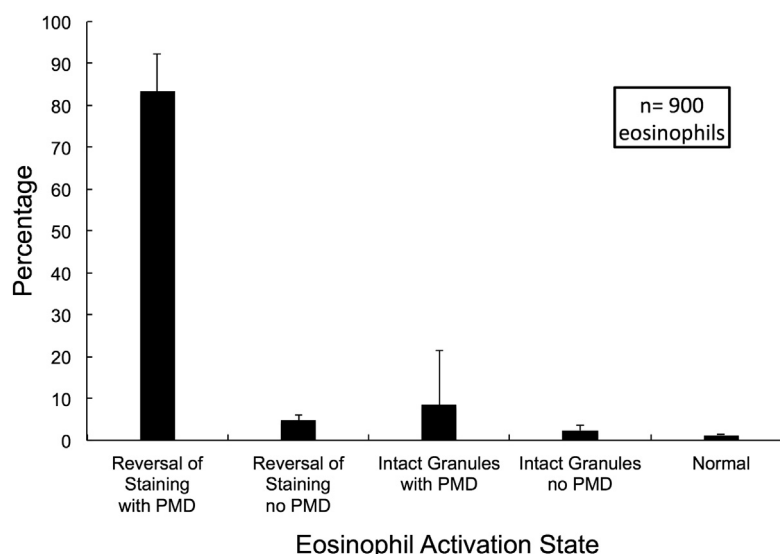


FIG 7. Percentage of eosinophils with cytoplasmic vesicles and granule abnormalities from electron microscopy of 9 patients with EoE. The averages and SDs (error bars) of the 3 graders (H.S., L.H.H., and G.J.G.) are reported. *PMD*, Piecemeal degranulation.

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TABLE E1. Averages and SDs of 1672 electron photomicrographs (9 patients with EoE, 900 eosinophils) graded by 3 researchers based on membrane integrity; evidence of cytoplasmic vesiculation, including sombrero vesicles; granule morphology; and extracellular granule location

	Free granules	Sombrero vesicles	Cytoplasmic vesiculation	Normal eosinophils
Average $\pm$ SD	1185 $\pm$ 68	641 $\pm$ 55	483 $\pm$ 66	11 $\pm$ 2
Eosinophils: Reversal of staining (cell activation) with piecemeal degranulation				
	Intact membrane	Mainly intact membrane	Partially disrupted membrane	Fully disrupted membrane
Average $\pm$ SD	116 $\pm$ 34	124 $\pm$ 21	175 $\pm$ 42	334 $\pm$ 26
Eosinophils: Reversal of staining (cell activation) without piecemeal degranulation				
	Intact membrane	Mainly intact membrane	Partially disrupted membrane	Fully disrupted membrane
Average $\pm$ SD	18 $\pm$ 7	4 $\pm$ 2	6 $\pm$ 3	15 $\pm$ 3
Eosinophils: Normal granules with piecemeal degranulation				
	Intact membrane	Mainly intact membrane	Partially disrupted membrane	Fully disrupted membrane
Average $\pm$ SD	16 $\pm$ 20	18 $\pm$ 22	6 $\pm$ 7	36 $\pm$ 47
Eosinophils: Normal granules without piecemeal degranulation				
	Intact membrane	Mainly intact membrane	Partially disrupted membrane	Fully disrupted membrane
Average $\pm$ SD	13 $\pm$ 4	6 $\pm$ 4	1 $\pm$ 0	2 $\pm$ 2

Cytoplasmic membrane integrity was classified as intact (no membrane loss), mainly intact ($\leq 20\%$ membrane loss), partially disrupted ($>20\%$ membrane loss), or fully disrupted (no recognizable membrane).