

Small Bowel of Patients with Asthma and Allergic Rhinitis: Absence of Inflammation Despite the Presence of Major Cellular Components of Allergic Inflammation

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ABSTRACT

Eosinophils participate in the pathogenesis of inflammatory diseases of the respiratory tract and the gut. We investigated the constitutive presence of eosinophils and mononuclear cells in the macroscopically normal duodenal mucosa of patients with asthma and allergic rhinitis. Macroscopically normal duodenal specimens were obtained at routine endoscopy for upper gastrointestinal symptoms from 16 patients with asthma and 13 patients with allergic rhinitis. Twelve nonatopic patients with irritable bowel syndrome were studied as controls. Specimens were analyzed by immunohistochemistry using a panel of antibodies to human eosinophil cationic protein clone EG1 (EG1) and clone EG2 (EG2), anti-human interleukin (anti-hIL)-5, anti-hIL-4, anti-CD4, and anti-CD68. Significantly increased numbers of eosinophils stained

with EG1 and EG2 were found in the duodenum of patients with asthma and allergic rhinitis compared with controls. IL-5⁺ cells and IL-4⁺ cells were detected in significantly increased numbers in the duodenal mucosa of patients with asthma and rhinitis compared with controls. Mononuclear cells expressing CD4 (helper T cells) and CD68 (macrophages) also were significantly increased in the duodenal mucosa of asthma and rhinitis compared with controls. Accumulation of eosinophils in conjunction with IL-4⁺ cells and IL-5⁺ cells in the noninflamed duodenal mucosa may reflect a predominant T helper cell subset 2 systemic immune response in patients with asthma and allergic rhinitis. The absence of intestinal inflammation despite the marked presence of cells implicated in the allergic inflammation suggests that local mechanisms might determine the state of nonresponsiveness in the gut mucosa of patients with asthma and allergic rhinitis. (Allergy and Asthma Proc 25:253–259, 2004)

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Eosinophils are effector components of the inflammatory cell infiltrate present in numerous allergic and inflammatory processes. These cells circulate through the peripheral blood to localize in mucosal sites where they participate in the inflammatory response by releasing their granule contents. Eosinophils can modulate the immune response by

means of interaction with other leukocytes through the production and secretion of cytokines and lipid mediators, and they also are able to control their own function.¹

Activated eosinophils have been implicated in the injury observed in both acute and chronic tissue inflammation, and their role in the pathogenesis of bronchial asthma² and host immunity to parasitic infections are well established. Nevertheless, infiltration and degranulation of eosinophils normally are not seen in healthy tissues, but the gastrointestinal tract shows a peculiar eosinophil infiltration. These cells are constitutively present in the gastrointestinal mucosa, and their numbers are significantly higher than that seen in hematopoietic sites, especially under inflammatory conditions.¹ Increased numbers of activated eosinophils have been detected in the gastrointestinal mucosa of patients with disorders such as food allergy and eosinophilic gastroenteritis, in which they are implicated as the main effector cells.^{3,4} In a previous study, the small bowel of patients with asthma showed increased numbers of eosinophils and other immunocompetent cells, constituting a cellular infiltration in the gut mucosa similar to that found in the bronchi.⁵

Asthma often is associated with allergic rhinitis, conjunctivitis, eczema, and other cutaneomucosal conditions. Most of these clinical manifestations are associated with pathological findings typical of allergic inflammation, including eosinophilic infiltration and degranulation. Although the larger area of intestinal mucosa may be relatively preserved from this phenomenon, it is possible that the involvement of the gastrointestinal tract in patients with bronchial asthma occurs more frequently than expected. Abdominal symptomatology has been reported in patients with asthma, but usually it does not result in a specific diagnosis. However, the eventual concomitant finding of either eosinophilic gastroenteritis or celiac disease suggests that these conditions may be considered in the investigation of patients with asthma presenting with gastrointestinal symptoms.⁶ Mechanisms involved in this relative lack of participation of the gut mucosa in the overall inflammation are not fully understood and could be related to the development of tight immune responses to ingested antigens because of tolerance, different patterns of molecular recognition after partial antigen digestion, or evolutionary immune and inflammatory responses to luminal parasites. The aim of this study was to investigate the constitutive presence of immunocompetent cells in the intestinal mucosa of patients with chronic respiratory tract diseases, to achieve a novel understanding of the common mucosal immune system.

MATERIALS AND METHODS

Patients

Sixteen patients with bronchial asthma and 13 patients with allergic rhinitis were included in the study. Bronchial asthma was defined according to the criteria of the Global Initiative for Asthma.⁷ Patients with asthma comprised 14 women and 2 men, with a mean age of 39.2 years

(range, 25–64 years). The clinical severity of asthma was assessed according to the Global Initiative for Asthma, with all cases classified as mild persistent. Regular inhaled steroids were taken by 4 of the 16 patients; all patients also received intermittent inhaled short-acting β 2-agonist, as needed.

Rhinitis was defined as inflammation of the lining of the nose, characterized by one or more of the following symptoms: nasal congestion, rhinorrhea, sneezing, and itching.⁸ Eight women and five men, with a mean age of 37.8 years (range, 21–63 years) were included in the study. Patients received intermittent oral antihistamines ($n = 3$) or inhaled nasal steroids ($n = 2$). None of the patients with rhinitis were taking medication on a regular basis.

All patients with asthma and rhinitis were atopic. Diagnosis of atopy was based on clinical history and at least two positive immediate-response prick tests. Positive skin tests were defined as the presence of a papule of at least 3 mm in transverse diameter. The extracts used were *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blomia tropicalis*, *Blattella germanica* (IPI-ASAC, Rio de Janeiro, Brazil), and *Aspergillus fumigatus* (FDA Allergenic Ltda, Rio de Janeiro, Brazil). Controls were performed with the use of normal saline (negative) and histamine (positive; FDA Allergenic Ltda). All patients with asthma or rhinitis had normal sinus and chest x-ray films.

Twelve patients with irritable bowel syndrome were studied as controls. This group consisted of six men and six women, with a mean age of 44.2 years (range, 23–63 years). None of the controls were taking any medication at the time of the study and had no previous history of either pulmonary disease or allergy and negative immediate-response prick tests. All of the subjects in the study had specimens of peripheral blood collected for total and differential white blood cell counting. All test and control patients had at least three stool specimens negative for ova and parasites at the time of the study. All patients had mild dyspeptic symptoms, with normal upper gastrointestinal endoscopy. Characteristics of the patients and the control group are summarized in Table I.

The study protocol was approved by the Institutional Review Board of the Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro, Brazil (Federal-wide Assurance number for Clementino Fraga Filho University Hospital/Federal University of Rio de Janeiro: 00000377. Institutional Review Board number for Clementino Fraga Filho University Hospital/Federal University of Rio de Janeiro: 00001048) and informed consent was obtained from all subjects.

METHODS

Endoscopic duodenal specimens were taken from the normal noninflamed mucosa of all patients. Biopsy specimens were divided into two portions for histological and immunohistochemical studies. The first portion was

TABLE I

Characteristics of Patients with Asthma, Allergic Rhinitis, and Nonatopic Controls (Irritable Bowel Syndrome)			
	Asthma	Allergic Rhinitis	Nonatopic Controls
Number	16	13	12
Sex ratio (F/M)	14/2	8/5	6/6
Age (mean with ranges) (yrs)	39.2 (25–64)	37.8 (21–63)	44.2 (23–63)
Positive skin-prick tests	16	13	0
Blood eosinophils per cubic millimeter	455.5*	249.5	96.0
Median with ranges	64.0–1645.6	0.0–600.0	0.0–306.0
Oral steroid therapy	0	0	0
Inhaled steroids	4	2	0

*Value is significantly different from that of the control group ($p < 0.001$).

fixed in 10% formol saline, embedded in paraffin, cut into 5- μ m sections, and stained with hematoxylin and eosin for the histological assessment.

The second portion was immediately covered with Tissue Tek O.C.T. compound (Miles Scientific Laboratories, Ltd., Naperville, IL) and snap frozen in isopentane in a liquid nitrogen bath. They were subsequently stored at -80°C before processing and cut into 5- μ m sections in a cryostat maintained at -20°C . Tissue sections were air-dried and fixed for 10 minutes in a 1:1 chloroform/acetone solution. Cell subsets were characterized by the indirect immunalkaline phosphatase technique. Immunohistochemical staining of eosinophils was performed using the mouse monoclonal antibodies to anti-human eosinophil cationic protein clone EG1 (EG1) (1:50) and clone EG2 (EG2) (1:50; Pharmacia, Upsala, Sweden). A rabbit polyclonal antibody anti-interleukin (IL)-5 (1:10) and a mouse monoclonal antibody anti-IL-4 (1:10; R & D Systems, Minneapolis, MN) were used as primary antibodies to stain IL-5⁺ and IL-4⁺ cells, respectively. Helper T cells and macrophages were stained with a mouse monoclonal antibody to CD4 (1:50) and with a mouse monoclonal antibody to CD68 (1:20; Dako A/S, Glostrup, Denmark), respectively. Mouse immunoglobulin G1 (IgG1) or rabbit IgG (concentration matched; Dako A/S) were used as negative controls, as appropriate. Secondary antibodies used were alkaline phosphatase-conjugated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) or alkaline phosphatase-conjugated goat anti-rabbit IgG (Vector Laboratories, Inc.).

Briefly, all sections were incubated for 30 minutes with a human normal serum to block nonspecific reactions. After rinsing in Tris-buffered saline (TBS) for 10 minutes, tissue sections were incubated at room temperature with each primary antibody for at least 1 hour. A second section of the same sample from each patient was incubated with either TBS or an isotype IgG1 and served as negative controls. After another rinse in TBS, secondary antibodies were added to sections for 30 minutes. Further rinsing was followed by the development with an alkaline phosphatase substrate buffer (Tris buffer, naphthol phosphate, and fast red TR salt (4-chloro-2-methylbenzenediazonium salt), with

levamisole to inhibit endogenous alkaline phosphatase). All slides were counterstained with Harris' hematoxylin, rinsed with distilled water, and coverslip mounted in Immu-mount (Shandon, Pittsburgh, PA).

Quantitative analysis of tissue sections was performed by two independent observers under light microscopy at 400 $\times$ magnification. Lamina propria cells exhibiting identifiable reactivity on their surface distinct from background were regarded as positive. All cells within the lamina propria were assessed by a quantitative grading system in at least five distinct high-power fields. The results were expressed as either positive cells (immunoreactive) or total cell (immunoreactive and nonimmunoreactive) numbers per square millimeter of lamina propria.

Statistical analysis was performed using the statistical software GraphPad InStat (1998, Version 3.01; GraphPad Software, Inc., San Diego, CA). Statistical differences among the experimental groups were evaluated with the Kruskal-Wallis Test in which multiple comparisons were performed using the Dunn's test. Spearman rank correlation was performed to correlate data from peripheral blood with intestinal mucosa. All values are expressed as median with ranges. The level of significance was set at $p < 0.05$.

RESULTS

Peripheral Blood Eosinophils

The peripheral blood eosinophils were found to be significantly increased in patients with asthma compared with controls (Table I). No significant correlation was found between the numbers of peripheral blood eosinophils and EG1⁺ cells in the intestinal mucosa of both patients and controls (Spearman's correlation coefficient = 0.302; $p = 0.055$).

Histopathological Studies

No histopathological alteration was found in the duodenal mucosa of patients with asthma and rhinitis compared with the control group.

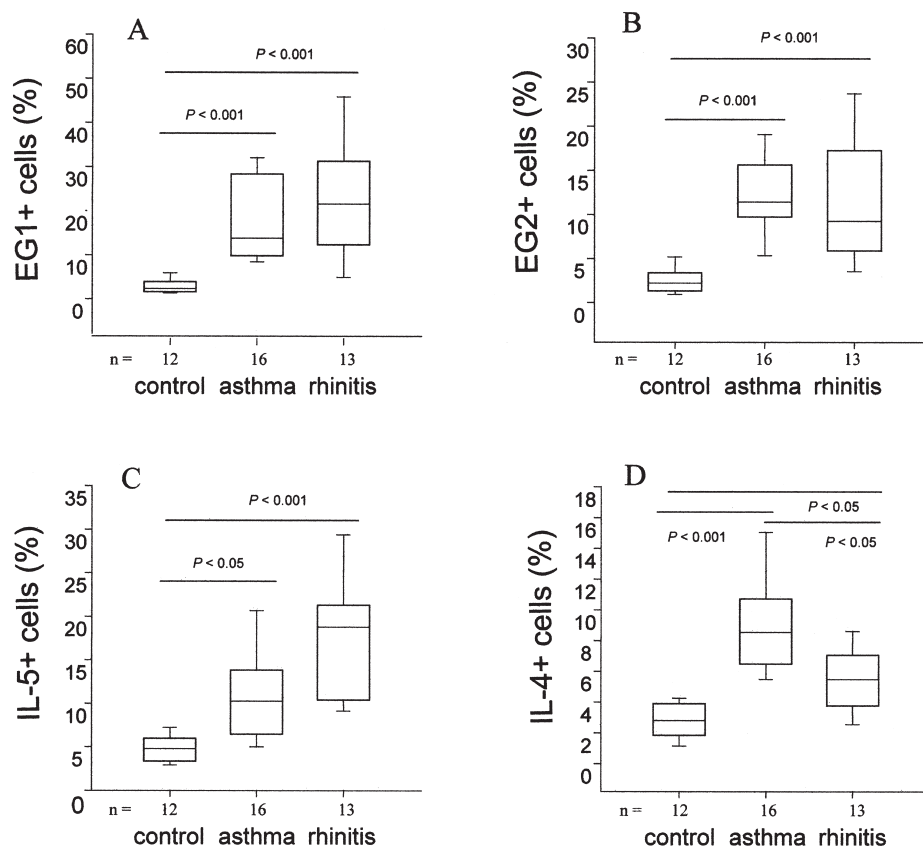


Figure 1. Differential expression of immunoreactive cells in the duodenal mucosa of the control, asthma, and allergic rhinitis groups. Values are percentages of positive cells in the lamina propria. Horizontal bars are medians, percentiles 25 and 75, respectively. Vertical bars represent ranges. The p values are indicated for significance; n represents the number of subjects in each group. (A) $EG1^+$ cells, (B) $EG2^+$ cells, (C) $IL-5^+$ cells, (D) $IL-4^+$ cells are represented.

TABLE II

Analysis of Mononuclear Cell Subpopulations of the Duodenal Lamina Propria

Positive Cells	Asthma	Allergic Rhinitis	Nonatopic Controls
CD4	10.24 (6.69–16.93)*	10.83 (5.34–22.47)*	4.15 (3.11–6.12)
CD68	8.19 (4.24–15.88)*	9.41 (4.28–19.52)*	3.14 (2.09–4.87)

CD4, helper T-cell marker; CD68, macrophage marker. Numbers are percentages of positive mononuclear cells in the lamina propria (median with ranges).

*Values are significantly different from those of the control group ($p < 0.001$).

Immunohistochemical Studies

The percentages of all immunoreactive cells in the intestinal lamina propria of patients with asthma and allergic rhinitis were significantly greater than in the control group. Data are summarized in Fig. 1.

Increased percentages of $EG1^+$ cells, a pan-eosinophil marker, were seen in the duodenal mucosa from patients with asthma and allergic rhinitis when compared with the control group. Staining with another eosinophil marker indicated that the percentages of $EG2^+$ cells from patients with asthma and allergic rhinitis also were significantly increased compared with controls. The $EG2:EG1$ ratio was

not significantly different among the patients and control group (data not shown). Significantly increased percentages of $IL-5^+$ cells and $IL-4^+$ cells were detected in the mucosa from patients with asthma and rhinitis compared with the control group.

The percentages of mononuclear cells expressing CD4 and CD68 in the duodenal lamina propria also were increased in both patients with asthma and allergic rhinitis compared with controls (Table II). Statistical assessment of the immunopathological results did not show any significant correlation with treatment, activity, and duration of the disease.

DISCUSSION

In this study we examined the number and state of the activation of eosinophils in the lamina propria of the intestinal mucosa of patients with asthma and allergic rhinitis. The cellular expression of IL-4 and IL-5 cytokines and the numbers of macrophages and helper T cells were analyzed also. All immunoreactive cells analyzed in the lamina propria were significantly increased in patients with asthma and allergic rhinitis, suggesting an airway-like cell infiltration of the duodenal mucosa, although without evidence of inflammation.

Previous observations showed that the bronchial epithelium and lamina propria of patients with mild, stable asthma display increased numbers of eosinophils, T cells, mast cells, and intraepithelial lymphocytes.⁹ In another study with asthmatic patients, cell infiltration also was detected in the intestinal mucosa, mimicking the inflammatory process in the bronchi.⁵ The results of our study are similar to those concerning eosinophils and IL-5 immunoreactivity in the duodenal mucosa. However, we did not find any architectural mucosal abnormalities or local inflammation.

The biological functions of eosinophils are attributed mainly to the secretion of their granule contents and not only to their constitutive presence in tissue. The degranulation process comprises the release of cytotoxic proteins including major basic protein (MBP), eosinophil cationic protein (ECP), and peroxidase and eosinophil-derived neurotoxin (EPX), cytokines, lipid mediators, and reactive oxygen metabolites.¹ The antibody EG1 recognizes both the storage and the secreted forms of ECP, whereas the antibody EG2 binds to both ECP during secretion and EPX.¹⁰ Interestingly, we found increased percentages of both EG1⁺ and EG2⁺ cells in the noninflamed intestinal mucosa of patients with asthma and allergic rhinitis compared with nonatopic controls. Nevertheless, although the antibody EG2 recognizes epitopes of ECP and EPX during secretion, counting cells was feasible only when the protein staining was intracellular. This fact raises the possibility that the increased numbers of eosinophils found in the gut might not necessarily be followed by increased cell degranulation. On the other hand, it is likely that small amounts of eosinophil granules eventually released in tissue could explain at least in part the relatively common vague gastrointestinal symptomatology of those patients. Gastrointestinal symptoms without any evidence of tissue injury are not uncommon in patients with asthma, and usually they are attributed to side effects of antiasthmatic medication. However, there have been some reports of possible associations between asthma and celiac disease, dermatitis herpetiformis, food allergy, and eosinophilic gastroenteropathy.⁶ Gut abnormalities observed in both celiac disease and food allergy are attributed mainly to abnormal local T-cell activation. Because bronchial asthma is also consistent with T-cell-mediated activity, it is possible that a similar mechanism may be responsible for duodenal alterations found in patients with asthma.

A common characteristic feature of allergic diseases is the presence of increased numbers of circulating eosinophils. Eosinophilia is a T-cell-mediated response as indicated by its absence in athymic or T-cell-depleted animals. In this study, peripheral blood eosinophils were found to be significantly increased in patients with asthma compared with the control group. However, no significant correlation could be established between the peripheral blood and intestinal percentages of eosinophils. One may infer that the atopic patients that participated in this study had only mild disease activity that might not be sufficient to produce or maintain systemic alterations.

IL-4 protein has been localized to tissue eosinophils in the airway of patients with allergic inflammation.¹¹ Therefore, it is likely that a subpopulation of eosinophils from allergic patients produces IL-4 and this may be important in local IgE production and other IL-4-dependent events associated with allergic response. In our study, IL-4⁺ cells also were detected in tissues from patients with both allergic rhinitis and asthma but at a distant site from inflammation.

By *in situ* hybridization, IL-5 mRNA was detected in the intestinal tissue of patients with celiac disease, eosinophilic cystitis, and hypereosinophilic syndrome.¹² Results of recent studies also established a significant increase of IL-5 immunolabeling in the duodenal mucosa from food-allergic patients.¹³ In a recent report, we also observed an increased expression of IL-5 in the inflamed colonic mucosa from patients with inflammatory bowel disease.¹⁰ In addition to stimulating eosinophil production, IL-5 is chemotactic for eosinophils and activates mature eosinophils, inducing secretion, and enhanced antibody-dependent cytotoxicity and it prolongs eosinophil survival by blocking apoptosis.² IL-5 is the most important eosinophilopoietin and in conjunction with granulocyte-macrophage colony-stimulating factor and IL-3, it is capable of activating and prolonging the survival of mature eosinophils. In this study, it is possible that the increased expression of both IL-4 and IL-5 found in the noninflamed gut of patients with respiratory diseases will reflect trafficking of immunoreactive T helper subset 2 (Th2) cells to another mucosal site of the common mucosal immune system. Previous evidence showed that CD4⁺ T cells and eosinophils may share the same cell adhesion molecules, such as $\alpha 4 \beta 7$ -integrin, indicating the existence of a common pathway for homing into the gut through the interaction with mucosal addressin cell adhesion molecule-1 (MAdCAM-1).¹ It is interesting to notice that although localized to the same compartment, accumulation of T cells and eosinophils did not result in gut inflammation in this study. However, the finding of an altered expression of EG1, EG2, IL-4, IL-5, CD4, and CD68 in the noninflamed gut, distant from the inflamed respiratory sites, suggests that the intestinal mucosa may not be entirely normal in patients with asthma or allergic rhinitis.

Bronchial biopsy specimens of patients with allergic asthma¹⁴ and nasal mucosa of patients with allergic rhinitis are characterized by Th lymphocytes displaying a predom-

inant Th2-like cytokine profile. On the other hand, immune response to allergens in nonallergic subjects is characterized by Th1-like lymphocyte responses.¹⁵ The absence of inflammation in normal subjects is maintained primarily by influences that promote the development of tolerance. One influence contributing to immune nonresponsiveness is diminished accessory cell function. In contrast to asthmatic lungs, in the healthy lung, the resident antigen-presenting cell population lacks B7, is unable to present allergen to Th lymphocytes, and can not stimulate cellular activation and proliferation.¹⁶ Moreover, the cytokine milieu of the non-asthmatic respiratory tract is characterized by elevated concentrations of IL-10 and transforming growth factor β , which can contribute to the down-regulation of accessory cells and to the inhibition of cytokine release. In the gastrointestinal tract, the normal, nonresponsive state is thought to be maintained by factors such as transforming growth factor β , which may similarly prevent inflammation in the respiratory tract.¹⁷ Thus, it is reasonable to suppose that the intestinal mucosa from patients with asthma or allergic rhinitis might also lack essential costimulatory signals rendering antigen-presenting cells unable to present allergens to T cells as well.

An important feature of the mucosal immune system is that immunocompetent cells primed in one specific mucosal site can migrate to other distinct mucosal sites. Inflammatory cells from the bronchial-associated lymphoid tissue proliferate in response to a local antigen challenge but also at distant mucosal sites. Thus, cellular homing to the intestinal mucosa could result from a bronchi-to-gut axis, through which there may be a continuous trafficking of immunocompetent cells. The evidence that food antigens exposed to the gut may have systemic effects on T cells corroborates the theory of a bidirectional communication between distinct mucosal sites. This also could explain why patients with asthma or allergic rhinitis eventually present with exacerbations of respiratory symptoms after food ingestion.

In this study, it was shown that there is a relative preservation of the intestinal mucosa of patients with either asthma or allergic rhinitis, despite the marked local presence of the same immunocompetent cells that participate in the allergic inflammation of the respiratory mucosa. In contrast to other mucosal sites, the mucosa-associated lymphoid tissue of the gastrointestinal tract appears to be regulated by unique mechanisms, and this is reflected in phenomena such as oral tolerance and physiological inflammation. Specific lymphoid populations that respond to alternative pathways of activation and the novel antigen-presenting intestinal epithelial cells constitute the background for distinct immune responses in the gut.¹⁸ In addition to these intrinsic regulatory factors, there is increasing evidence that the resident flora also participates in this process whether the response at the major interface with the external environment will be tolerance or active immunity.¹⁹ In some cases, the breakdown of this finely regulated mucosal immune

system leads to pathological responses to antigenic components of the normal flora. Consequently, disorders such as allergic gastroenteropathy or inflammatory bowel disease can be elicited in the gastrointestinal tissues.

Results of recent studies support a relationship between the composition of the intestinal microflora and allergic inflammation and have suggested a possible role for probiotics in the down-regulation of atopic disease.²⁰ These findings indicate the participation of the resident gut flora in potentially antiallergenic responses and support the need for new insights on the role of probiotics in modulating mucosal immunity and tolerance, which might become useful in future treatment strategies for atopic diseases.

Finally, although we can not exclude completely the possibility of a primary defect inherent to the whole mucosal immune system in patients with asthma and allergic rhinitis, this hypothesis seems unlikely because there is no intestinal inflammation in this study as opposed to overt airways inflammation. Future studies should be aimed at determining the expression of costimulatory molecules and Toll-like receptors, transcriptional polarization of T cells, the master factors such as GATA-binding transcription factor-3 (GATA-3) and T-bet, and also the function of accessory cells in the gut.

In conclusion, our results show that the accumulation of activated eosinophils in conjunction with IL-4⁺ cells and IL-5⁺ cells in the noninflamed duodenal mucosa may reflect a predominant Th2 systemic immune response in patients with asthma and allergic rhinitis. The absence of intestinal inflammation despite the marked presence of cells implicated in the allergic inflammation of the airways suggests that local specific regulatory mechanisms might sustain the predominant state of nonresponsiveness in the gut mucosa of the patients with asthma and allergic rhinitis. This information can be used in the development of novel therapeutic approaches based on the induction of oral tolerance for the modulation of allergic disorders.

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COLUMBUS AND THE NEW WORLD : MEDICAL IMPLICATIONS

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