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ORIGINAL CONTRIBUTIONS

Esophagus

Treatment With Topical Steroids Downregulates IL-5, Eotaxin-1/CCL11, and Eotaxin-3/CCL26 Gene Expression in Eosinophilic Esophagitis

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OBJECTIVES: Our aim was to evaluate the changes induced by topical steroid treatment to the esophageal epithelial inflammatory eosinophilic and T-cell infiltrate and to IL-5, eotaxin-1/CCL11, and eotaxin-3/CCL26 esophageal gene expression levels in patients with eosinophilic esophagitis (EE).

METHODS: Esophageal biopsies were taken from eight adult patients at the moment of diagnosis and after 3-month treatment with fluticasone propionate. Eosinophils, CD8, and CD4 T cells were examined by immunohistochemistry. IL-5, eotaxin-1/CCL11, and eotaxin-3/CCL26 gene expression levels were measured by real-time PCR. Eight control samples were also analyzed.

RESULTS: A significant decrease in the eosinophil infiltrate and in $CD8^+$ T-cell density was observed in the esophageal epithelium from the patients upon steroid treatment. IL-5 was not detected in control samples, and expression levels were variably downregulated after treatment in six of the patients. Gene expression of eotaxin-1/CCL11 showed relevant downregulation in four cases and a modest twofold decrease in three of the patients studied. Mean CCL11 expression values upon steroid treatment were similar to control samples (19.4 ± 28.6 *vs* 8.42 ± 5, $P = 0.7$). Eotaxin-3/CCL26 gene expression levels were significantly increased in EE. Although they were significantly downregulated upon steroid treatment, control expression levels were not reached in any of the cases analyzed (580.9 ± 943.9 *vs* 1.45 ± 1.0, $P = 0.001$).

CONCLUSIONS: Our results confirm that eotaxin-3/CCL26 is significantly increased in EE esophageal samples. However, the individual analysis of IL-5, CCL11, and CCL26 expression data suggests that several cytokines and chemokines could participate in the physiopathology of EE in humans.

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INTRODUCTION

Eosinophilic esophagitis (EE) is a clinicopathologic disease characterized by esophagus-related symptoms and dense esophageal eosinophilia both of which persist despite treatment with prolonged proton pump inhibitors (1). This eosinophil infiltrate causes morphologic and functional alterations that result in symptoms like dysphagia. EE presents characteristics similar to bronchial asthma, and similar molecular and cellular mechanisms have been involved in the physiopathology of both diseases in which eosinophils seem to play a central role (2–5). Interleukin (IL)-5, a cytokine typically produced by Th2 $CD4^+$ lymphocytes, has been shown to play a crucial role in the recruitment of eosinophils to the lung (6) as it is essential for the proliferation, differentiation, survival, and activation of eosinophils in chronic allergic reactions. The similarities between EE and bronchial asthma strongly suggest a Th2 immune response during pathogenesis that would involve the accumulation of eosinophils through as-yet undefined mechanisms that seem to involve the production of eosinophil-specific chemokines which are known as eotaxins (7). Eotaxins are a subfamily of chemokines that act through the chemokine receptor CCR3, which is expressed predominately in eosinophils (8). Eotaxin-1 /CCL11 is the best-studied eosinophil-attracting chemokine in the digestive tract, where it is expressed ubiquitously (9, 10). Eotaxin-1/CCL11-specific mRNA can be detected in mononuclear cells resident in the lamina propria of the small intestine, which contains the main population of eosinophils in the digestive tract in healthy individuals.

IL-5 and eotaxin functions in eosinophil-associated gastrointestinal hypersensitivity disorders have been explored through different strategies: (a) IL-5-deficient mice and transgenic mice expression of IL-5 and eotaxin (5, 9, 11), (b)

studying the ability of T cells from EE patients to produce IL-5 upon stimulation with various allergens (2, 12), (c) quantifying gene expression of several cytokines in the esophageal epithelium from patients with EE as compared to healthy donors (13–15).

Topical steroid treatment in EE achieves the elimination of the eosinophilic infiltrate and restores architectural changes in the epithelial tissue, which results in a clear symptomatic improvement of the patients (16). However, few data exist regarding the effect of steroid drugs on lymphocytes present in the esophageal epithelium and concerning the molecular signals regulating tissue accumulation of eosinophils.

We herein analyzed changes in the esophageal lymphoid infiltrate induced by the topical steroid treatment in patients with EE in which an improvement of the clinical symptoms and substantial reduction of the eosinophilic infiltrate was achieved, and investigated the effect of such treatment on the gene expression levels of IL-5, eotaxin-1/CCL11, and eotaxin-3/CCL26, which have been clearly involved in the development of experimental and human EE. Variations in gene expression levels and changes in the density of epithelial leukocytes upon resolution of the eosinophil infiltrate induced by the topical steroid treatment are shown and compared to control esophageal samples.

MATERIALS AND METHODS

Our study included 8 male patients (mean age 31.6-yr-old, SD $\pm$6.05) with esophageal symptoms, manifested in the form of dysphagia or food impaction, who were diagnosed as suffering from EE, after endoscopic biopsies were performed in upper and lower esophageal thirds, according to the following criteria (17): (a) infiltration of the esophageal epithelium by $>$24 eosinophilic leukocytes per high-power field (HPF); (b) absence of eosinophilic infiltration in biopsies obtained in gastric and duodenal mucosa; (c) exclusion of gastroesophageal reflux as a cause of eosinophilia by means of ambulatory 24-h pH-metry. In the case of pathologic recording, persistence of the eosinophilic infiltrate after an 8-wk treatment with an antisecretor strength drug (Omeprazole 20 mg/twice a day); and (d) exclusion by clinical history of drug intake, parasites, caustications, hematological neoplasm, or other illnesses that could give rise to esophageal eosinophilia. Control esophageal samples were defined as having 0 eosinophils per HPF and were obtained from 8 patients (2 men and 6 women, mean ages 32.5-yr-old, SD $\pm$11.41) with suspected gastroduodenal ulcers, in which symptoms of gastroesophageal reflux, hiatus hernia, incompetent cardias, and esophageal peptic lesions were ruled out, and were found to have normal endoscopic appearance and microscopic analysis. The study was conducted according to the declaration of Helsinki principles and informed consent was obtained from all patients. Table 1 shows clinical data and results of allergic sensitization tests in the EE patients.

Histological Study

Endoscopic biopsies, taken in upper and lower oesophageal thirds, at a minimum of four specimens per location (18) were fixed in 4% formalin and routinely processed: 5-μm sections were cut from formalin-fixed and paraffin-embedded blocks, placed on microscope slides and stained with hematoxylin and eosin.

To identify the presence of lymphocytes in epithelial biopsies, the paraffin-embedded tissue sections were deparaffinated in xylene, and rehydrated gradually adding concentrates of ethanol and balanced in an aqueous buffer. Endogenous peroxidase activity was inactivated by incubation in 3% hydrogen peroxide in aqueous solution for 15 min at room temperature. Antigen recovery was achieved by heating at 150°C in EDTA for 45 min (pH 8.2). Next, the sections were incubated using specific primary monoclonal antibodies against human antigens for 1 h at room temperature, and were washed in a buffer solution. Antibodies used included: anti-CD3 (Clone F7.2.38, Dakocytomation, Glostrup, Denmark, dilution 1/100), anti-CD4 (Clone 4B12, Novocastra, Newcastle Upon Tyne, UK, dilution 1/50), and anti-CD8 (Clone 144-B, Dakocytomation, dilution 1/100) and antimast-cell tryptase (Clone AA1, Dakocytomation, dilution 1/100). The four monoclonal antibodies are of the IgG1 isotype. An IgG1 isotype control antibody was included in the study. After immunofixation of the primary antibody, a secondary antibody targeted against the constant fraction of murine IgG (ChemMate Dako Envision, Dakocytomation) was incubated for 30 min at room temperature. Finally, the specific signal was detected using the avidin-biotin colorimetric system with peroxidase as a detection enzyme. Diaminobenzidine was used as a color substrate. The preparations were slightly counterstained with hematoxylin and mounted for subsequent microscopic examination.

Cellular components of the mucosal infiltrate were exclusively counted into the epithelial or squamous stratum using a stereological microscope (Olympus BX 51) connected to a solid-state JVC TK-C1380 video camera. The control of the stage movements and the interactive test grids were provided by the CAST stereological software package (Visionpharm, Hørsholm, Denmark, and Olympus, Madrid, Spain) running on a Dell OptiPlex computer. We counted immunostained cells using an optical dissector (19, 20), randomly selecting 50 fields of each histologic preparation, using a 1,482 μm^2 unbiased frame and a dissector height of 5 μm (the volume of each counting "brick" was 7,412 μm^3). To determine cell densities, the Cavalieri method was used (21, 22). Final results were expressed as the number of cells per mm^3 of tissue (Table 2).

RNA Extraction and Real-Time PCR

Two samples of the mucosa of the upper oesophageal third were collected during endoscopies in each of the EE patients, before and after steroid treatment, which were conserved in an RNA stabilization solution (RNAlater, Ambion Inc., Austin, TX) at a –20°C until processing.

Table 1. Clinical and Allergic Characteristics of Patients

	Sex and Age	Duration of Symptoms	Type of Digestive Symptoms	Blood Eosinphil Count*	Family History of Atopy	Personal History of Atopy	Positive Prick Tests		Ig E (KU/L)	
							Inhalants	Food Allergens	Total†	Specific (CAP)‡
Case 1	Male, 35	12 yr	Solid food impaction pyrosis	180	Yes	Rhinoconjunctivitis and seasonal and epithelia-induced bronchial asthma	Cat and dog epithelia	Peach, sunflower seeds, lentils	73	Dog 1.22; cat 7.22
Case 2	Male, 38	30 yr	Frequent food impaction	520	Yes	Rhinoconjunctivitis	None	None	55.1	All tested <0.35
Case 3	Male, 37	3 months	Occasional food impaction	550	No	Pollen rhinoconjunctivitis. Baker's asthma	Grass	Cereals, leguminous	827	Lollium 7.94; soy 0.36; chick peas 0.49; lentils 0.46; oat 5.52; barley 15.2; rye 39.2; corm 1.66; wheat 20.1; rice 0.83
Case 4	Male, 25	3 months	Dysphagia to solid food. Food impaction	940	Yes	Seasonal bronchial asthma. Rhinoconjunctivitis	Lollium, platanus	Leguminous, nuts, melon	69	Lollium 18.4; platanus 0.6; peanut 0.41
Case 5	Male, 27	3 yr	Impaction with sausage	190	No	Seasonal allergic rhinitis	Grass, olive, Cupressus arizonica	None	340	C. arizonica 9.7; olive 1.96; lollium 39.2
Case 6	Male, 22	15 yr	Impaction with different kinds of meat: chicken, beef, pork	300	Yes	Pollen rhinoconjunctivitis	Grass, olive, Cupressus arizonica, platanus and cat	Fish, melon, water melon, leguminous, nuts, cereals	714	C. arizonica 15.40; olive 22.3; alternaria 2.94; cat 2.47; dog 0.79; lollium 98.6
Case 7	Male, 35	14 months	Dysphagia to solid food. Food impaction	250	No	Seasonal bronchial asthma	Pollen. Cat and dog epithelia	Leguminous, nuts, cereals	58	Lollium 7.85; olive 1.76; plantago 0.6; lentils 1.82; soy 0.72; peanut 1.67; dog 6.78; cat 3.0
Case 8	Male, 34	4 months	Dysphagia to solid and liquid food	210	Yes	Pollen rhinoconjunctivitis	Dog epithelium. Pollen	None	127	Lollium 24.4; artemisa 1.85; C. arizonica 1.22; olive 3.73; dog < 0.035

*Normal <350 cells mm^3.
†Normal <100 KU/L.
‡Normal <0.35 KU/L.

Table 2. Densities of Leukocyte Subpopulations

	Cell Count*				
	Eosinophils (EE Pre/ Post-Treatment)	CD3 (EE Pre/ Post-Treatment)	CD4 (EE Pre/ Post-Treatment)	CD8 (EE Pre/ Post-Treatment)	Mast Cells (EE Pre/ Post-Treatment)
Patient 1	302,600/0	84,300/10,000	3,000/28,100	60,000/5,390	33,700/5,290
Patient 2	61,300/0	121,000/63,400	44,000/18,800	60,300/50,900	92,300/2,760
Patient 3	195,000/6,420	97,900/68,900	25,000/36,200	65,600/34,900	25,800/4,500
Patient 4	29,100/0	105,000/33,000	15,300/0	102,000/14,000	92,300/2,590
Patient 5	161,000/0	148,000/19,700	34,400/2,760	97,100/6,270	17,600/12,800
Patient 6	65,400/2,490	135,000/4,000	47,000/0	102,000/3,740	88,000/43,900
Patient 7	77,800/0	63,900/13,400	3,370/1,880	47,000/5,090	41,200/21,400
Patient 8	42,900/0	135,000/4,000	47,000/0	102,000/3,740	117,000/2,970
Control 1	0	23,800	6,710	15,600	3,000
Control 2	0	15,340	6,240	10,300	3,290
Control 3	0	6,540	0	5,400	8,400
Control 4	0	15,600	3,940	10,630	11,300
Control 5	0	15,700	2,980	10,870	4,820
Control 6	0	9,060	2,980	5,360	12,400
Control 7	0	25,100	8,940	20,980	5,090
Control 8	0	15,870	5,440	11,300	6,900

*Expressed as cells/mm^3.
Hematoxylin-eosin, immunostaining, and subsequent stereology were performed in serial sections of endoscopic biopsies obtained from the upper third of the esophagus.

Total RNA was extracted in TriReagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. After treatment with DNAse I, 1 μg of total RNA was reverse-transcribed with random hexamers and avian myeloblastosis virus reverse transcriptase (Roche) for 1 h at 42°C.

Quantitative analysis of human IL-5, eotaxin-1/CCL11, and eotaxin-3/CCL26 gene expression levels was performed by polymerase chain reaction (PCR) amplification of the resulting cDNAs in a Light Cycler with FastStart DNA Master SYBR Green I (Roche, Penzburg, Germany). Primers used were as follows: IL-5 forward primer: 5′-GCT TCT GCA TTT GAG TTT GCT-3′; IL-5 reverse primer: 5′-CCG TCA ATG TAT TTC TTT ATT AAG-3′; eotaxin-1 (CCL11) forward primer: 5′-CCA TCA ATG TAT TTC TTT ATT AAG-3′; eotaxin-1 (CCL11) reverse primer: 5′-CTT GAA GAT CAC AGC TTT CTG-3′; eotaxin-3 (CCL26) forward primer 5′-CTG TGA TAT TCA CTA CCA AAA G-3′; eotaxin-3 (CCL26) reverse primer5′-GTA GCC TTC AGA AAA GAT TCC-3′; β-2 microglobulin forward primer: 5-CCA GCA GAG AAT GGA AGG TC-3′; β-2 microglobulin reverse primer: 5′-GAT GCT GCT TAC ATG TCT CG-3′.

Standard curves for target mRNA expression were generated using serial dilutions (1/10) of known quantities of amplified products of the studied genes. Quantification of IL-5, eotaxin-1/CCL11, and eotaxin-3/CCL26 gene expression was obtained using Light Cycler system software (Roche), by interpolation into their respective standard curves. RNA molecules estimated from the quantification were normalized to β-2-microglobulin gene expression by calculating the ratio between specific mRNA molecules/μl cDNA and molecules of β-2-microglobulin/μL cDNA.

Statistical Analysis

The software package SPSS 11.0 was used for statistical analysis (SPSS, Inc., Cary, NC). The data were analyzed using linear regression and were expressed as mean ± SD. All the *P* values and confidence intervals were calculated and evaluated using a 95% bilateral confidence interval. A nonparametric Wilcoxon signed rank test was used to analyze pretreatmet and post-treatment gene expression data. Comparison between post-treatment and control data was also performed by Mann–Whitney nonparametric test for independent samples.

RESULTS

The esophageal epithelial infiltrate of the patients with EE is characterized not only by a dense population of eosinophils, but also by the presence of T lymphocytes, most of which (75%) are CD8$^+$, and a significant increase of mast cells compared to control samples (2, 23–25). As summarized in Figure 1, treatment with topical steroids led to a significant reduction not only of the eosinophil infiltrate, but also of the density of intraepithelial T cells and mast cells to levels similar to those found in control samples, as measured by specific immunostaining of esophageal biopsies and stereology. Both CD4$^+$ and CD8$^+$ T-cell subpopulations were proportionally reduced. Immunostaining of esophageal biopsies from a representative EE patient before and after steroid treatment, and of a control esophageal sample is shown in Figure 2. Cell counts from each patient and from control samples are shown in Table 2.

As IL-5, eotaxin-1/CCL11, and eotaxin-3/CCL26 have been involved in the recruitment of eosinophils to

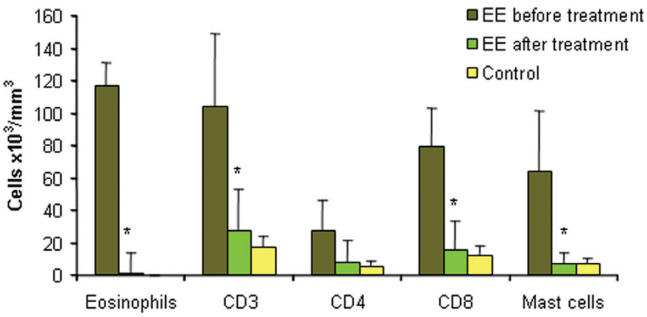

Figure 1. Density of epithelial eosinophilic leukocytes and the different types of T lymphocytes and mast cells determined by stereology in endoscopic biopsies of the upper third of the esophagus of EE patients and controls, shown as thousands of cells/mm^3 and expressed as mean values ± SD. "*" denotes significant statistical differences ($P < 0.05$) between pretreatment and post-treatment EE.

inflammation sites, we next analyzed their gene expression levels in esophageal biopsies taken from the patients before and after topical steroid treatment. Quantitative PCR analysis was performed and normalized mRNA levels for each gene were compared in the two biopsies obtained from each patient. As shown in Figure 3, gene expression levels were downregulated in most of the patients upon treatment with topical steroids. IL-5 expression was downregulated to different levels in six patients and did not show substantial changes in two of them, despite the resolution of the clinical symptoms, the histologic lesions and the reduction of the eosinophilic infiltrate in all cases studied (see Table 2). However, the absence of IL-5 expression in control esophageal samples suggests that in most cases IL-5 is highly overexpressed in EE. Eotaxin-1/CCL11 gene expression levels were downregulated post-treatment by more than 10-fold in three of the patients studied, fourfold in one of them, and about twofold in three of them, and we did not detect important changes in one of the cases studied (Fig. 3B). Expression of eotaxin-3/CCL26 was always higher prior to treatment; although in cases 1, 2, and 3 the downregulation was really modest. In patients 4, 6, 7, and 8, the decrease in expression was around 10-fold, and in case 5 the levels descend about two thousand times after treatment (Fig. 3C). Interestingly, the patients with higher modifications in expression of eotaxin-3/CCL26 were those with lower decrease in IL-5 and especially in eotaxin-1 expression, suggesting that both eotaxins might have a complementary role. Individual expression data for every patient are shown for each gene normalized to β-2-microglobulin in Table 3. Gene expression levels were also analyzed in eight control esophageal samples for comparative purposes. As shown in Table 3, while IL-5 expression was not detected in these samples, CCL11/eotaxin-1 expression was also variable between samples, showing mRNA levels in control samples lower but comparable to those found in patients upon steroid treatment (mean relative expression values 8.42 ± 5 in control samples *vs* 19.4 ± 28.6 in patients upon steroid treatment, $P = 0.7$). On the contrary, although CCL26/eotaxin-3 was also detected in control esophageal samples, mRNA levels were notably lower than those measured in EE patients treated with topical steroids (mean relative expression values of 1.45 ± 1.0 in control samples *vs* 580.9 ± 943.9 in patients upon steroid treatment, $P = 0.001$). The results shown in Table 3 are in agreement with those reported by Blanchard *et al*. (14) and Bhattacharya *et al*. (15) as eotaxin-3 is induced at much higher levels in EE than eotaxin-1 compared with control samples (mean relative values 5,033.26 ± 10,260.3 in EE *vs* 1.45 ± 1.0 in control samples for eotaxin-3, and 46.92 ± 63.05 in EE *vs* 8.42 ± 5 in control samples for eotaxin-1). Nonetheless eotaxin-1 expression seems to be more sensitive to steroid treatment in the eight patients studied.

DISCUSSION

General stimuli that regulate the biology of eosinophils also govern the signaling processes leading to the recruitment of these leukocytes to the digestive tract in gastrointestinal diseases associated with local eosinophilia (9, 26–28). IL-5 has clearly been shown to be involved in the physiopathology of several allergic pulmonary or systemic disorders, as well as asthma (6, 29), and in fibrous tissue remodeling in chronic bronchial (30) or cutaneous (31) inflammation. It has been reported that IL-5 production by peripheral blood lymphocytes upon *in vitro* stimulation with different allergens is increased in EE patients compared to control subjects (12). In a murine model overexpression of IL-5 results in an increase in circulating eosinophils and an intense accumulation of these cells in the lamina propria of the esophagus is observed (9, 26). A recent study analyzed the gene expression of several cytokines in the esophageal epithelium of children suffering from EE and of healthy donor controls, and shows that average expression of IL-5 increased in the patients, although individual expression data are not provided (13). Real-time PCR data obtained in endoscopic biopsies from our patients showed variable expression of IL-5 in the esophageal epithelium of adult patients with EE and downregulation of gene expression levels upon treatment with topical steroids in six out of the eight patients studied, which correlated with a decrease in the number of eosinophils infiltrating the esophageal epithelium. In the remaining two patients, IL-5 levels did not change upon treatment, despite the resolution of the eosinophilic infiltration and clinical symptoms. This could indicate that, at least in a small subgroup of patients, IL-5 would not be sufficient in itself to cause the development of the eosinophilic inflammation and the participation of other cytokines could be required. In this sense, it has been shown that the accumulation of eosinophils in the digestive tract induced by antigens may happen in the absence of IL-5 (32), which indicates that under some circumstances, its participation in EE might not be critical. However, the absence of IL-5 gene expression in control esophageal biopsies suggests that it might play an important role in EE.

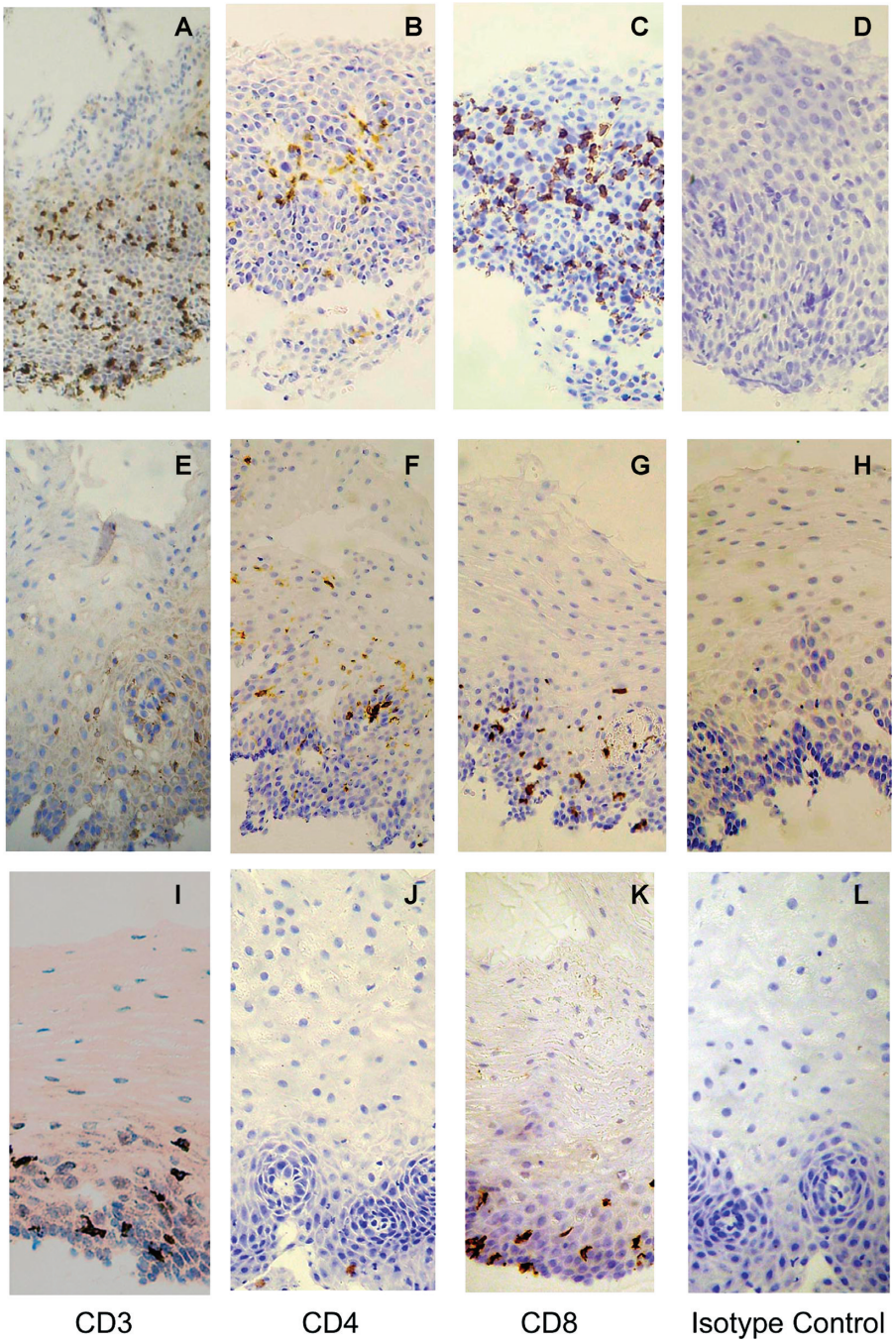

Figure 2. Immunohistochemical analysis of CD3 (*A,E,I*), CD4 (*B,F,J*), and CD8 (*C,G,K*) expression in esophageal epithelia from a representative EE patient before (*A–D*) and after (*E–H*) successful treatment with fluticasone propionate, and from a control esophageal sample (*I–L*). IgG1 isotype control staining is shown (*D,H,L*). Original magnification 200×.

Eotaxins are a family of chemokines attracting mainly eosinophils, acting over the chemokine receptor CCR3, expressed mainly by these leukocytes, but also by mast cells (33, 34). Eotaxin-1/CCL11 was the first member of the family identified (35), and therefore is the best-studied eosinophil-attracting chemokine in the digestive tract. In mice lacking eotaxin-1 eosinophils are not recruited to the gastrointestinal tract, but normal levels of eosinophils are found in the hematopoietic compartments; therefore, eotaxin-1/CCL11 was found to be critical for the recruitment of eosinophils to the gastrointestinal tract in mice even in the presence of elevated levels of IL-5 (9), which suggests that eotaxin-1/CCL11 acts in a tissue-specific manner and that its expression does not influence eosinophilopoyesis or circulating eosinophils. Elevated levels of eotaxin-1/CCL11 have been associated with inflammatory diseases of the human digestive and respiratory tract and have been correlated with their clinical severity (10, 36, 37). Although eotaxin-1 could be relevant

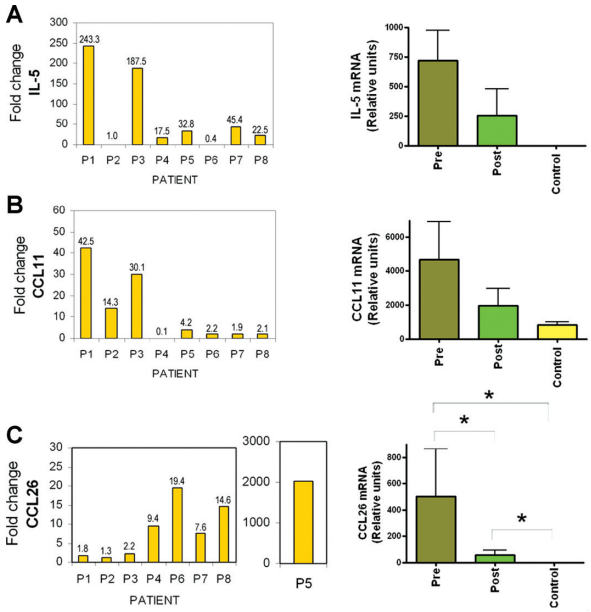

Figure 3. Changes in IL-5 (*A*), eotaxin-1/CCL11 (*B*), and eotaxin-3/CCL26 (*C*) gene expression induced by topical steroid treatment in esophageal epithelium. Real-time PCR was performed to determine specific mRNA levels in esophageal biopsies taken from each of the patients previously and upon treatment with fluticasone propionate and the consequent resolution of the clinical symptoms. The results were normalized to β-2 microglobulin gene expression in the same samples. The resulting normalized expression levels for each gene upon treatment were compared to normalized expression levels previous to steroid treatment for each patient and are expressed as fold change. Mean expression values ± SD for the samples analyzed pre- and post-treatment and in control esophageal biopsies are also shown. "*" denotes significant statistical differences ($P < 0.05$)

in the physiopathology of EE in humans (13), and in spite of the immunohistochemical staining with antieotaxin-1 in EE, no increase in the serum levels of eotaxin-1 was detected in one case of EE reported (38).

We found the results regarding eotaxin-1/CCL11 expression in esophageal biopsies from the patients interesting. Half of them showed a notably decreased expression of eotaxin-1 upon steroid treatment. The other half showed only moderate or no change in gene expression levels. These data oblige us to consider the putative action of other chemokines in the recruitment of eosinophils to the esophagus. As we detected resolution of the eosinophil infiltrate in all the patients studied, the data suggest that eotaxin-1/CCL11 is not likely the only chemokine involved in the recruitment of eosinophils to the esophagus in human EE. Some researches have addressed the putative role of RANTES/CCL5, but no differences were found in its expression in patients and control esophageal samples (13). Eotaxin-2/CCL24 identified in mice and humans (39, 40), and eotaxin-3/CCL26, which has been identified only in humans (41, 42), have been also involved in the development of human EE. However, contradictory results have been reported regarding the involvement of eotaxin-2 and eotaxin-3 in EE. While some authors describe downregulated expression of eotaxin-2 and eotaxin-3 compared to healthy donors (13), a more profound study has found that eotaxin-3/CCL26 is the gene with the highest level of induction in patients with EE compared to control individuals (14). Besides, esophageal mRNA and protein levels strongly correlated with tissue eosinophilia in EE; although increased levels of eotaxin-1/CCL11 mRNA expression were found in the esophageal samples from the same patients as well (14, 15). We found that CCL26/eotaxin-3 mRNA levels were also downregulated in the patients upon steroid treatment. However, when we analyzed control samples with no eosinophilia we found that eotaxin-1 levels were similar to those found in the patients upon treatment and improvement of the clinical symptoms. In contrast, expression of eotaxin-3 mRNA was also downregulated upon treatment but levels did not reach those found in control esophageal samples. Our gene expression data in EE samples before treatment and control biopsies are in agreement with the data reported by Blanchard *et al*. (14) and Bhattacharya *et al*. (15). Nevertheless, gene expression, histology, and clinical data upon steroid treatment suggest that although both eotaxin genes studied are upregulated at different levels, eotaxin-1/CCL11 might also cooperate

Table 3. Reverse Transcription Real-Time PCR Analysis of mRNA Levels in Esophageal Epithelia

	Normalized mRNA Levels*								
	IL-5 ($\times 10^4$)			CCL11 ($\times 10^4$)			CCL26 ($\times 10^4$)		
Sample	EE Pre-Treatment	EE Post-Treatment	Control Samples	EE Pre-Treatment	EE Post-Treatment	Control Samples	EE Pre-Treatment	EE Post-Treatment	Control Samples
P1/C1	0.41	0.001	n.d.	101.76	2.39	17.38	2,824.9	1568.9	1.3
P2/C2	0.28	0.294	n.d.	3.27	2.29	9.34	3,339.4	2,522.8	2.0
P3/C3	0.60	0.003	n.d.	11.60	0.38	7.07	588.6	261.7	3.8
P4/C4	4.45	0.254	n.d.	3.20	28.54	1.02	1,840	195.0	0.6
P5/C5	12.63	0.384	n.d.	6.64	1.58	5.78	30,253.8	15.0	0.7
P6/C6	6.47	18.154	n.d.	12.38	5.60	8.39	1,182.8	61.0	0.5
P7/C7	12.05	0.265	n.d.	60.15	31.48	13.11	107.7	14.3	1.5
P8/C8	20.35	0.893	n.d.	176.43	83.14	5.33	128.9	8.8	1.2

*Results are expressed as the ratio between the number of molecules of the specific gene and the number of molecules of β2-microglobulin in the same cDNA sample (see Methods).
n.d. = not detectable.
Relative expression levels are shown in arbitrary units from eight EE patients (P1–P8) and from eight unrelated control esophageal biopsies (C1–C8).

with eotaxin-3/CCL26 the recruitment of eosinophils to the esophagus in the patients.

Th2 responses are generally mediated by $CD4^+$ T-helper lymphocytes. Our results show that in EE the lymphocytic infiltrate is composed primarily of $CD8^+$ T cells, which is in agreement with previous observations (23, 25). Classically, $CD8^+$ T cells have been associated with a Th1 pattern of cytokine secretion represented mainly by the production of IFN-γ and TNF-α. Straumann *et al*. found high levels of TNF-α in esophageal biopsies from eight adult patients with EE (2), and Gupta *et al*. reported increased IFN-γ gene expression in a cohort of children suffering from EE (13). Therefore, it is tempting to speculate that the inflammatory cascade triggered by the local production of Th1 cytokines could play a role in the pathogenesis of EE. Topical steroid treatment achieved significant reduction of the lymphocytic infiltration into esophageal epithelium in all patients (see Fig. 1 and Table 2), but this reduction was accompanied by a variable reduction in gene expression of analyzed cytokines, whose production is dependent on T lymphocytes' function.

The heterogeneity of our and other data reported in literature is probably a reflection of the interindividual heterogeneity of the molecular mechanisms involved in the physiopathology of EE, in which IL-5 and different eotaxins would exert synergistic or cooperative effects among each other and with other not so well-studied cytokines in the regulation of the gastrointestinal eosinophilia; although according to murine models IL-5 seems to be essential for the accumulation of eosinophils in the esophageal epithelium (6, 11). In this sense, a humanized monoclonal antibody against human IL-5 seems to be a promising therapeutic intervention for EE (43).

Our data also suggest that the expression of the three genes analyzed and the effect of these molecules in the physiopathology of EE are independent processes, and are likely due to diverse regulatory mechanisms. Although the inflammatory phenotype observed was similar in all patients, the cascade of inflammatory mediators leading to morphologic and functional disorders observed in EE may not be identical in all cases.

Our small series of eight patients suffering from EE showed a significant reduction in epithelial eosinophilic infiltrate after a 3-month treatment period with topical fluticasone propionate and avoiding exposure to allergens in those cases in which we were able to demonstrate sensitization. In our experience, only few patients remained symptomatic after this 3-month treatment, and eosinophilia was still found in biopsies. They probably need a more prolonged treatment, guaranteeing a good therapeutic completion before they could achieve total esophageal recovery. Although the global efficacy of fluticasone propionate in a series of 36 children treated during 3 months was only 50% (24), these differences in final results could be explained in several ways. The first one is that in order to achieve the maximal effect of the drug, we need to guarantee the correct administration. Due to the specific presentation in an inhaler system, fluticasone propionate for EE must be applied over the tongue and then swallowed during several minutes. This could be too difficult in kids, particularly in younger children. For this reason, some authors have recommended other alternatives in this kind of patients to improve the drug intake, like viscous budesonide (44). We should also consider that the heterogeneity of the results could be related to the evolution of the disease and the time exposed to a possible allergen responsible for the epithelial inflammation. Moreover IL-5, CCL11, and CCL26 may be subjected to additional regulatory mechanisms leading to final protein expression levels. These questions deserve further study.

STUDY HIGHLIGHTS

What Is Current Knowledge

- Human esophagus contains a small amount of T lymphocytes in the epithelium, whose number increases in gastroesophageal reflux disease and especially in eosinophilic esophagitis (EE).
- EE is characterized by a dense inflammatory infiltrate composed by eosinophils and T lymphocytes in esophageal epithelium.
- Several eosinophil-attractant molecules have been implicated in the recruitment of eosinophils to the epithelium in murine and human models of EE. Among them, IL-5, eotaxin-1/CCL11, and eotaxin-3/CCL26 are described to play a critical role.

What Is New Here

- We evaluate for the first time the effect of topical steroids over the inflammatory infiltrate of adult patients with EE in parallel with pre- and post-treatment changes induced over gene expression levels of IL-5, eotaxin-1/CCL11, and eotaxin-3/CCL26.
- A wide variability exists in gene expression patterns of eosinophil-attractant molecules between the different patients, in which IL-5 and eotaxins could act in a synergistic way.

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CONFLICT OF INTEREST

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