

Cytokine Expression in Normal and Inflamed Esophageal Mucosa: A Study into the Pathogenesis of Allergic Eosinophilic Esophagitis

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ABSTRACT

Objectives: We studied the expression of cytokines and inflammatory cells in normal and inflamed esophageal mucosa of children with the aim of furthering our understanding of the pathophysiology of allergic eosinophilic esophagitis (AEE).

Methods: Controls and AEE patients (≥ 15 eosinophils/high-power field on esophageal mucosal biopsies) between the ages of 1 and 18 years were recruited. Esophageal biopsies were obtained for histologic examination, immunohistochemical studies, and cytokine analysis.

Results: Eight controls (4 males; mean age 9.99 years) and 11 AEE patients (8 males; mean age 7.15 years) were studied. mRNA expression of interferon (IFN)- γ , interleukin (IL)-4, IL-5, IL-13, eotaxin-1, eotaxin-2, eotaxin-3, and RANTES was

studied. IFN- γ and IL-5 expressions were significantly up-regulated in AEE patients compared with controls. Expressions of IL-4 and IL-13 were similar between AEE patients and controls. Eotaxin-1 expression was significantly up-regulated in AEE patients, whereas eotaxin-2 was up-regulated in controls. Expression of RANTES and eotaxin-3 was similar between the two groups. There was increased staining for mast cells in AEE patients compared with controls.

Conclusions: Our data suggests that AEE is primarily an IL-5 selective TH2 response, with a possible TH1 component, and a differential role of eosinophilic chemoattractants. The role of mast cells in the pathogenesis of AEE needs additional study. *JPGN* 42:22–26, 2006. **Key Words:** Allergic eosinophilic esophagitis—Pediatrics—Cytokines. © 2006 Lippincott Williams & Wilkins

INTRODUCTION

Allergic eosinophilic esophagitis (AEE) is an emerging, incompletely understood disease. It is being increasingly encountered in children of all ages and in adults (1–3). The initial descriptions of this entity date back to the 1970s (1), although wider appreciation of this disease occurred in the mid to late 1990s after a report by Kelly et al. (2) in which an elemental diet was used to treat 10 children with eosinophilic esophagitis who had failed antireflux therapy. All 10 patients demonstrated a resolution or improvement of symptoms and attenuation of the eosinophilic inflammation.

The diagnostic criteria of AEE are not completely established but usually include the presence of more than 15 eosinophils/ high-power field (hpf) on esophageal mucosal biopsies and the absence of pathologic gastroesophageal reflux on 24 hour continuous monitoring of intraesophageal pH (4). At endoscopy, vertical lines in

the esophageal mucosa, circular rings in the esophagus, and whitish specks adherent to the esophageal mucosa highly suggest AEE (5,6). The main therapies for AEE are corticosteroids (either systemic or topical) and an elemental diet (2,7–10). Patients may, however, suffer disease relapse on discontinuation of therapy (2,7).

The esophageal inflammation in AEE is thought to be allergen mediated rather than acid mediated. The culprit allergen is presumed to be a food protein because improvement may occur after institution of an elemental diet (2,10). There is some data to support a role for aeroallergens (11). Broad gaps exist in our knowledge of the pathophysiology of this entity, including how the putative allergen(s) produce the intense eosinophilic inflammation. We studied the expression of cytokines and inflammatory cells in normal and inflamed esophageal mucosa of children with the aim of furthering our understanding of the pathophysiology of AEE.

MATERIALS AND METHODS

Patients

Patients between 1 and 18 years who underwent esophago-gastroduodenoscopy through the Division of Pediatric Gastroenterology at the James Whitcomb Riley Hospital for Children,

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These data were presented at the 16th Annual Meeting of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition, October 2003 and received the Young Faculty Clinical Investigator Award.

Indiana University School of Medicine (Indianapolis, IN) were considered for the study. We recruited controls (with normal endoscopic and histologic appearance of the upper gastrointestinal tract) and patients with AEE (with ≥ 15 eosinophils/hpf in esophageal mucosal biopsies).

Two biopsies of the distal esophagus were secured from controls for histologic study. In patients with AEE, at least four esophageal biopsies (2 of the distal and 2 of the mid esophagus) were examined histologically. Esophageal epithelial eosinophils were counted, and the maximal number per hpf recorded. Biopsies of stomach and duodenum were secured from all study subjects for histologic examination and were free of significant histologic abnormalities. The subjects were not on anti-inflammatory agents such as corticosteroids or leukotriene receptor antagonists at the time of the endoscopy.

Three additional esophageal biopsies were obtained for cytokine analysis. These were snap frozen in liquid nitrogen and stored at -70°C . The study was approved by the institutional review board of our institution.

Cytokine Analysis

Total RNA Isolation

RNA extraction from tissue biopsies was performed using a modification of original methods of Chomczynski and Sacchi (12). The average yield of total RNA was $1.37 \mu\text{g}/\text{mg}$ tissue.

Realtime Polymerase Chain Reaction

Total RNA ($1\text{--}2 \mu\text{g}$) was reverse transcribed with Superscript II Plus Reverse Transcriptase (Gibco BRL Life Technologies, Gaithersburg, MD) using $100 \mu\text{M}$ random hexamer primers (Pharmacia Biotech, Uppsala, Sweden) according to manufacturer's instructions. Realtime polymerase chain reaction (PCR) was performed using specific primers for human cytokines and chemokines designed using NCBI BLAST search program. The following primers were used to examine expression of the cytokines at the mRNA in both controls and patients with AEE: interferon (IFN)- γ , interleukin (IL)-4, IL-5, IL-13, eotaxin-1, 2, and 3, regulated on activation normal T-cell expressed and presumable secreted (RANTES):

IFN- γ forward primer, 5'-CTCTTCGACCTCGAAACAGC-3'
 IFN- γ reverse primer, 5'-GTCCAACGCAAAGCAATACA-3'
 IL-4 forward primer, 5'-CTCTTCGACCTCGAAACAGC-3'
 IL-4 reverse primer, 5'-GAGTCAACGGATTGTCGT-3'
 IL-5 forward primer, 5'-GCAGTGCCAAGGTCTCTTTC-3'
 IL-5 reverse primer, 5'-CAAACGCAGAACGTTTCAGA-3'
 IL-13 forward primer, 5'-TTTCAGTTGAACCGTCCCTC-3'
 IL-13 reverse primer, 5'-GTCTCAGCTGGGCAGTTTC-3'
 Eotaxin -1 forward primer, 5'-ACTTCATGGAATCCTGCACC-3'
 Eotaxin -1 reverse primer, 5'-ACAGGAGAATCAC-CAGTGGC-3'
 Eotaxin -2 forward primer, 5'-CCATAGTAACCAGCCTTC-3'
 Eotaxin -2 reverse primer, 5'-CAGGTTCTTCATGTACCTC-3'
 Eotaxin -3 forward primer, 5'-AACTCCGAAACAATTGTGACTCAGCTG-3'
 Eotaxin -3 reverse primer, 5'-GTAACCTCTGGGAGGAAACACCTCTCC-3'
 RANTES forward primer, 5'-CTCAAGTGATCCACCACCT-3'

RANTES reverse primer, 5'-GAGGCTTCCCCTCAC-TATCC-3'

18S rRNA forward primer, 5'-CGGGATCCATCAGAC-CAAAACCAACC-3'

18S rRNA reverse primer, 5'-CCCAAGCTTGACAGACAGTACCATCG-3'.

cDNA was synthesized after DNase I treatment from total RNA. cDNA ($1\text{--}2 \mu\text{L}$) was used in quantitative realtime PCRs using fluorescent dye SYBR Green (Applied Biosystems) and the Perkin-Elmer 5700 GeneAmp Sequence Detection System. The threshold cycles C_T of cytokines and chemokines gene expression from control and AEE samples were compared. The C_T is when the system begins to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase. This is the point on the growth curve when the log-linear phase begins and represents the greatest rate of change along the growth curve. The higher the initial amount of gene expression, the sooner accumulated product is detected in the PCR process and the lower the C_T value. The results are derived from three independent experiments and quantitated using 18S rRNA as the internal control.

Immunohistochemistry

Paraffin sections of esophageal biopsies were dewaxed in xylene, rehydrated, and subjected to protease K (Dako, Carpinteria, CA) digestion for antigen retrieval. The sections were then incubated with mouse anti-mast cell tryptase (Dako) followed by biotinylated anti-mouse immunoglobulin G and horseradish peroxidase-labeled avidin (LSAB2 system, Dako). Enzyme activity was revealed using aminoethylcarbazole as substrate.

Statistical Analysis

The C_T values for all patients in each group were averaged and the means compared between the two groups. Statistical analysis was performed using SigmaStat software, version 2.03, from SPSS, Inc. (Chicago, IL). Analysis between the two groups for the analytic assays was performed by unpaired two-tailed Student's t test. A P value of less than 0.05 was considered significant.

RESULTS

Eight controls (4 males; mean age 9.99 years; age range 3.25–15.08 years) and 11 AEE patients (8 males; mean age 7.15 years; age range 2.08–13.92 years) were studied. The main presenting symptoms in controls were abdominal pain ($n = 8$) and emesis ($n = 2$). In AEE patients, the main presenting symptoms were emesis ($n = 6$), abdominal pain ($n = 2$), dysphagia ($n = 2$), and heartburn ($n = 1$). Esophageal biopsies were histologically normal in all controls. Patients with AEE had a mean esophageal epithelial eosinophilia of 37 eosinophils/hpf (range 20–70 eosinophils/hpf); the esophageal inflammation was diffuse in some and patchy in others.

The expression of cytokines IFN- γ , IL-5, IL-4, IL-13, eotaxin-1, eotaxin-2, eotaxin-3, and RANTES at the mRNA level are shown in Figure 1. Expression of IFN- γ was significantly up-regulated in the disease group compared with controls ($P = 0.005$), as was IL-5 ($P = 0.015$). Expressions of

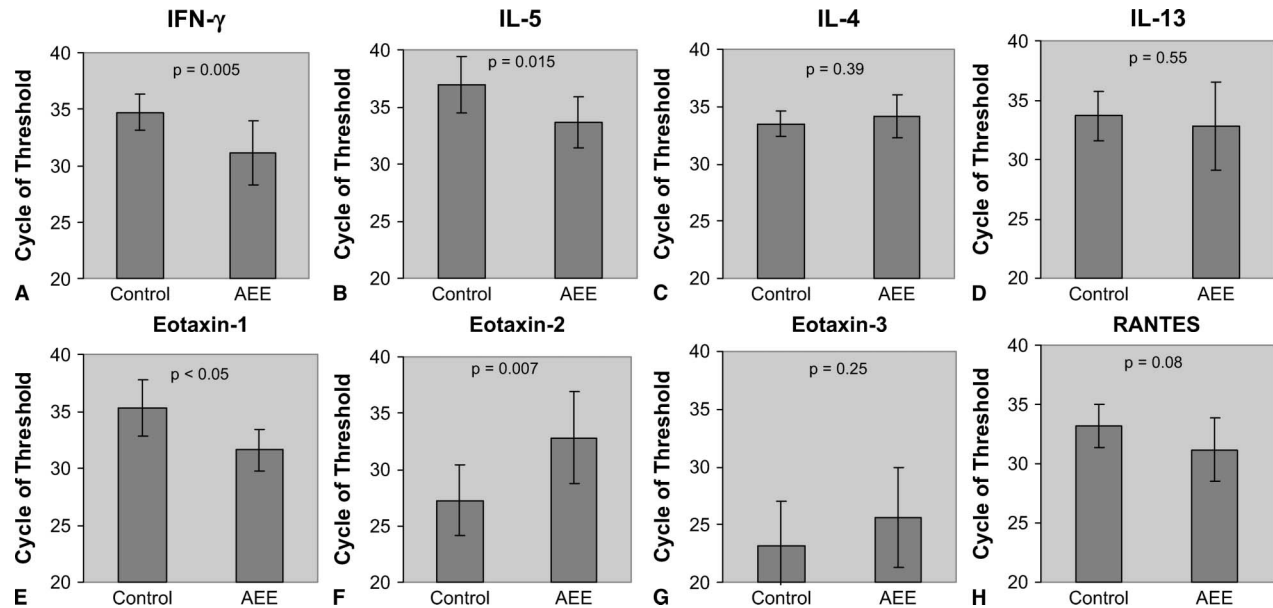


FIG. 1. Expression of the various cytokines in controls and patients with allergic eosinophilic esophagitis. The higher the initial amount of gene expression, the sooner accumulated product is detected in the polymerase chain reaction process, and the lower the cycle of threshold (C_T) value.

IL-4 and IL-13 were similar between the groups ($P = 0.39$ and 0.55 , respectively). Eotaxin-1 expression was significantly up-regulated in patients with AEE versus controls ($P < 0.05$). Expression of RANTES was higher in the diseased group, but this did not reach statistical significance ($P = 0.08$). Expression of eotaxin-2 was up-regulated in controls ($P = 0.007$), whereas that of eotaxin-3 was similar between the two groups ($P = 0.25$). We found increased staining for mast cells in AEE patients compared with controls (Figures 2 and 3).

DISCUSSION

In this study, we focussed on the cellular responses involved in the pathogenesis of AEE based on cytokine

levels and immunohistochemistry techniques. Expression of IL-5 was significantly up-regulated in the esophageal mucosa of our patients with AEE compared with controls. IL-5 has several stimulatory effects on eosinophils and promotes their proliferation, maturation, migration, activation, degranulation, and survival, and its upregulation would suggest that AEE is a TH2 response. Although we did not find up-regulation of other TH2-specific cytokines, such as IL-4 and IL-13 expression, this is not entirely unexpected. IL-4 and IL-13 tend to modulate other cellular components of the TH2 response, specifically B cells, rather than eosinophils (13,14). Current knowledge and data do not support a role for humoral response/B cells in the pathogenesis of AEE.

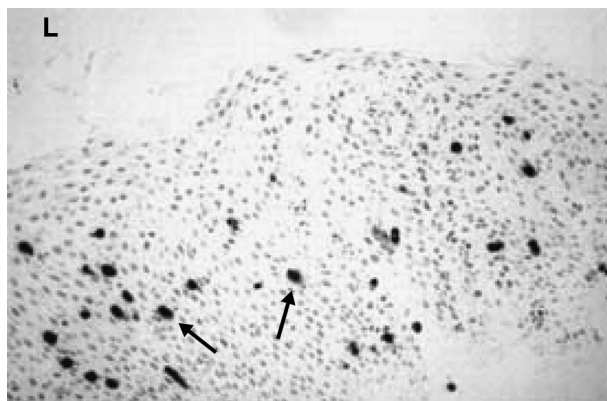


FIG. 2. Immunohistochemistry for mast cell tryptase reveals numerous mast cells (arrow) in the esophageal epithelium of a patient with allergic eosinophilic esophagitis. L, lumen.

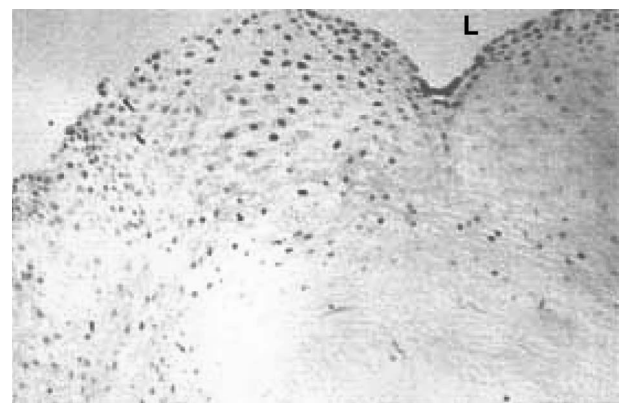


FIG. 3. Immunohistochemistry for mast cell tryptase did not reveal any mast cells in control esophageal epithelium. L, lumen.

Compared with controls, patients with AEE had up-regulated expression of IFN- γ , a TH1 cytokine. A number of reasons could explain a secondary role for TH1 responses in allergic diseases. IFN- γ is known to positively affect eosinophils. Specifically, IFN- γ augments expression of CD69 on the eosinophil surface, activates eosinophils, and enhances their survival (15). Teitelbaum et al. (16) found a significantly greater number of esophageal mucosal CD3+ and CD8+ T lymphocytes in patients with AEE. CD8+ T lymphocytes classically secrete TH1 cytokines. Straumann et al. (17) reported elevated levels of tumor necrosis factor- α , a classic TH1 cytokine, in the esophageal biopsies of eight adult patients with AEE.

We examined the expression of eosinophil chemoattractants to investigate how inflammation is sustained in AEE. The eosinophil chemoattractants eotaxin-1 and RANTES were up-regulated in AEE patients compared with controls. Straumann et al. (17) reported increased levels of RANTES, but not eotaxin, in eight adults with AEE; however, they did not specify which eotaxin was examined. We found no difference in the expression of eotaxin-3 between the groups. Interestingly, eotaxin-2 was preferentially up-regulated in controls compared with AEE patients. These results raise several intriguing possibilities. It is plausible that eotaxin-2, which is constitutively expressed (18), has a negative feedback effect on eotaxin-1 and is able to attenuate eotaxin-1 expression in control states but not in inflamed conditions. Murine eotaxin-2 is involved in IL-4 associated immune responses (18), and it may not be induced in the absence of IL-4 upregulation, as was seen in our AEE patients. Last, expression of the three eotaxins occurs at distinct times during the course of an allergic inflammatory reaction, and each eotaxin may be responsible for different phases of leukocyte/eosinophil recruitment (19).

Immunohistochemistry staining of esophageal biopsies was conducted to further elucidate the cellular basis of AEE. Increased staining for mast cells was observed in AEE patients compared with controls. The increased staining for mast cells is in keeping with earlier data (17) and would suggest a role for these cells in the pathogenesis of AEE. Similar to eosinophils, activated mast cells release potent inflammatory mediators such as leukotrienes, histamine, and platelet-activating factor (17). Mast cells can also activate eosinophils through the generation of eosinophil-directed cytokines, such as IL-5 (20).

This is a preliminary study and among the first such studies in children with AEE. Other TH1 cytokines, in addition to IFN- γ , need to be examined to clarify the role of the TH1 cellular cascade in the pathogenesis of AEE. Even though our immunohistochemistry data is in keeping with previous reports (17), further study is necessary. Although we found an increase in the numbers of mast cells by immunohistochemistry, we are unable to definitively implicate mast cells in the pathogenesis of AEE because IL-4, a key regulator of mast cells (21), was not up-regulated in our patients with AEE.

In summary, this study scrutinizes the molecular and immunologic basis of AEE. Our data suggest that AEE is primarily an IL-5 selective TH2 response, with a possible TH1 component and a differential role for eosinophilic chemoattractants. It is plausible that a dietary allergen is taken up by a resident antigen-presenting cell (APC) (e.g., a dendritic cell or macrophage) within the esophageal mucosa. The APC stimulates TH2 cells residing in lymphoid tissue by way of adhesion molecules and unleashes an intense inflammatory response with release of cytokines, mainly IL-5. The combined chemokinetic actions of IL-5 and the chemotactic effects of eotaxin act in synergy to induce and sustain a considerable mucosal eosinophilia. The role of mast cells in the pathogenesis of AEE deserves further investigation. This study raises several new questions. What is the cellular source of eotaxin and RANTES; what is the significance of the different subtypes of eotaxins; what are the concentrations of other TH1 cytokines in esophageal mucosa of AEE; what is the role of mast cells in AEE; and is there an identifiable APC?

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