

Expression of the cysteinyl leukotriene receptors cysLT₁ and cysLT₂ in aspirin-sensitive and aspirin-tolerant chronic rhinosinusitis

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Background: Cysteinyl leukotrienes play a disease-regulating role in rhinosinusitis and asthma, particularly aspirin-sensitive disease. They act through 2 G-protein coupled receptors termed cysteinyl leukotriene type 1 receptor (cysLT₁) and cysteinyl leukotriene type 2 receptor (cysLT₂). We previously compared expression of cysLT₁ on mucosal leukocytes in patients with aspirin-sensitive and aspirin-tolerant rhinosinusitis.

Objective: To compare expression of cysLT₁ and cysLT₂ on leukocytes, mucus glands, and epithelium in 32 patients with chronic polypoid rhinosinusitis (21 aspirin-sensitive, 11 aspirin-tolerant) and 9 normal controls.

Methods: Total numbers of CD45⁺ leukocytes, percentages of these cells expressing cysLT₁ or cysLT₂, and percentages of the total epithelial and glandular areas expressing cysLT₁ or cysLT₂ were measured in sections of nasal biopsies by using immunohistochemistry and image analysis.

Results: The percentages of mucosal CD45⁺ leukocytes expressing cysLT₁ were significantly ($P < .0001$) elevated in the aspirin-sensitive but not the aspirin-tolerant patients compared with the controls. In contrast, the percentages of leukocytes expressing cysLT₂ did not differ significantly in the 3 groups. On epithelial and glandular cells, expression of cysLT₂ significantly exceeded that of cysLT₁ in both the patients with rhinosinusitis and the controls ($P \leq .004$), although there was no significant difference in the expression of either receptor in the patients with rhinosinusitis (aspirin-sensitive or aspirin-tolerant) and the controls.

Conclusion: Although cysLT₁ expression predominates on inflammatory leukocytes in patients with aspirin-sensitive rhinosinusitis, the effects of cysteinyl leukotrienes on glands and epithelium may be mediated predominantly through cysLT₂. This has potentially important therapeutic implications. (J Allergy Clin Immunol 2005;115:316-22.)

Abbreviations used

cysLT: Cysteinyl leukotriene
cysLT₁: Cysteinyl leukotriene type 1 receptor
cysLT₂: Cysteinyl leukotriene type 2 receptor
LT: Leukotriene

Key words: Rhinosinusitis, aspirin, cysteinyl leukotriene, cysLT receptor

Cysteinyl leukotrienes (cysLTs) are formed by the action of 5-lipoxygenase and its activating protein on arachidonic acid released from cell membrane phospholipids by the enzyme phospholipase A₂. This produces leukotriene (LT) A₄, which is hydrolyzed in neutrophils and monocytes to form LTB₄ and conjugated to glutathione in mast cells, basophils, eosinophils, and structural cells such as epithelial cells by the enzyme LTC₄ synthase to form LTC₄, and then enzymatically converted to LTD₄ and LTE₄. The cysLTs LTC₄, LTD₄, and LTE₄ likely contribute to the pathogenesis of chronic rhinosinusitis through their effects on microvascular leakage, epithelial cell activation, elevated mucus secretion, and mucosal inflammation.¹⁻⁵

The cysLTs exert their actions on target cells through specific receptors. So far, 2 G-protein-coupled receptors for the cysLTs, termed cysLT type 1 receptor (cysLT₁) and cysLT type 2 receptor (cysLT₂), have been cloned.^{6,7} Both receptors bind to LTC₄, LTD₄, and LTE₄ with affinity order LTD₄ $\gg$ LTC₄ $>$ LTE₄ (cysLT₁) and LTD₄ = LTC₄ $\gg$ LTE₄ (cysLT₂), and both have been described to be expressed, at least at the level of mRNA, in various tissues (cysLT₁, lung including smooth muscle, small intestine, pancreas; cysLT₂, cardiac and vascular smooth muscle, adrenal medulla, central nervous system) as well as inflammatory leukocytes. Potent cysLT₁ antagonists have been shown to ameliorate the clinical symptoms of chronic rhinosinusitis,^{8,9} suggesting that at least some of the disease regulatory actions of the cysLTs are mediated through cysLT₁. In contrast, the precise function of cysLT₂ in both health and disease is unknown. Furthermore, there have been no detailed studies of its tissue distribution in allergic disease.

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Although elevated production of cysLTs is characteristic of rhinosinusitis of diverse etiology, patients with aspirin-sensitive rhinosinusitis show even higher levels of cysLTs in the nasal mucosa, which are further enhanced by aspirin challenge.^{10,11} Elevated production of cysLTs in these patients may reflect enhanced activity of key synthetic enzymes,¹² which may in part be genetically determined.¹³ Alternatively, or in addition, it may reflect unique sensitivity of LT-producing cells, particularly mast cells and eosinophils, to the direct stimulatory effects of aspirin and related salicylates.¹⁴ In a recent study,¹⁵ we showed that the percentages of inflammatory leukocytes expressing cysLT₁ were significantly elevated in the nasal mucosa of patients with aspirin-sensitive compared with aspirin-tolerant chronic rhinosinusitis. This may be one mechanism for increased target organ responsiveness to cysLTs in aspirin-sensitive rhinosinusitis, as we have previously observed in aspirin-sensitive asthma.¹⁶ Clearly, altered cysLT₂ expression could also influence such responsiveness.

Our aim was to extend our previous study¹⁵ by comparing the expression of cysLT₁ and cysLT₂ on inflammatory cells, epithelium, and mucus glands in the nasal mucosa of a group of patients with chronic rhinosinusitis, both aspirin-sensitive and aspirin-tolerant, as well as a group of healthy controls. We hypothesized that cysLT₂ as well as cysLT₁ expression is upregulated in nasal mucosal inflammatory and structural cells in patients with chronic rhinosinusitis compared with controls, and further upregulated in aspirin-sensitive compared with aspirin-tolerant patients.

METHODS

Patients

Nasal biopsy specimens were obtained from 32 patients with chronic rhinosinusitis, as defined by the presence of nasal polyps, and 9 normal control subjects with a lifelong absence of perennial or seasonal nasal symptoms. Of the patients with rhinosinusitis, 21 were aspirin-sensitive, and 11 were aspirin-tolerant. Eighteen of the 21 aspirin-sensitive patients, 8 of the 11 tolerant-patients, and 7 of the 9 normal controls were atopic as defined by a positive skin prick test to 1 or more of a panel of 10 standard aeroallergens in the presence of positive (histamine) and negative (diluent) controls. The ages of the patients ranged from 20 to 69 years and were not significantly different in the 3 groups. None of the patients smoked, and none had taken oral or intranasal corticosteroids or LT antagonists for at least 1 month before the taking of the biopsies. Data on leukocyte cysLT₁ expression in the patients with rhinosinusitis have previously been reported,¹⁵ but all immunohistochemical data have been generated de novo both to extend the work and to ensure compatibility of staining runs between the antibodies used in this study.

Diagnosis of aspirin sensitivity

If a subject could confirm that he or she had taken aspirin or a related nonsteroidal anti-inflammatory drug at clinical dosages within the previous 6 months without any untoward reaction (exacerbation of rhinitis and/or asthma, urticaria/angioedema, anaphylaxis, nausea/vomiting/diarrhea within 4 hours after ingestion), the subject was considered aspirin-tolerant. All of the normal control subjects without rhinosinusitis were tolerant by this definition. All

other patients underwent bilateral intranasal challenge with incremental dosages of lysine aspirin in a single-blind, placebo-controlled fashion.¹⁷ Reaction to the instillation was assessed subjectively with the use of symptom scores and objectively by means of acoustic rhinometry. A response was defined as a reduction in nasal patency (in terms of minimal cross-sectional area and volume, as determined by rhinometry) of at least 25%. Such a response was usually accompanied by symptoms of rhinorrhea and sneezing within 45 to 60 minutes after instillation. Aspirin tolerance was defined as absence of these changes in response to 16 mg intranasal lysine aspirin. In the event, all patients who reported a history of a clinical reaction to aspirin had a positive response to the lysine aspirin challenge.

Nasal biopsies and immunohistochemistry

Nasal biopsies were obtained by using Gerritsma forceps under local cocaine anesthesia. All patients provided written informed consent to provide these biopsies, and the Ethics Committees of Guy's Hospital and the Royal National Throat, Nose and Ear Hospital approved the study. Biopsies were processed for immunohistochemistry as previously described.¹⁸ Polyclonal rabbit antibodies selective and specific for cysLT₁ or cysLT₂ (Cayman Chemical, Ann Arbor, Mich) and extensively validated both by the manufacturers and others¹⁹ were used for immunohistochemical analysis. These antibodies showed no crossreactivity of staining. Immunostaining with avidin-biotin was performed as described previously.¹⁸ In brief, primary antibodies specific for cysLT₁ and cysLT₂ were used at dilutions of 1:50. The primary antibodies were detected with swine antirabbit biotinylated secondary antibody (Dako, High Wycombe, United Kingdom) used at a dilution of 1:200, and subsequent application of avidin/biotin/peroxidase complex solution (Dako). The brown immunoperoxidase reaction was developed with diaminobenzidine, and the sections counterstained with hematoxylin. A similar technique was used to detect leukocytes by using the panleukocyte marker CD45. These sections were stained with murine monoclonal antihuman CD45 primary antibody (Dako) at a dilution of 1:100. The primary antibodies were then detected with a biotin-conjugated, second-layer rabbit antimouse antibody (Dako) at 1:400 dilution, with subsequent application of avidin/biotin/peroxidase as before.

Double immunostaining to detect the phenotypes of leukocytes expressing cysLT₁ and cysLT₂ was performed as previously described.¹⁸ In brief, murine mAbs against CD3 (1:50), CD68 (1:50), mast cell tryptase (1:50), neutrophil elastase (1:100), and eosinophil major basic protein (1:10) were used to identify T cells, macrophages, mast cells, neutrophils, and eosinophils, respectively. All antibodies were from Dako, with the exception of antieosinophil major basic protein, which was a kind gift from Professor A. B. Kay (Imperial College, University of London, United Kingdom). Alkaline phosphatase conjugated donkey antimouse secondary antibody (1:50 dilution; Jackson ImmunoResearch Europe Ltd, Cambridgeshire, United Kingdom) followed by alkaline phosphatase-antialkaline phosphatase (1:50; Dako) was used to detect these antibodies. A red reaction was developed with Fast Red (Sigma, Poole, United Kingdom). The rabbit polyclonal antibodies were detected as described above. Sections were counterstained with hematoxylin. Double-stained cells were identified by expression of both a red and a brown precipitate.

Cells in the coded biopsy sections were counted by an investigator without previous knowledge of the provenance of the samples. Positive cells were counted by using a microscope (Olympus UK Ltd, London, United Kingdom) connected an image analyzer and associated software (KS300; Zeiss; Imaging Associates Ltd, Oxfordshire, United Kingdom). This analysis system was used to measure the areas of the epithelium, glands, and submucosa. The

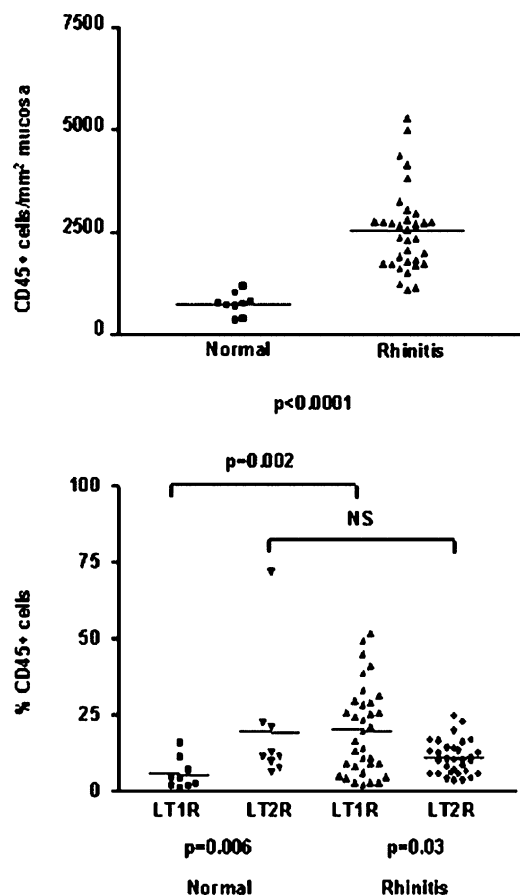


FIG 1. Total CD45⁺ leukocytes per unit area of nasal submucosa (top) and percentages of CD45⁺ leukocytes expressing cysLT₁ (LTR1) and cysLT₂ (LTR2) (bottom) in all patients with chronic rhinosinusitis and normal controls. Mann-Whitney *U* test. NS, Not significant.

areas of epithelium and glands in the biopsies showing immunoreactivity for cysLT₁ or cysLT₂ were also determined objectively by using a published strategy²⁰ to differentiate the color of hematoxylin and diaminobenzidine by threshold adjustment of the hue-light saturation-scale image after setting a shading correction for each slide by using a low pass filtered blank image. Data were expressed as percentages of CD45⁺ leukocytes per unit area of the nasal mucosa, or as percentages of total epithelial and/or glandular areas showing positive immunoreactivity. In sections from some patients (epithelium, 3 aspirin-sensitive, 4 aspirin-tolerant, 1 normal control; glands, 8 aspirin-sensitive, 3 aspirin-tolerant, 4 normal controls), no epithelium and/or glandular structures were visible, so these could not be included in analysis.

Statistical analysis

All statistical analyses were nonparametric. Between-group comparisons were performed by using the Mann-Whitney *U* test. Differences (2-sided) were considered significant at $P < .05$.

RESULTS

Expression of cysLT₁ and cysLT₂ on nasal mucosal leukocytes

The absolute numbers of CD45⁺ leukocytes as well as percentages of these cells expressing cysLT₁ and cysLT₂

in all of the patients with chronic rhinosinusitis assessed as a single group and normal controls are shown in Fig 1. Significantly elevated percentages of nasal mucosal leukocytes expressed cysLT₁ but not cysLT₂ in the patients with rhinosinusitis compared with the normal controls (cysLT₁ controls: median, 4.2; range, 0.9-15.6; rhinitis: median, 18.0; range, 2.2-51.8; $P = .002$; cysLT₂ controls: median, 11.3; range, 6.2-71.9; rhinitis: median, 10.5; range, 3.5-24.7; $P = .22$). In addition, in the biopsies from the normal controls, significantly elevated percentages of leukocytes expressed cysLT₂ compared with cysLT₁ ($P = .006$). This situation was reversed in the patients with chronic rhinosinusitis, in whom significantly elevated percentages of leukocytes expressed cysLT₁ compared with cysLT₂ ($P = .03$).

The data in Fig 2 show that elevated expression of cysLT₁ in nasal mucosal leukocytes in patients with chronic rhinosinusitis is restricted to aspirin-sensitive patients. The percentages of leukocytes expressing both cysLT₁ and cysLT₂ were not significantly elevated in the biopsies from aspirin-tolerant patients compared with normal controls. In the aspirin-tolerant patients, as in the normal controls, the median percentage of leukocytes expressing cysLT₂ exceeded that of cells expressing cysLT₁, although not significantly so.

Expression of cysLT₁ and cysLT₂ on mucus glands and epithelial cells

In both the patients with chronic rhinosinusitis and the normal controls, significantly increased percentages of the total areas of the mucus gland structures expressed immunoreactivity for cysLT₂ compared with cysLT₁ (controls: cysLT₂, median, 9.6; range, 5.2-14.2; cysLT₁, median, 0.1; range, 0.0-6.2; $P = .009$; rhinitis: cysLT₂, median, 14.6; range, 0.2-55.9; cysLT₁, median, 1.5; range, 0.0-27.8; $P = .004$; Fig 3). Similarly, significantly elevated areas of the nasal mucosal epithelium expressed cysLT₂ compared with cysLT₁ in both the patients and the controls (controls: cysLT₂, median, 21.2; range, 9.3-29.6; cysLT₁, median, 0.3; range, 0.0-2.9; $P = .0002$; rhinitis: cysLT₂, median, 14.0; range, 0.1-49.3; cysLT₁, median, 3.2; range, 0.0-31.9; $P = .002$; Fig 3). However, there was no significant difference in the amount of expression of both cysLT₁ and cysLT₂ on both glands and epithelial cells in the patients with chronic rhinosinusitis compared with the controls (Fig 3). Furthermore, there was no significant difference in the amount of expression of both receptors on both glands and epithelial cells in the patients with aspirin-sensitive compared with aspirin-tolerant rhinosinusitis (Fig 3).

Some examples of stained mucosal inflammatory cells, epithelial cells, and glands are shown in Fig 4.

Phenotypes of nasal mucosal inflammatory cells expressing cysLT₂

The percentages of the total cysLT₂ immunoreactive cells other than epithelial and glandular cells costaining

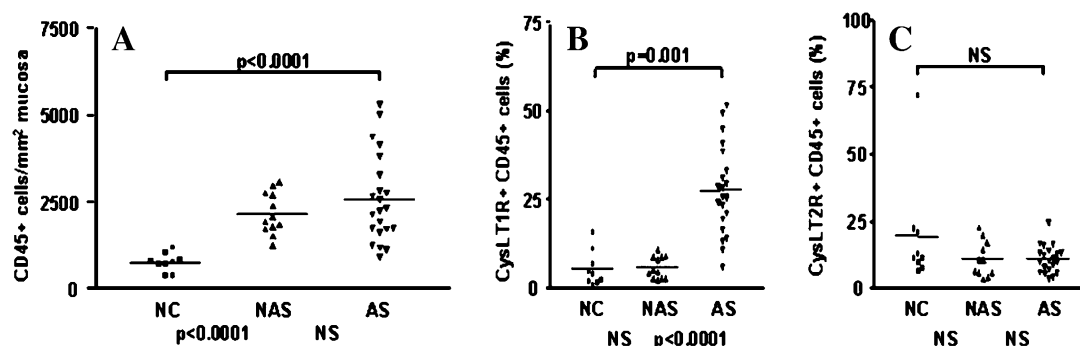


FIG 2. A, Total CD45⁺ leukocytes per unit area of nasal submucosa in patients with aspirin-sensitive (AS) or nonaspirin-sensitive (NAS) chronic rhinosinusitis and normal controls (NC). Percentages of these CD45⁺ leukocytes expressing cysLT₁ (B) and cysLT₂ (C). Mann-Whitney *U* test. NS, Not significant.

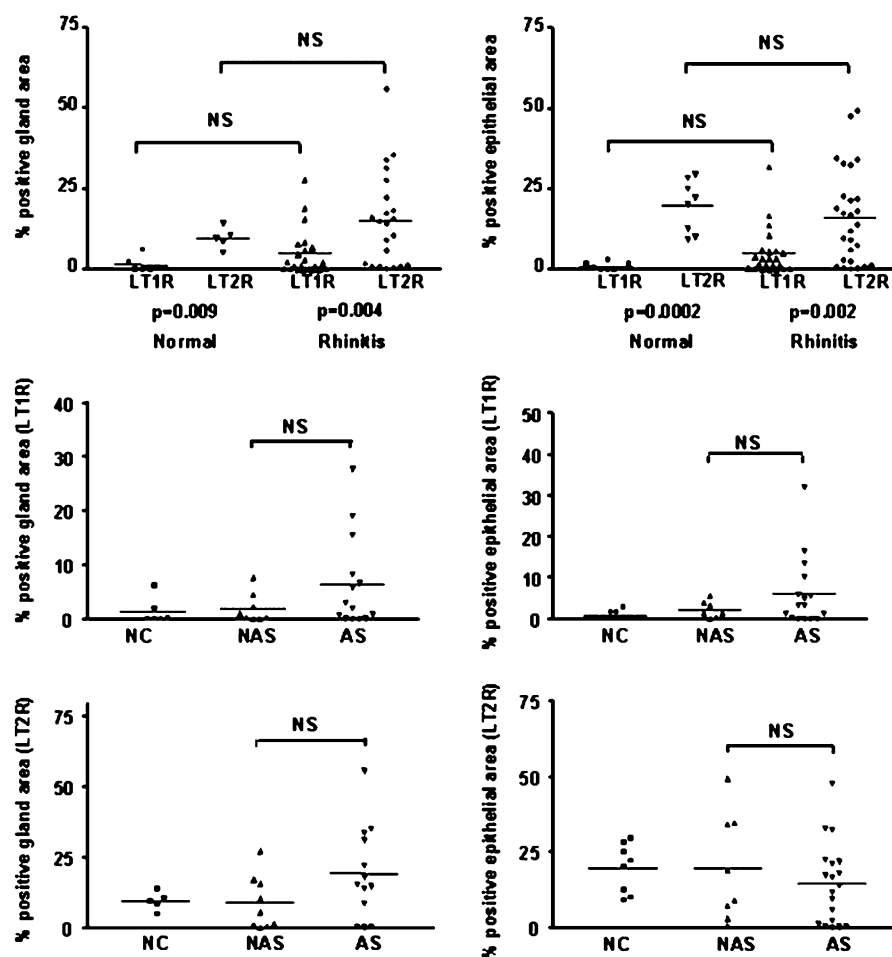


FIG 3. Percentages of areas of glands (left) and epithelium (right) expressing cysLT₁ (LT1R) and cysLT₂ (LT2R) in all patients with rhinosinusitis and normal controls (top panel). *P* values under the ordinates represent within-group comparisons. CysLT₁ data (middle panel) and cysLT₂ data (bottom panel) shown separately for the patients with aspirin-sensitive (AS) or nonaspirin-sensitive (NAS) chronic rhinosinusitis and normal controls (NC). Mann-Whitney *U* test. NS, Not significant.

with phenotypic markers for macrophages, T cells, eosinophils, neutrophils, and mast cells in representative biopsy sections from 6 aspirin-sensitive and 6 aspirin-tolerant patients with rhinosinusitis are shown in Table I.

There was clear evidence of expression of cysLT₂ immunoreactivity on all mucosal leukocytes. Aside from the glandular and epithelial cells, leukocytes accounted for most if not all of the remaining cysLT₂ immunoreactive

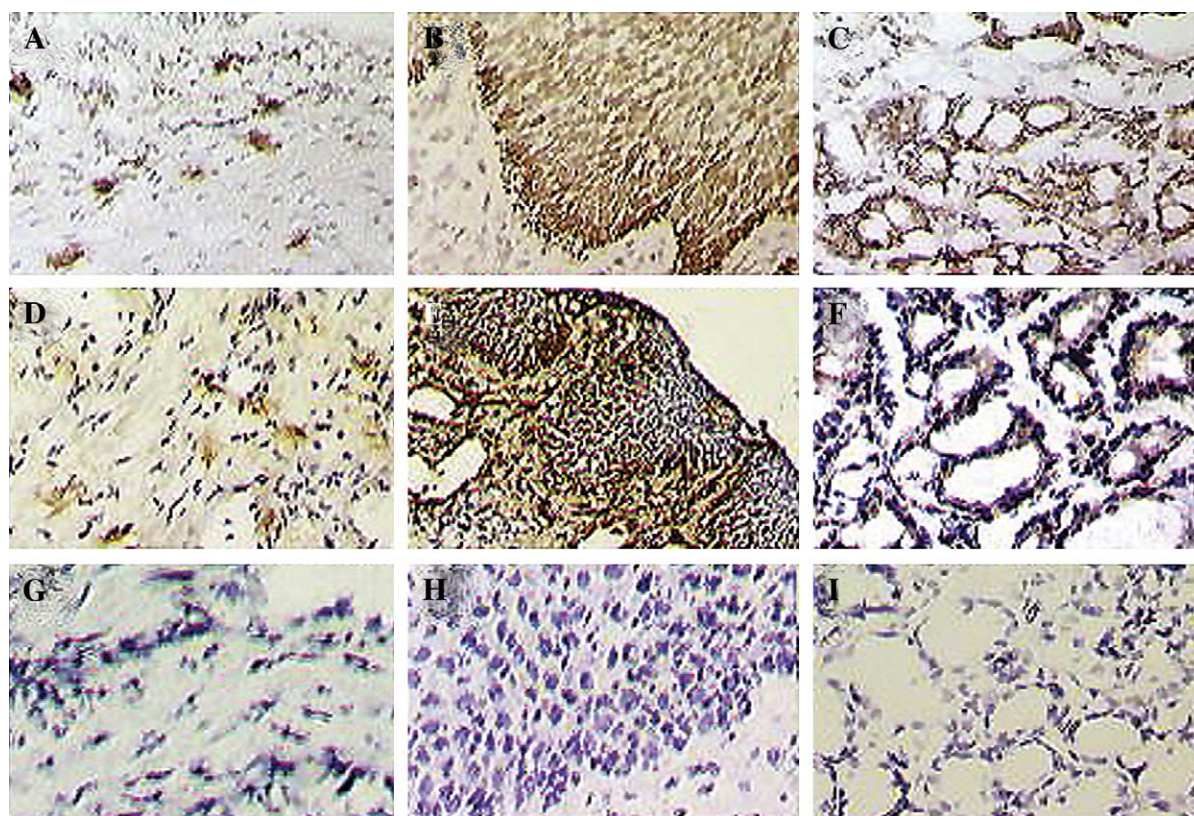


FIG 4. cysLT₂ (A-C) and cysLT₁ (D-F) receptor immunoreactivity and irrelevant isotype negative control (G-I) staining on submucosal cells, epithelium, and glands in nasal biopsy sections, respectively.

TABLE I. Percentages (mean, range) of cysLT₂ receptor immunoreactive cells (other than glandular and epithelial cells) accounted for by individual inflammatory leukocytes from 6 patients with aspirin-sensitive and 6 patients with aspirin-tolerant rhinosinusitis

Type of leukocyte	Fraction of total cysLT ₂ positive cells accounted for by cell type (%)	
	Aspirin-sensitive (n = 6)	Aspirin-tolerant (n = 6)
Macrophage	31.0 (24.9-34.1)	31.8 (10.8-53.4)
T cell	10.1 (1.2-19.3)*	4.2 (1.9-7.5)
Eosinophil	23.9 (0.0-58.0)	13.3 (2.9-28.6)
Neutrophil	22.7 (13.4-32.6)	35.3 (3.5-58.3)
Mast cell	20.7 (10.6-44.3)	37.0 (8.7-83.8)

* $P = .04$ (Mann-Whitney U test).

cells in the mucosa. T cells accounted for a significantly greater proportion of these cells in the aspirin-sensitive compared with the aspirin-tolerant patients ($P = .04$; Table I). Conversely, minority subsets of all leukocytes expressed cysLT₂ (Table II).

DISCUSSION

We have shown in this study that elevated expression of cysLT₁ on nasal mucosal inflammatory leukocytes is

TABLE II. Percentages (mean, range) of inflammatory cells expressing cysLT₂ immunoreactivity in nasal biopsy sections from 6 patients with aspirin-sensitive and 6 patients with aspirin-tolerant rhinosinusitis

Type of leukocyte	Fraction of each cell type expressing cysLT ₂ (%)	
	Aspirin sensitive (n = 6)	Aspirin tolerant (n = 6)
Macrophage	31.9 (20.3-42.7)	18.9 (4.5-41.2)
T cell	12.2 (6.8-27.1)	4.4 (0.0-8.7)
Eosinophil	36.7 (0.0-59.1)	30.3 (6.7-58.9)
Neutrophil	39.3 (18.1-68.2)	32.9 (11.6-47.5)
Mast cell	39.7 (25.0-46.4)	35.8 (20.6-53.1)

a phenomenon confined to aspirin-sensitive patients with rhinosinusitis. In contrast, these same cells showed no evidence of upregulation of cysLT₂ in patients with rhinosinusitis, whether aspirin-sensitive or aspirin-tolerant, compared with controls. These data considerably extend our previous study.¹⁵

The data in this and our previous study¹⁵ confirm that subsets of leukocytes of all phenotypes express both cysLT₁ and cysLT₂ in chronic rhinosinusitis. This is in accord with a recent study on leukocytes obtained by nasal lavage of patients with seasonal allergic rhinitis,²¹ in which

it was shown that subsets of macrophages, mast cells, eosinophils, and neutrophils expressed both receptors.

The mechanisms of preferential upregulation of *cysLT₁* compared with *cysLT₂* expression on leukocytes in aspirin-sensitive rhinosinusitis remain obscure. Proinflammatory cytokines such as IL-5, IL-4, and IL-13 have been shown to upregulate *cysLT₁* expression on an eosinophilic cell line and monocyte/macrophages^{19,22} and *cysLT₂* expression on cultured human mast cells,²³ and the expression of such cytokines is increased in the target mucosa of patients with aspirin-sensitive rhinosinusitis and asthma compared with aspirin-tolerant patients of comparable disease severity.^{24,25} Aspirin and other COX-1 inhibitors, as well as endogenous salicylates, might also activate granulocytes directly in these patients, thereby altering *cysLT* receptor expression. For example, we have provided evidence that endobronchial aspirin challenge of aspirin-sensitive patients with asthma directly activates mast cells,¹⁴ and that desensitization of patients with aspirin-sensitive rhinosinusitis with repeated topical application of lysine aspirin downregulates *cysLT₁* expression.¹⁵ As far as we are aware, the hypothesis that aspirin directly alters expression of *cysLT₁* and *cysLT₂* on cells such as mast cells and eosinophils in aspirin-sensitive subjects has never been investigated. Selective recruitment of leukocytes expressing *cysLT₁* to the nasal mucosa of aspirin-sensitive subjects is also a possibility that has been postulated²¹ on the basis of discrepancies in receptor expression on blood and nasal mucosal leukocytes in patients with rhinitis. Although the mechanism of this selective recruitment is unclear, it is possible that local elaboration of *cysLTs* in the inflamed tissues selectively promotes chemotaxis and/or survival of cells bearing the highest concentrations of *cysLT* receptors.

Elevated production of *cysLTs* in asthma and rhinitis in general and aspirin-sensitive disease in particular^{10,11,26,27} has been implicated in causing epithelial cell activation and excessive mucus secretion, which characterize these diseases.^{2,28} It is of particular interest, therefore, that we have been able to show for the first time that the distribution of *cysLT₂* in the nasal mucosa, both in health and disease, differs from that of *cysLT₁*, with a predominance of *cysLT₂* on glands and epithelium (and, indeed, mucosal leukocytes in the normal controls). Presumably, separate mechanisms ultimately result in elevated expression of *cysLT₁* on leukocytes in aspirin-sensitive rhinosinusitis and elevated expression of *cysLT₂* on structural cells of the mucosa.

Our findings raise intriguing implications for the treatment of rhinosinusitis, centered on the relative roles of *cysLT₁* and *cysLT₂* in disease pathogenesis. In some reports,^{29,30} functional activation of key cells such as eosinophils by *cysLTs* seems to be blocked extensively by specific *cysLT₁* antagonists alone, despite the fact that these cells express both *cysLT₁* and *cysLT₂*. This has led some researchers²¹ to go as far as to suggest possible redundancy of *cysLT₁* and *cysLT₂* signaling in cell types in which both receptors are expressed. Set against this is clear evidence for fundamentally different roles for *cysLT₁* and

cysLT₂ signaling. For example, in IL-4-primed cultured human mast cells, which express *cysLT₁* and *cysLT₂*, a *cysLT₁* antagonist did not inhibit IL-8 production in response to stimulation with *cysLT* but did inhibit associated production of IL-5 and phosphorylation of extracellular signal-related kinase.²³ This led the authors to suggest a possible role for *cysLT₂* receptor in mediating neutrophil as opposed to eosinophil infiltration. In another recent study,³¹ bleomycin-induced pulmonary fibrosis in mice was reduced by targeted ablation of LTC₄ synthase, the pivotal enzyme for *cysLT* biosynthesis, but increased by targeted ablation of *cysLT₁*, suggesting a role for *cysLT₂* in mediating airways remodeling, and a role for *cysLT₁* in inhibiting it. Consequently, although at first sight it is tempting to speculate from our data that combined antagonism of *cysLT₁* and *cysLT₂* or the use of 5-lipoxygenase inhibitors may offer additional therapeutic benefits over a *cysLT₁* antagonist used alone in these diseases,^{32,33} this may not necessarily be the case if *cysLT₁* and *cysLT₂* mediate distinct, possibly antagonistic effects. Further studies will be necessary to resolve these issues.

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