

An Improved Method to Visualize Eosinophils in Eosinophilic Esophagitis

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Abstract. We previously found in Giemsa-stained colorectal sections from IBD patients that eosinophilic granulocytes turned fluorescent when excited with indirect fluorescent light, while other inflammatory cells were non-fluorescent. We now studied with that method, the frequency of eosinophilic granulocytes in sections from patients with eosinophilic esophagitis (EE). Cell counting was done in consecutive sections stained with Giemsa stain using indirect fluorescence light (G-IFL setting) and with hematoxylin-eosin using transmitted light (HE-TL setting) in 5 cases of EE and in 10 consecutive cases of reflux esophagitis (RE) grade 2. In EE 45.0 eosinophils/case (range 39-51) were recorded with the G-IFL setting but only 33.4 eosinophils/case (range 28-39) with the HE-TL setting ($p < 0.05$). In RE cases, 3 eosinophils/case (range 2-4) were found with the G-IFL setting and 2 eosinophil/case (range 1-3) with the HE-TL setting. The G-IFL method is not only more sensitive in detecting eosinophils than the conventional HE-TL method but also quicker, since a differential cell counting is not necessary.

The eosinophilic infiltration of the esophageal epithelium, initially considered to be a manifestation of eosinophilic gastroenteritis is today regarded as a distinct clinical entity. Already in 1981 Picus and Frank (1) described a case of eosinophilic esophagitis (EE) and one year later Munch *et al.* (2) reported a second case of EE. In 1983, Feczko *et al.* (3) described 3 cases of EE and in 1985, Lee (4) found 11 cases of EE among young patients, with an average age of 14.6 years. Three of their patients (4) had an esophageal stricture and one peripheral eosinophilia. The critical review of the literature by Lee in 1985 (4) disclosed three additional cases. In 1993 Attwood *et al.* (5) also recorded the infiltration of eosinophilic granulocytes in the distal

esophagus in pediatric patients. This subset of esophageal inflammation was later reported in adults (6-8).

Patients with EE usually consult for symptoms that may mimic gastro-esophageal reflux (GER), but medications used to treat gastric reflux are not effective (9). Peripheral eosinophilia is found in about 40% of the patients with EE and some of them develop asthma (10). Other EE patients have symptoms related to the thickening of the esophageal wall with narrowing of the lumen (11-15), symptoms that may be confused with those of an infiltrating tumour in the distal esophagus. In this respect, Evrard *et al.* (16) reported in a 72-year-old man with severe dysphagia and weight loss, an endoscopical lesion compatible with an infiltrating esophageal tumor. The surgical resection of the esophagus revealed, however, only EE at histology.

EE is a chronic process. Straumann *et al.* (17) studied 8 patients with EE having an average disease-duration of 13.6 years (range 2-26 yr).

Recent developments indicate that eosinophils express several growth factors, among them TGF- β 1, important in connective tissue remodelling (17). Among other effects or functions, TGF- β 1 stimulates fibroblast proliferation, enhances fibroblast collagen synthesis, and inhibits expression of the collagenase gene, thereby decreasing collagen degradation (18).

Although the cause(s) for the gathering of eosinophils within the squamous epithelium of the distal esophagus remains unclear some authors claim that EE is part of an allergic response to food antigens (9, 19). It should be mentioned that antigen-IgE complexes activate eosinophilic granulocytes (20). Eosinophils may bind IgE, and can elaborate many inflammatory mediators (19). Because of these characteristics eosinophils may be directly implicated in the inflammatory process (21).

To diagnose EE, biopsies of the esophagus are necessary. Pathologists diagnose EE in hematoxylin and eosin (HE) stained sections using transmitted light (TL). There is, however, disagreement regarding the number of eosinophils per high power field that are required to diagnose EE at histology. Orenstein *et al.* (22) define EE when ≥ 5 eosinophils

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Table I. Clinical data and histological findings in 5 patients having eosinophilic esophagitis (EE).

Patient #	Age	Gender	GI symptoms/ other diseases	Endoscopy	Eosinoph. /HPF (HE-TL)	Eosinoph. /HPF (G-ILF)	Initial histologic diagnosis	Follow-up
1	68	Female	Protracted dysphagia, achalasia, hypertension, hyperthyreosis	Easy bleeding distal mucosa	39	51	EE	Renewed biopsy 4 month later: EE
2	15	Male	Weight loss, increased fatigue, high levels of calprotectin (IBD?)		34	46	Esophagitis grade 2	--
3	5	Female	Vomits (GERD?, achalasia?)	Slight inflamm. distal esophagus covered with fibrin	28	39	Esophagitis grade 2	--
4	59	Male	Diarrhea (celiac disease?) Eosinophilia	distal esophagus: slightly bleeding	35	47	EE	Colonoscopy Normal histology
5	15	Male	Nocturnal vomits. Peri-umbilical pain, HP positive serology	distal esophagus and stomach: slightly bleeding	31	42	Esophagitis grade 2	One year previously: No esophagitis

HPF: high power field, TL: transmitted light, ILF: indirect light fluorescence, H&E: hematoxylin-eosin stain, G: Giemsa stain.

per high power field are present in the distal epithelium of the esophagus, Lim *et al.* (23) and Potter *et al.* (8) when ≥ 15 eosinophils are found, Arora *et al.* (12) Sant'Ana *et al.* (24) and Zimmermann *et al.* (25) if > 20 eosinophils are recorded and Noel *et al.* (26) if ≥ 24 eosinophils per high power field are found.

In a previous study we described a novel method to detect eosinophilic granulocytes in the colonic mucosa (27). Using the same method of observation we investigated 5 cases of EE recently found at this department.

Materials and Methods

The material comprised esophageal biopsies from 15 patients: 5 with EE and 10 unselected controls with reflux esophagitis (RE) grade 2 (*i.e.*, showing architectural changes and granulocytic infiltration) seen at this department during 2003 and 2005. Cell counting was done at high power examination (400x) in consecutive sections stained with hematoxylin and eosin (H&E) seen with transmission light (TL, Labophot-2, Nikon microscope) and in Giemsa (G) excited with indirect fluorescent light (IFL, Axioscope, Leitz, fluorescent microscope green H filter, 546 nm wave length) (26). By the aid of the Giemsa sections and IFL (G-ILF setting) we recently demonstrated that eosinophilic granulocytes in the colonic mucosa of patients with IBD (26) turned fluorescent and were easily discernable against the non-fluorescent background. No other inflammatory cell turned fluorescent with that method (26). When HE stained sections were observed with IFL, eosinophils were not fluorescent. The only structures that were fluorescent were the basement membrane (28) and erythrocytes.

Following the recommendations of Wang, Mangano and Antonioli (29) for intraepithelial lymphocytes in the esophagus, the

counting of intraepithelial eosinophils was done at high power examination in the most densely populated well-oriented field. According to those authors (29) the selection of one field is better than results based on the average count of three or five fields for two reasons. First, the number of cells to be counted may vary from one field to another and the average count might not sufficiently reflect the severity of changes in the entire biopsy. The second reason is that the average number of eosinophils derives from varying number of fields as some of the biopsies may contain less than three well-oriented fields at high power examination. Before counting all sections in the 15 cases were coded to avoid bias. After counting, all sections were decoded.

PAS stain was done in all cases. Data were analysed by the nonparametric Wilcoxon test: $p < 0.05$ was considered significant.

Results

The blind review of coded sections indicated that there were two groups of patients: one having high numbers of eosinophils infiltrating the squamous epithelium of the esophagus ($n=5$ cases) and the other infiltrated by occasional eosinophils ($n=10$ cases). The decoding of the sections in the 15 cases showed that 10 corresponded to cases of RE and that the remaining 5 cases corresponded to those with EE. In EE intraepithelial eosinophils could be found in all cell layers of the squamous epithelium but particularly in superficial cell layers (Figures 1 and 2). In RE the few eosinophils present were found haphazardly distributed within the entire thickness of the squamous epithelium. PAS stain revealed no *candida albicans*.

Table I shows that when counting was done with the G-ILF setting as many as 45.0 eosinophils/case (mean, range

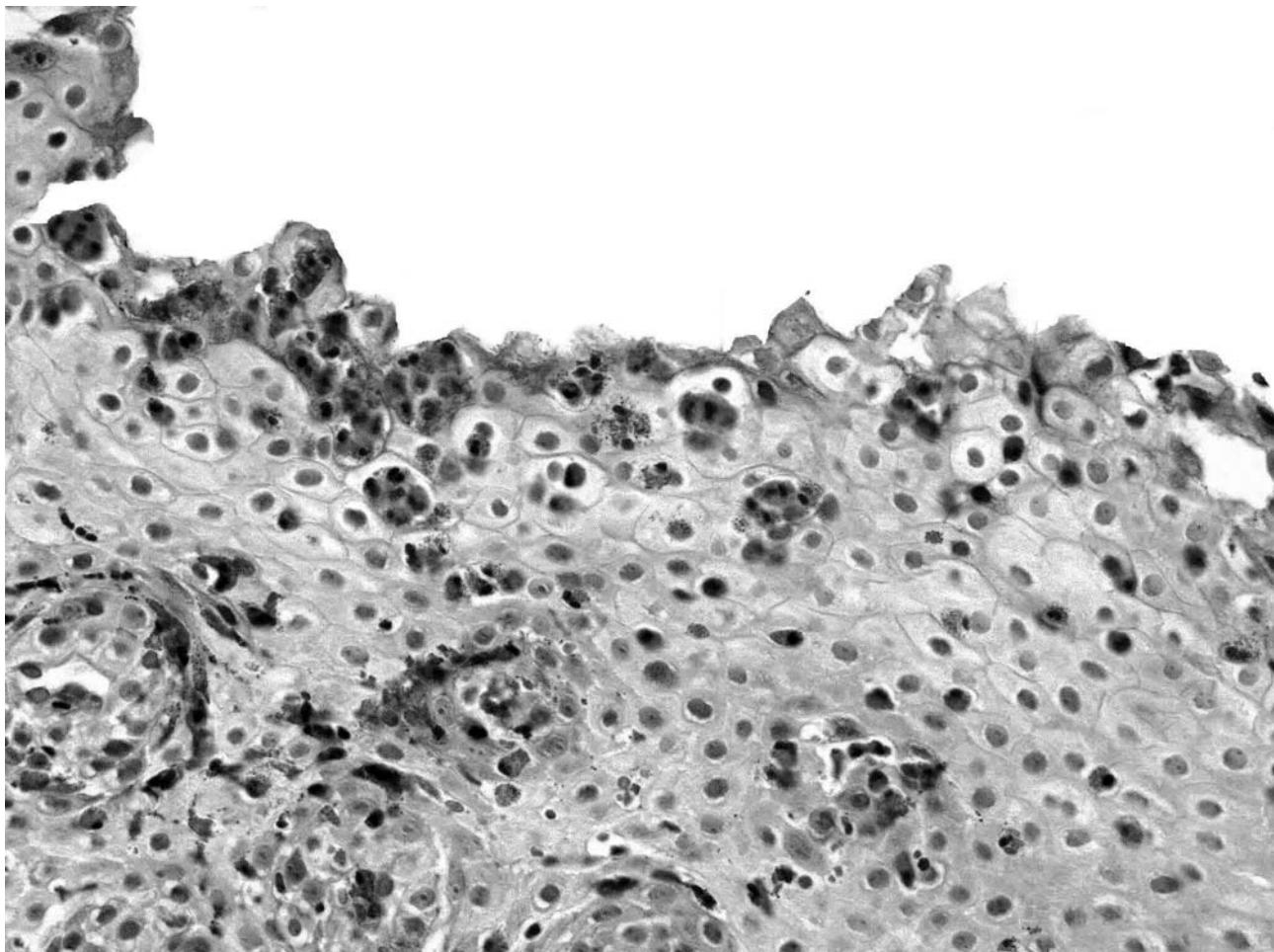


Figure 1. *Esophagus epithelium in EE. Note epithelial infiltration by eosinophilic granulocytes, particularly in the superficial layers of the epithelium (H&E, transmitted light, 20x, actual size).*

39-51) were recorded but when using the HE-TL setting only 33.4 eosinophils/case (range 28-39) were found. The difference was significant ($p<0.05$). In RE 3 eosinophils/case (range 2-4) were found with the G-IFL setting and 2 eosinophil/case (range 1-3) with the HE-TL setting.

The clinical data in the 5 patients with EE are shown in Table I. It is seen that 3 patients were males and the remaining 2 patients females. The mean age was 32.4 years (range 5-68 years). Three out of the 5 patients (67%) were ≤ 15 years of age. The Table also shows that out of the 5 patients 2 had symptoms compatible with gastro-esophageal reflux disease. In addition, one patient had vomiting and peri-umbilical pain and in the other, *achalasia cardiae* was suspected. Two patients had weight loss and fatigue (one had increased levels of calcoprotectin, and the other showed eosinophilia in peripheral blood), one had both *achalasia*, hypertension and hyperthyreosis.

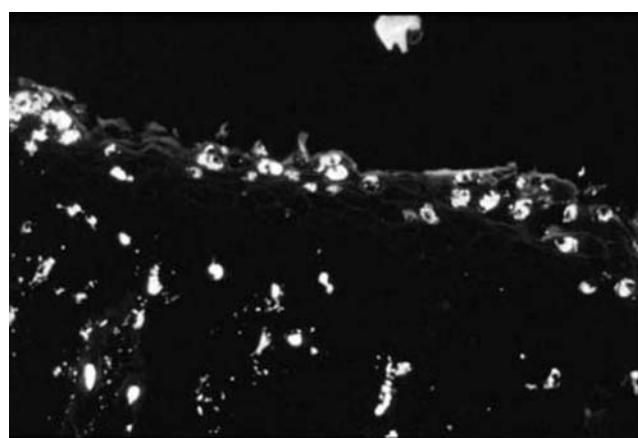


Figure 2. *Esophagus epithelium in EE. Note fluorescent eosinophils, particularly infiltrating the upper layers of the epithelium (Giemsa, indirect fluorescent light, 20x, actual size).*

During the same endoscopy biopsies from other organs of the GI tract were taken in 4 out of the 5 patients. In 3 out of the 4 patients, biopsies were taken from the corpus, from the antrum pylori and from the duodenum (in one of them biopsies from the distal ileum, the colon at 9 different levels and the rectum were also available) and in the fourth patient a biopsy from the duodenum. Eosinophils were not increased in any of the biopsies taken from those mucosas of the GI tract in patients with EE.

In one out of the 5 patients esophageal biopsies were repeated 4 months later; that biopsy showed EE. In another patient, a biopsy of the esophagus taken 12 months previously showed esophagitis with occasional eosinophils.

Discussion

The results demonstrated that in EE the number of eosinophils/high power field was higher when counting was done with the G-IFL setting than when using the conventional HE-TL setting. Could the counting of eosinophils in the two consecutive sections – one stained with HE and the other with G – explain the discrepancy in results? This possibility was rejected, as the number of eosinophils was repeatedly higher in the G-IFL setting than in the conventional HE-TL setting, using differential cell counting. With the G-IFL setting eosinophils were more obvious against the non-fluorescent dark background.

In blood smears the nucleus of eosinophilic granulocytes is bi-lobulated, but in tissue sections, depending upon the level at which the nuclei had been sectioned, some eosinophils appear as having a small, non-lobulated nucleus and others with two minor nuclei. Some observers may disregard in differential cell counting in HE-stains, sectioned eosinophils displaying uni-lobulated, small nuclei or two minor nuclei and a minor fraction of cytoplasm. Against that background we counted 1,000 consecutive cells having uni-lobulated, small nuclei or two minor nuclei and a small fragment of cytoplasm carrying eosinophilic granules in the HE-TL setting. As many as 19.3% (193/1000) eosinophils had these characteristics. The question arises: Could this circumstance partly explain the discrepancy in the number of eosinophils required to define EE reported by various authors, from ≥ 5 (22) to > 20 (12,24,25) and to ≥ 24 (26) per high power field?

EE is a distinct histological and clinical therapeutic entity, at variance with RE. The histological difference between RE and EE is based on the number of eosinophilic granulocytes infiltrating the squamous epithelium.

In conclusion, in differential cell counting of conventional HE sections using TL, eosinophils are counted amidst neutrophils, lymphocytes, plasma cells, monocytes, mast cells and macrophages. Which can be time consuming. On the other hand, the herein described technique of counting

only fluorescent eosinophils permits the easy identification of those cells without applying differential cell counting.

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