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Research Article

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Overexpression of Leukotriene C_4 Synthase in Bronchial Biopsies from Patients with Aspirin-intolerant Asthma

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Abstract

Aspirin causes bronchoconstriction in aspirin-intolerant asthma (AIA) patients by triggering cysteinyl-leukotriene (cys-LT) production, probably by removing PGE_2-dependent inhibition. To investigate why aspirin does not cause bronchoconstriction in all individuals, we immunostained enzymes of the leukotriene and prostanoid pathways in bronchial biopsies from AIA patients, aspirin-tolerant asthma (ATA) patients, and normal (N) subjects. Counts of cells expressing the terminal enzyme for cys-LT synthesis, LTC_4 synthase, were fivefold higher in AIA biopsies (11.5 ± 2.2 cells/mm^2, $n = 10$) than in ATA biopsies (2.2 ± 0.7, $n = 10$; $P = 0.0006$) and 18-fold higher than in N biopsies (0.6 ± 0.4, $n = 9$; $P = 0.0002$). Immunostaining for 5-lipoxygenase, its activating protein (FLAP), LTA_4 hydrolase, cyclooxygenase (COX)-1, and COX-2 did not differ. Enhanced baseline cys-LT levels in bronchoalveolar lavage (BAL) fluid of AIA patients correlated uniquely with bronchial counts of LTC_4 synthase$^+$ cells ($\rho = 0.83$, $P = 0.01$). Lysine-aspirin challenge released additional cys-LTs into BAL fluid in AIA patients (200 ± 120 pg/ml, $n = 8$) but not in ATA patients (0.7 ± 5.1, $n = 5$; $P = 0.007$). Bronchial responsiveness to lysine-aspirin correlated exclusively with LTC_4 synthase$^+$ cell counts ($\rho = -0.63$, $P = 0.049$, $n = 10$). Aspirin may remove PGE_2-dependent suppression in all subjects, but only in AIA patients does increased bronchial expression of LTC_4 synthase allow marked overproduction of cys-LTs leading to bronchoconstriction. (*J. Clin. Invest.* 1998. 101: 834–846.) Key words: asthma • aspirin • leukotriene C_4 synthase • 5-lipoxygenase • cyclooxygenase

Introduction

Aspirin-intolerant asthma (AIA)[1] is a distinct clinical syndrome characterized by adverse respiratory reactions to aspirin and other nonsteroidal antiinflammatory drugs (NSAIDs). In one study, oral challenge with aspirin caused bronchoconstriction in 19% of consecutive adult asthmatic patients (1), and other NSAID-challenge studies in both adults and children with asthma confirm a prevalence of 10–20% (2). The cysteinyl-leukotrienes (cys-LTs) have long been suspected to be important bronchoconstrictor and proinflammatory mediators in asthma (3), and this hypothesis has been confirmed recently by clinical trials with specific antagonists and synthesis inhibitors (4). Cys-LTs may be particularly prominent in NSAID-induced respiratory reactions (5).

During stimulus-specific cell activation, AA released by cytosolic phospholipase A_2 ($cPLA_2$) (6) and translocated to the 5-lipoxygenase activating protein (FLAP) (7) is converted in two steps to leukotriene (LT) A_4 by 5-lipoxygenase (5-LO) (8). LTA_4 is converted to the dihydroxy leukotriene LTB_4 by cells expressing LTA_4 hydrolase (9) and/or to the cys-LT LTC_4 by cells expressing LTC_4 synthase, which conjugates LTA_4 to reduced glutathione (10). After carrier-mediated cellular export of LTC_4 (11), the sequential cleavage of Glu and Gly provides the extracellular, receptor-active metabolites LTD_4 and LTE_4, respectively (12, 13). Elevated levels of LTE_4 in the urine of patients with aspirin intolerance provide evidence of constitutive chronic activation of the 5-LO/LTC_4 synthase pathway (14–16). After oral aspirin, inhaled lysine-aspirin (lys-aspirin), or bronchoscopic lys-aspirin challenge, a large increase in production of these cys-LTs is detectable in the bronchoalveolar lavage (BAL) fluid and/or urine of patients with AIA but not those with aspirin-tolerant asthma (ATA) (14, 15, 17–19). LT synthesis inhibitors and selective cys-LT receptor antagonists markedly attenuate aspirin-induced respiratory reactions (5, 20–23), whereas selective histamine H_1 antagonists have little effect (24).

In prostanoid biosynthesis, the released AA is converted directly by constitutive prostaglandin endoperoxide synthase (PGHS-1/cyclooxygenase [COX]-1) or by induced PGHS-2 (COX-2) in two steps to the intermediate prostaglandin PGH_2, which is common to the terminal prostanoid synthases and thromboxane synthase (25–27). Oral aspirin and inhaled lys-aspirin challenges of AIA patients reduce levels of prostanoids in BAL fluid and urine at the same time that cys-LT levels rise markedly (17–19). The COX product PGE_2 has been proposed as an important downregulatory mediator in asthma (28) be-

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1. *Abbreviations used in this paper:* AIA, aspirin-intolerant asthma; ATA, aspirin-tolerant asthma; BAL, bronchoalveolar lavage; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; cys-LT, cysteinyl-leukotriene; ECP, eosinophil cationic protein; EIA, enzyme immunoassay; FEV_1, forced expiratory volume in 1 s; FLAP, 5-LO activating protein; GMA, glycol methacrylate; 5-LO, 5-lipoxygenase; LT, leukotriene; lys-aspirin, lysine-aspirin; N, nonatopic normal; NSAID, nonsteroidal antiinflammatory drug; PD_{20}, provocation dose producing a 20% decrease in FEV_1.

cause NSAIDs enhance, and PGE_2 reduces, LT synthesis in a number of inflammatory cell types in vitro, including human eosinophils and neutrophils and rat macrophages (29–32). In AIA patients, both the aspirin-induced rise in urinary LTE_4 and the consequent bronchoconstriction can be abrogated completely by prior inhalation of PGE_2 (33). Thus, NSAIDs could trigger asthma in susceptible patients by reducing the PGE_2-dependent suppression of cys-LT synthesis in the lung. However, while plausible, this concept fails to explain why NSAIDs do not trigger a similar rise in cys-LTs in ATA patients (15, 18, 19), nor why AIA patients have persistently elevated levels of urinary LTE_4 and chronic asthma even in the absence of NSAID exposure (14–16).

The 5-LO/LTC_4 synthase pathway is limited to cells of bone marrow origin (34, 35), and the chronic defect subject to exacerbation with aspirin challenge could be the overexpression of some step in the pathway in the infiltrating eosinophils/basophils or resident mast cells/macrophages. Alternatively, or in addition, there could be underrepresentation of some control function of the prostanoid pathways, which are widely distributed in diverse lineage cell types (28, 36). Therefore, we have identified and quantitated the cells expressing the principal enzymes of the COX and 5-LO pathways in biopsies of bronchial mucosa from patients with AIA and with ATA and from nonatopic, normal (N) individuals after local placebo and lys-aspirin challenges. We report here a unique overrepresentation in bronchial biopsies from AIA patients of cells expressing LTC_4 synthase, the essential enzyme for cys-LT synthesis, and the correlation of this finding with the release of cys-LT into BAL fluid and with bronchial responsiveness to inhaled lys-aspirin.

Methods

Reagents. mAbs to mast cell tryptase (AA1), CD3 (UCTH1), CD8, and the macrophage-restricted form of CD68 (PG-M1), biotinylated swine anti–rabbit IgG, biotinylated rabbit anti–mouse Fab fragments, and streptavidin-biotin–horseradish peroxidase conjugate were from DAKO Ltd. (High Wycombe, Bucks, UK). mAbs EG1 and EG2 to granule-associated and secretory eosinophil cationic protein (ECP), respectively (Pharmacia Biosystems Ltd., Milton Keynes, UK), to CD4 (Becton Dickinson Ltd., Oxford, UK), to recombinant human IL-3 (R & D Systems, Abingdon, Oxfordshire, UK), and to human COX-1 and COX-2 (Cayman Chemical Co., Inc., Ann Arbor, MI) were purchased as indicated. Drs. P. Hissey and L. McNamee (Glaxo Wellcome PLC, Greenford, Middlesex, UK) provided mAbs to recombinant human IL-5 (MAB7) and GM-CSF (101 BB.2.86), respectively. Dr. J. Evans (Merck Frosst Canada Inc., Quebec, Canada) generously provided rabbit polyclonal antibodies to human leukocyte 5-LO (LO-32), FLAP (H4), and LTA_4 hydrolase. Affinity-purified rabbit polyclonal antibody to human LTC_4 synthase was generated as described (35). Peptidoleukotriene EIA kits (Cayman Chemical Co., Inc.), ^{3}H-LTC_4 (DuPont-NEN, Boston, MA), glycol methacrylate (GMA) embedding kits (Park Scientific Ltd., Northampton, UK), aminoethylcarbazole (Muratech Scientific, Aylesbury, Bucks, UK), PMSF, iodoacetamide, poly-L-lysine, BSA, and DME (Sigma Chemical Co., Poole, Dorset, UK), hydrogen peroxide, sodium azide, acetate and Tris buffers (Merck Ltd., Lutterworth, Leicester, UK), and L-lys-aspirin (Aspisol; Bayer AG, Leverkusen, Germany) were purchased as indicated.

Subjects. Patients with AIA (n = 10) and with ATA (n = 10) were in the long-term care of the Department of Medicine, University School of Medicine, Krakow, Poland. All were nonsmokers, were clinically stable at the time of study, and had a diagnosis established by previous oral aspirin challenge tests. Bronchial responsiveness to inhaled lys-aspirin was assessed with a dosimeter-controlled nebulizer driven by compressed air (Spiro Electro 2; Hengityshoitokeskus Co., Hameenlinna, Finland) 2–6 wk before bronchoscopy. The nebulizer output was 10.3 μl/breath with a particle mass median diameter of 1.6 μm. Doses of lys-aspirin are expressed as equivalent doses of aspirin in milligrams. The solution was inhaled every 30 min with exponential dose increments up to 200 mg aspirin equivalents, with forced expiratory volume in 1 s (FEV_1) measured at 10, 20, and 30 min after each challenge. AIA patients had a provocation dose of aspirin producing a 20% fall (PD_{20}) in FEV_1 ranging from 0.5 to 199 mg (Table I). ATA patients did not show a 20% fall in FEV_1 even at the maximal dose ($PD_{20} > 200$ mg). The two groups were similar in age and sex, with no significant differences ($P > 0.05$, Mann-Whitney) in the duration of asthma, spirometry, serum IgE, or duration and dosage of inhaled corticosteroid medication (Table I). N subjects (n = 9) were nonatopic nonsmokers (six male and three female; median age 21 yr, range 19–35) who had no history of chronic respiratory disease and were taking no medication.

Table I. Clinical Characteristics of Patients with AIA and ATA

	AIA	ATA
Number	10	10
Sex (male:female)	6:4	7:3
Aspirin PD_{20} FEV_1 (mg)*	3.5 (0.5–199)	> 200
Baseline FEV_1 (% predicted)‡	92±3	92±7
Age (yr)*	44.5 (33–70)	45 (20–59)
Duration of asthma (yr)*	6 (1–13)	8.5 (3–20)
Number with > 2 positive skin-prick tests	1/10	5/10
Serum IgE (IU/ml)§	156 (83–290)	107 (44–262)
Number receiving inhaled steroids	8/10	7/10
Dosage of inhaled steroids (μg/d)*	650 (0–1800)	800 (0–1400)
Duration of inhaled steroid use (yr)*	2 (0–9)	1.8 (0–14)
Number receiving oral prednisolone	2/10	3/10

*Median (range). ‡Mean±SEM. §Geometric mean (95% confidence interval).

Bronchoscopy. Bronchoscopy and BAL were carried out according to the American Thoracic Society guidelines (37). Bronchial mucosal biopsies were collected as described (38). All subjects were given supplemental oxygen by cannula, and arterial oxygen saturation and pulse rate were monitored by pulse oximeter. After premedication with 0.5 mg atropine, 2.5 mg midazolam, and topical anesthesia with 2–4% lidocaine, the fiberoptic bronchoscope (Olympus Optical Co., Ltd., Tokyo, Japan) was inserted through the nostril, and the lingula was entered. Application of lidocaine to the bronchi was minimized and was similar in all patients. In the AIA and ATA patients, four 50-ml portions of sterile 0.9% sodium chloride at 37°C were instilled and aspirated gently into a siliconized glass container on ice. Then, 10 mg of L-lys-aspirin or placebo dissolved in 5 ml sterile saline (0.9%) was instilled immediately into the right middle lobe segmental bronchus. Placebo consisted of lysine and glycine at the same pH and osmolarity used in lys-aspirin. With the bronchoscope in situ, the challenged segment was lavaged 15 min later as described above. In all subjects, bronchial mucosal biopsies were then taken from the right lower lobe carinae. 10 AIA and 10 ATA patients underwent placebo challenge, and 10 AIA and 6 ATA patients returned for lys-aspirin challenge on a separate day 4 wk later. Nine N subjects underwent bronchoscopy and biopsy on one occasion.

Immunohistochemistry. The biopsy specimens were fixed in acetone containing PMSF (2 mM) and iodoacetamide (20 mM) for 16–24 h

at −20°C and then embedded in GMA resin as described (39). Serial sections were cut at 2 μm, floated onto ammonia water (0.2%), and picked up on poly-L-lysine–coated glass slides. Immunostaining was performed as described (39); irreversible inhibition of endogenous peroxidase with sodium azide (0.1%) and hydrogen peroxide (0.3%) was confirmed by the lack of color development when the aminoethylcarbazole (0.03%) chromagen was added to washed sections in the absence of antibodies. Background staining was reduced by blocking with BSA (1% wt/vol) in DME. Sections were incubated with primary antibodies for 1 h (polyclonals) or overnight (monoclonals). Secondary antibodies were biotinylated swine anti–rabbit IgG (1:200) or rabbit anti–mouse Fab as appropriate. Staining was visualized with streptavidin-biotin–horseradish peroxidase conjugate (1:200) using aminoethylcarbazole (0.03%) in acetate buffer (pH 5.2) as chromagen. Sections were counterstained for 1–2 min with Mayer's hematoxylin and rinsed before mounting. Control sections were routinely immunostained with the primary antibody absent, or replaced with an unrelated isotype-matched mouse IgG or nonimmune rabbit serum; they always showed no color development.

Quantification of immunohistochemical staining. Positive immunostaining was defined conservatively as cell-associated red reaction product detectable at fixed illumination intensity against the background of the Mayer's hematoxylin counterstain and compared with a uniform reference standard, but no attempt was made to quantify the intensity of staining within each cell. Cell counts were performed at a magnification of 400 on coded sections, and data are expressed as the mean density of positively staining nucleated cells per square millimeter evaluated over the total submucosal area of two to eight nonadjacent biopsy sections, giving a minimum counted area of 3 mm^2, excluding mucous glands, blood vessels, and areas of forceps damage. Biopsy areas were quantified in square millimeters with an image analysis system (ColorVision 164SR; Analytical Measurement Systems, Cambridge, UK). Colocalization of immunostaining for two markers was performed on adjacent 2-μm sections with the camera lucida (Leica UK Ltd., Milton Keynes, UK) as described (40).

BAL fluid mediator assays. BAL fluid was filtered through gauze and centrifuged at 1,000 *g* for 10 min at 4°C to remove cells. The cell pellets were stained with Giemsa stain for differential counts. The total concentration of LTC_4, LTD_4, and LTE_4 in BAL fluid supernatants was determined by commercial EIA (Cayman Chemical Co., Inc.) after purification on a silica column as described (19). Recovery was determined with 4,000 cpm of tritiated LTC_4 as an internal standard. The range of the EIA standard curve was 7–1,000 pg/ml, with 50% binding at 53 pg/ml. The polyclonal antibody recognizes LTC_4 and LTD_4 (100%), LTE_4 (67%), and *N*-acetyl-LTE_4 (11%), but not LTB_4, 20-OH-LTB_4, monohydroxyeicosatetraenoic acids, or prostanoids (< 0.01%).

Statistical analyses. Normality of data was assessed by normal probability score using the Minitab statistical software package (Minitab, Inc., State College, PA), and results are expressed as mean±SEM or median (range) as appropriate. Parametric data sets were compared by paired or unpaired Student's *t* tests as appropriate, and nonparametric data were compared by Mann-Whitney U test (unpaired) or Wilcoxon signed rank test (paired). $P < 0.05$ was accepted as significant.

Results

Profile of leukocytes in bronchial biopsies after placebo challenge. Counts of mast cells, eosinophils, and macrophages in 10 AIA and 10 ATA biopsies after placebo challenge are

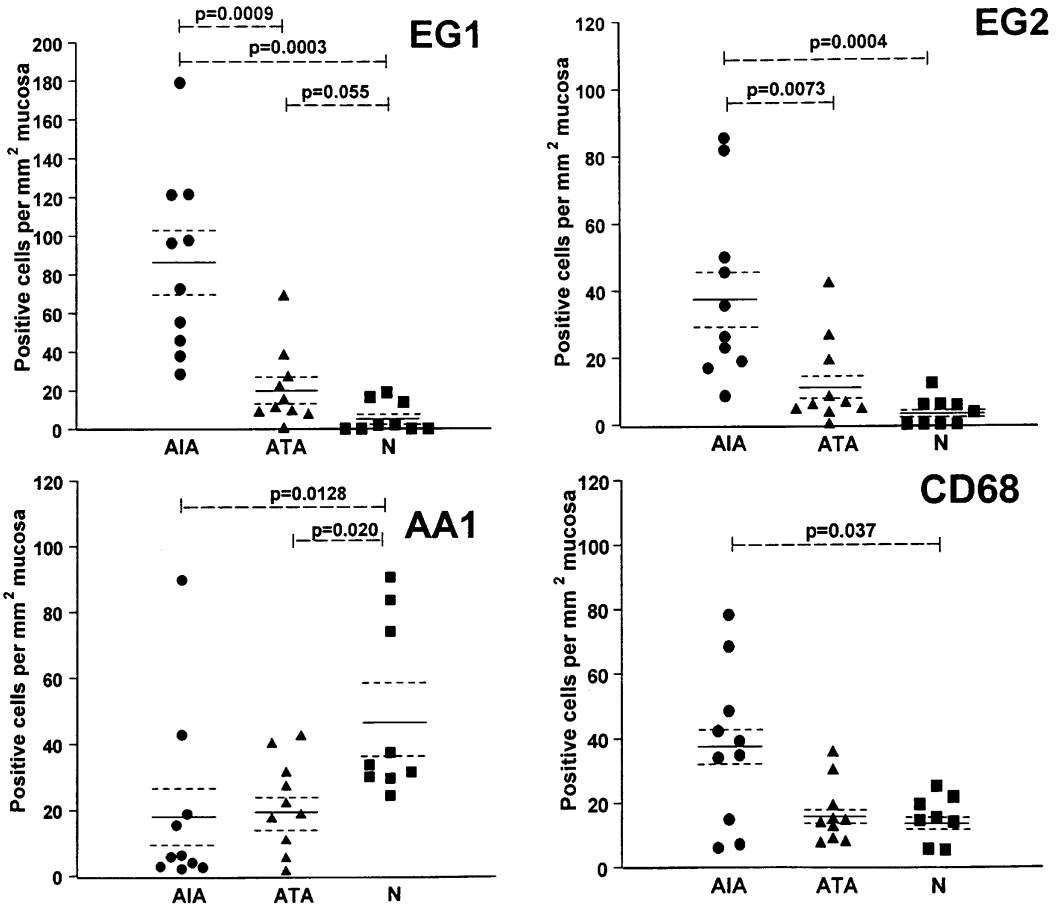

Figure 1. Immunostaining for myeloid cell markers in GMA-embedded bronchial mucosal biopsies from patients with AIA (●; $n = 10$), patients with ATA (▲; $n = 10$), and N subjects (■; $n = 9$), taken 20 min after bronchoscopic challenge with placebo solution. Cell markers are total cellular ECP (*EG1*, total eosinophils), translocated ECP (*EG2*, "activated" eosinophils), mast cell tryptase (*AA1*), and CD68 (PG-M1, CD68 macrophages). *Horizontal bars*, mean±SEM. All significant comparisons between subject groups ($P < 0.05$ Mann-Whitney) are indicated.

Table II. T Lymphocyte Counts in Biopsies of AIA and ATA Patients and N Controls

	Placebo			Lys-aspirin		
	CD3	CD4	CD8	CD3	CD4	CD8
AIA	46.9±12.3 (10)	22.7±7.4 (10)	16.6±4.8 (10)	74.3±13.3* (10)	30.6±5.9 (10)	34.9±8.3 (10)
ATA	54.1±13.7 (10)	26.3±6.9 (10)	21.0±8.3 (10)	31.4±13.1 (6)	18.0±10.4 (6)	16.6±8.5 (6)
N	33.6±6.4 (9)	18.6±3.4 (9)	17.1±3.5 (9)	—	—	—

Counts of cells immunostaining for the T lymphocyte markers CD3, CD4, and CD8 in bronchial mucosal biopsies of patients with AIA and ATA taken 20 min after bronchoscopic challenge with placebo or with lys-aspirin (5 mg aspirin equivalents). Counts of T cell markers are also shown in bronchial biopsies of unchallenged, N controls. Values are mean±SEM cells/mm^2 (n). There are no significant differences (Mann-Whitney) for any CD marker between AIA, ATA, and N biopsies after placebo challenge, and no CD marker changed significantly after lys-aspirin challenge compared with the placebo challenge of the same group (Wilcoxon). *$P = 0.03$ vs. ATA.

shown in Fig. 1, together with control values from 9 unchallenged N biopsies. The mast cell population was identified with the mAb AA1, which recognizes tryptase in the resting granule (41). There were no differences between AIA and ATA biopsies in counts of AA1$^+$ mast cells, although there were fewer AA1$^+$ mast cells in both asthmatic groups compared with the N biopsies ($P \leq 0.02$), possibly indicating ongoing mast cell degranulation. The total eosinophil population was identified with the mAb EG1, which recognizes granule-associated and secretory ECP and eosinophil-derived neurotoxin. The population of eosinophils expressing secretory ECP was identified with mAb EG2 (42). AIA biopsies contained fourfold more EG1$^+$ eosinophils (85.4±14.8 cells/mm^2) than ATA biopsies (20.5±6.4 cells/mm^2; $P = 0.0009$), and 15-fold more than N biopsies (5.8±2.6 cells/mm^2; $P = 0.0003$). EG2$^+$ eosinophils were threefold more numerous in AIA biopsies (39.4±8.4 cells/mm^2) than in ATA biopsies (12.1±4.2 cells/mm^2; $P = 0.0073$) and 10-fold more than in N biopsies (3.8±1.4 cells/mm^2; $P = 0.0004$). Macrophages were identified with mAb PG-M1, which recognizes a monocyte- and macrophage-restricted form of CD68 (43). Counts of CD68$^+$ macrophages in AIA biopsies (37.2±7.6 cells/mm^2) tended to be higher than in ATA biopsies (16.3±3.0 cells/mm^2; $P = 0.070$) and were significantly higher than in N biopsies (15.2±2.5 cells/mm^2, $n = 8$; $P = 0.037$).

Counts of T lymphocytes (CD3$^+$; UCTH1), T helper cells (CD4$^+$), and CD8$^+$ T cells did not differ among the AIA ($n = 10$), ATA ($n = 10$), and N ($n = 8$) groups (Table II). However, in the AIA but not in the ATA or N biopsies, the counts of CD4$^+$ cells correlated significantly with EG1$^+$ eosinophil counts ($\rho = 0.76$, $P = 0.01$, $n = 10$) and with EG2$^+$ eosinophil counts ($\rho = 0.71$, $P = 0.02$, $n = 10$). Eosinophil counts did not correlate with CD8$^+$ T cell counts in any subject group.

Eicosanoid enzyme expression after placebo challenge. Representative photomicrographs showing immunostaining for 5-LO, FLAP, LTA_4 hydrolase, COX-1, and COX-2 in one AIA biopsy are shown in Fig. 2. After placebo challenge, there were no significant differences between the AIA, ATA, and N groups in counts of cells immunostaining for COX-1 or COX-2 ($P > 0.05$, Mann-Whitney) (Table III) or in counts of cells immunostaining for 5-LO, FLAP, or LTA_4 hydrolase (Fig. 3). However, mean (±SEM) counts of cells immunostaining for LTC_4 synthase, the committed enzyme for cys-LT synthesis, were fivefold higher in the AIA biopsies (11.5±2.2 cells/mm^2; $n = 10$) than in the ATA biopsies (2.2±0.7 cells/mm^2, $n = 10$; $P = 0.0006$) and 18-fold higher than in the N biopsies (0.6±0.4 cells/mm^2, $n = 9$; $P = 0.0002$) (Figs. 3 and 4). The counts in ATA biopsies did not reach statistical significance compared with N biopsies ($P = 0.077$). No N and only one ATA biopsy had LTC_4 synthase$^+$ cell counts within the range of values in the AIA biopsies.

In the AIA biopsies, counts of LTC_4 synthase$^+$ cells did not correlate with counts of mast cells, eosinophils, macrophages, or CD8$^+$ T cells, but did correlate significantly with CD3$^+$ T cell counts ($\rho = 0.658$, $P = 0.039$, $n = 10$) and with CD4$^+$ T cell counts ($\rho = 0.648$, $P = 0.043$, $n = 10$). No correlations were observed between LTC_4 synthase$^+$ cells and the counts of any cell types in ATA or N biopsies.

Relationship of LTC_4 synthase$^+$ cell counts to BAL fluid levels of cys-LTs and eosinophil counts in BAL cell pellets. Mean (±SEM) baseline BAL fluid levels of total cys-LTs were significantly higher in AIA patients (41.9±7.8 pg/ml, $n = 8$) than in ATA patients (22.2±3.4 pg/ml, $n = 5$; $P = 0.046$). In the AIA group, baseline cys-LT levels correlated significantly with counts of bronchial mucosal LTC_4 synthase$^+$ cells ($\rho = 0.83$, $P = 0.01$, $n = 8$), but not with counts of cells expressing 5-LO, FLAP, LTA_4 hydrolase, COX-1, or COX-2. The correlation improved when the ATA subjects were included ($\rho = 0.86$, $P < 0.001$, $n = 13$) (Fig. 5), although the correlation

Table III. Expression of COX-1 and COX-2 in AIA, ATA, and N Biopsies

	Placebo		Lys-aspirin	
	COX-1	COX-2	COX-1	COX-2
AIA	35.8±11.2 (10)	6.2±1.2 (10)	30.5±3.0* (10)	19.8± 5.6 (10)
ATA	25.5±6.5 (10)	8.7±2.7 (10)	13.2±4.0 (5)	11.9±2.9 (5)
N	24.7±4.0 (8)	14.3±4.5 (8)	—	—

Counts of cells immunostaining for COX-1 and COX-2 in bronchial mucosal biopsies of patients with AIA and ATA taken 20 min after bronchoscopic challenge with placebo or with lys-aspirin (10 mg aspirin equivalents). Counts of cells immunostaining for COX-1 and COX-2 are also shown in bronchial biopsies of unchallenged, N controls. Values are mean±SEM cells/mm^2 (n). The expression of COX-1 and COX-2 did not differ between the AIA, ATA, and N biopsies after placebo challenge (Mann-Whitney), and did not change significantly after lys-aspirin challenge compared with the placebo challenge of the same group (Wilcoxon). *$P = 0.012$ vs. ATA.

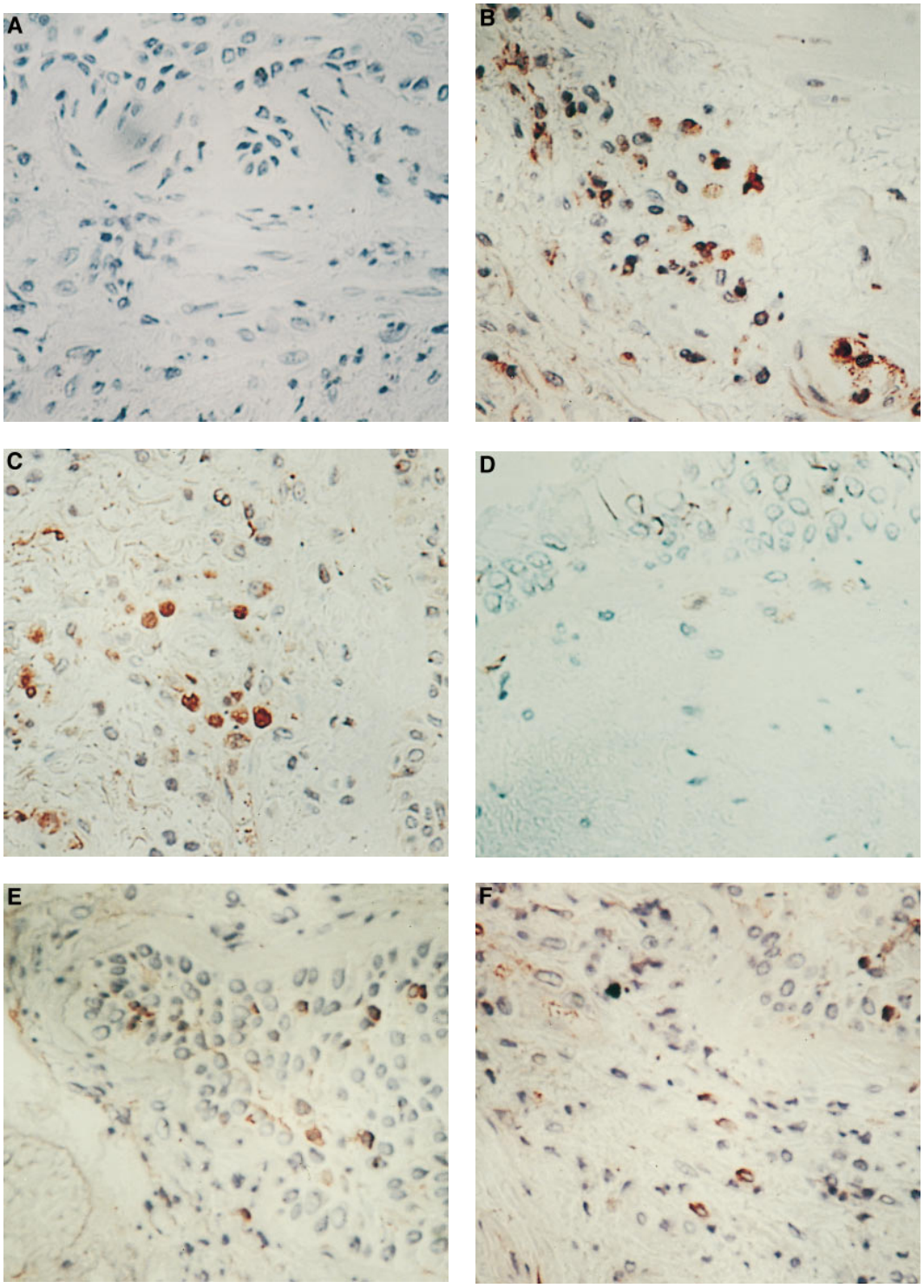

Figure 2. Photomicrographs (×400) of immunostaining for eicosanoid pathway enzymes in representative GMA-embedded bronchial biopsy from a patient with AIA after placebo challenge. Positive immunostaining appears red against blue background of Mayer's hematoxylin counterstain. Panels are (*A*) control (primary antibody absent), (*B*) 5-lipoxygenase (LO-32), (*C*) FLAP (H4), (*D*) LTA_4 hydrolase, (*E*) COX-1, and (*F*) COX-2.

within the ATA group itself was not significant ($\rho = 0.46$, $P = 0.4$, $n = 5$). The proportion of eosinophils in BAL cell pellets was significantly higher at baseline in the AIA group (2.2±0.7%, $n = 10$) than in the ATA group (0.2±0.1%, $n = 5$; $P = 0.03$).

Relationship of LTC_4 synthase$^+$ cell counts to bronchial responsiveness to inhaled lys-aspirin. Bronchial responsiveness to aspirin was determined as the PD_{20} FEV_1 to inhaled lys-aspirin 2–6 wk before bronchoscopy. PD_{20} FEV_1 to lys-aspirin is stable for at least 6 wk (44). In 10 AIA subjects, $\log_{10}$ PD_{20} FEV_1 to inhaled lys-aspirin showed a significant inverse relationship with the $\log_{10}$-transformed counts of LTC_4 synthase$^+$ cells in the bronchial mucosa ($\rho = -0.633$, $P = 0.049$) (Fig. 6). From this, a fivefold higher LTC_4 synthase$^+$ cell count in the AIA patients equalled an ~ 200-fold enhanced sensitivity to inhaled lys-aspirin. PD_{20} values showed no significant relation-

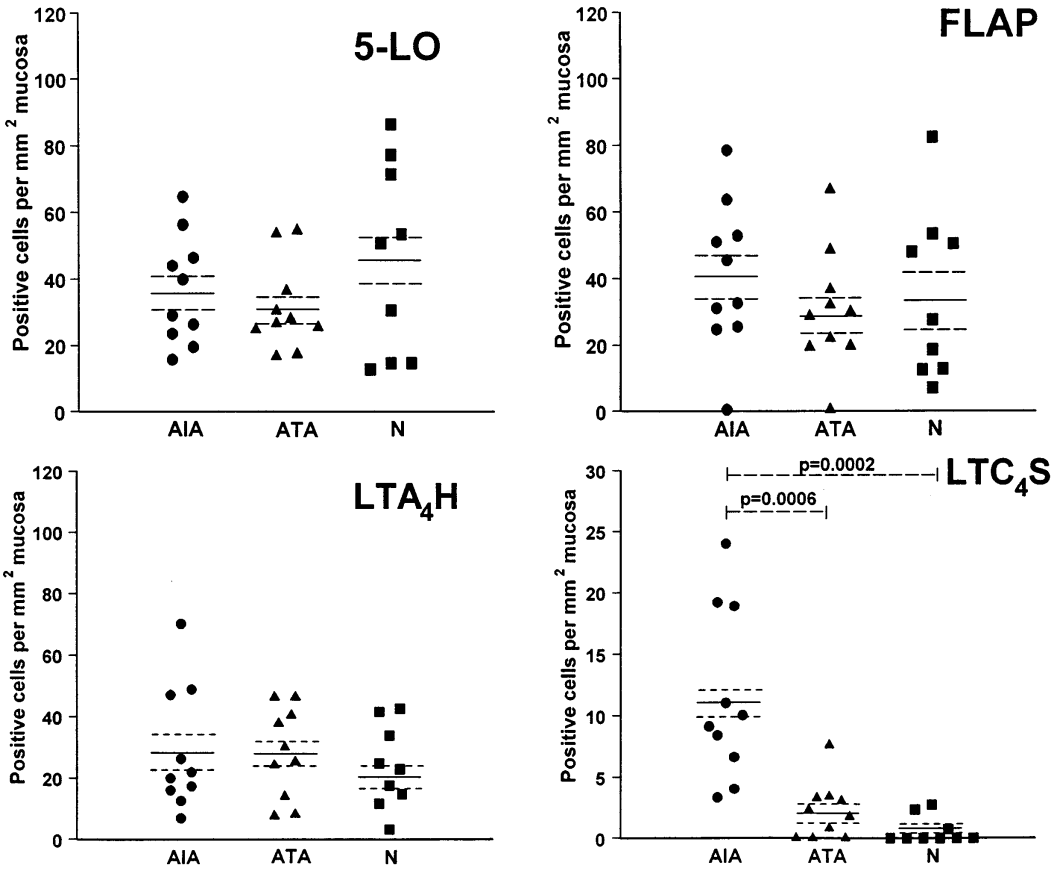

Figure 3. Counts of cells immunostaining for 5-LO pathway enzymes in bronchial mucosal biopsies from patients with AIA ($n = 10$), with ATA ($n = 10$), and N subjects ($n = 9$), taken 20 min after bronchoscopic challenge with placebo solution. Enzymes are 5-LO, FLAP, LTA_4 hydrolase (LTA_4H), and LTC_4 synthase (LTC_4S). *Horizontal bars*, mean±SEM. All significant comparisons between subject groups ($P < 0.05$ Mann-Whitney) are indicated.

ships to counts of cells immunostaining for other eicosanoid pathway enzymes (5-LO, FLAP, LTA_4 hydrolase, COX-1, or COX-2), to any cell markers, or to clinical characteristics.

Colocalization of eicosanoid enzymes to cell markers. Eicosanoid pathway enzymes were colocalized to cells by parallel staining of adjacent sections and optical superimposition by the camera lucida system (40). Counts of cells immunostaining for 5-LO or FLAP correlated closely in all subjects ($\rho = 0.84$, $P < 0.001$, $n = 20$), and colocalization confirmed that most 5-LO$^+$ cells were also FLAP$^+$. Most 5-LO and FLAP colocalized to macrophages, eosinophils, and mast cells, and there were no significant differences between the AIA, ATA, and N subjects in the proportion of each of these cell types which immunostained positively for 5-LO or FLAP. Immunostaining for LTA_4 hydrolase was apparent in macrophages, bronchial epithelium, and vascular endothelium. COX-1 and COX-2 colocalized particularly to mast cells, with lesser staining in eosinophils and macrophages. COX-1 was also present in vascular endothelium. There were no gross differences in the profile of colocalization between the subject groups for these enzymes.

A more detailed analysis was performed on LTC_4 synthase. LTC_4 synthase$^+$ cells were widely distributed in the submucosal area of all biopsies, with occasional clusters of positive cells colocalizing particularly to EG2$^+$ eosinophils. In the eight of ten AIA biopsies which had a density of LTC_4 synthase$^+$ cells sufficient for colocalization ($> 5/mm^2$), the LTC_4 synthase$^+$ cells were mostly EG2$^+$ eosinophils (mean±SEM: 70.6± 6.4%; range 50–100%), with smaller proportions of AA1$^+$ mast cells (10.6±4.3%; range 0–31%) and CD68$^+$ macrophages (2.4±1.7%; range 0–12.5%) (Fig. 7). LTC_4 synthase immunostaining did not colocalize to lymphocytes. In ATA patients, the small numbers of LTC_4 synthase$^+$ cells precluded quantitative colocalization in individual subjects, but pooled data from six biopsies suggest ~ 45% were EG2$^+$ eosinophils, 18% were mast cells, and < 4% were CD68$^+$ macrophages. The proportion of mast cells expressing LTC_4 synthase was relatively low in the AIA (12.0±7.7%) and ATA biopsies (5.2±3.2%), and the proportion of CD68 macrophages expressing LTC_4 synthase was also low in both groups (mean < 5%). The proportion of EG2$^+$ eosinophils expressing LTC_4 synthase was significantly higher in AIA biopsies (51.4±7.6%; $n = 10$) than in ATA biopsies (21.1±10.1%; $n = 6$; $P = 0.038$), with all AIA biopsies showing > 20% positivity compared with only three of the ATA biopsies. In the AIA but not the ATA biopsies, the proportion of EG2$^+$ eosinophils expressing LTC_4 synthase correlated strongly with the counts of CD4$^+$ lymphocytes ($\rho = 0.88$, $P < 0.001$, $n = 10$), but not with counts of CD8$^+$ or CD3$^+$ lymphocytes or other cell types or with cytokine expression.

Cytokine expression after placebo challenge. Immunostaining for the eosinophilopoietic cytokines GM-CSF, IL-3, and IL-5 was performed in biopsies from 10 patients with AIA and 10 with ATA after placebo challenge. Fourfold more cells immunostained for IL-5 in the AIA than in the ATA biopsies ($P = 0.031$), but there were no significant differences in immunostaining for IL-3 or GM-CSF (Table IV). The density of IL-5$^+$

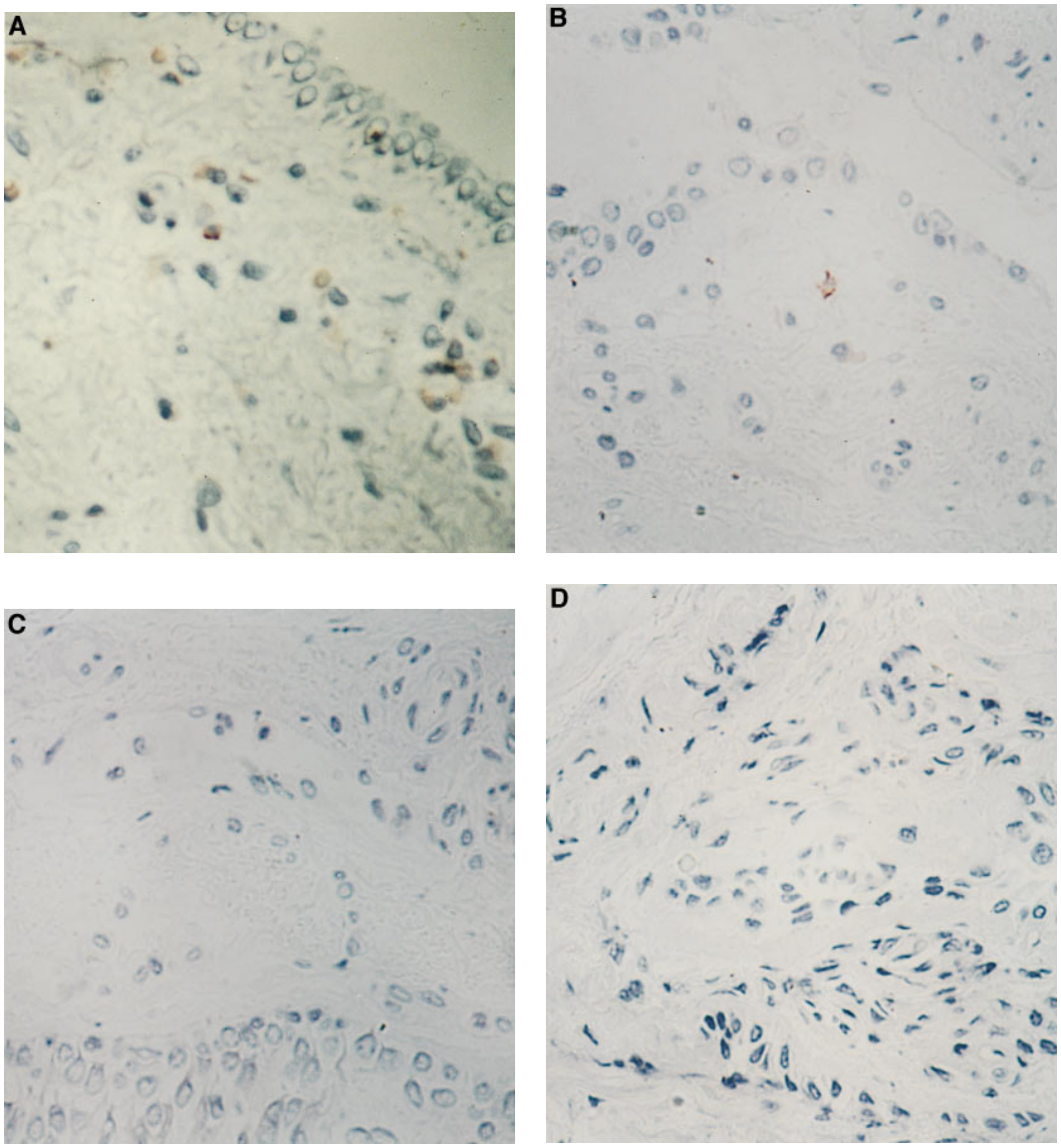

Figure 4. Photomicrographs (×400) illustrating distribution and density of immunostaining for LTC_4 synthase in representative GMA-embedded bronchial biopsies from (*A*) an AIA patient, (*B*) an ATA patient, and (*C*) an N subject. *D* is a control section from the AIA biopsy stained in the absence of primary antibody.

cells was insufficient for camera lucida colocalization to cell markers in most biopsies, but pooled data from six AIA and four ATA biopsies suggested that ~ 40–50% of IL-5$^+$ cells were AA1$^+$ mast cells in both subject groups, and 8–15% were EG2$^+$ eosinophils. The proportion of AA1$^+$ mast cells which were IL-5$^+$ was 20±3% in AIA (n = 6) and 10±3% in ATA biopsies (n = 4) (P = 0.07). The proportion of EG2$^+$ eosinophils that were IL-5$^+$ was very low in both groups (AIA, 6±3%; ATA, 5±3%; $P > 0.05$).

The numbers of eosinophils in the AIA biopsies after placebo challenge did not correlate significantly with the numbers of cells expressing IL-5 ($\rho = 0.55$, $P = 0.1$, $n = 10$), but this correlation was significant in the ATA biopsies ($\rho = 0.90$, $P < 0.001$, $n = 10$). The correlation remained significant when the

Table IV. Eosinophilopoietic Cytokine Immunostaining in Biopsies of AIA and ATA Patients

	Placebo			Lys-aspirin		
	IL-3	IL-5	GM-CSF	IL-3	IL-5	GM-CSF
AIA	3.4±1.3 (10)	5.0±1.3* (10)	4.0±1.5 (10)	4.2±1.1‡ (10)	9.3±1.7* (10)	5.6±1.1 (10)
ATA	2.1±0.7 (10)	1.3±0.4 (10)	5.7±1.8 (10)	0.3±0.1 (6)	3.9±0.7 (6)	7.3±2.6 (6)

Counts of cells immunostaining for IL-3, IL-5, and GM-CSF in bronchial mucosal biopsies of patients with AIA and ATA taken 20 min after bronchoscopic challenge with placebo or with lys-aspirin (5 mg aspirin equivalents). Values are mean±SEM cells/mm^2 (n). IL-5 expression was significantly higher in AIA than in ATA biopsies after placebo challenge (Mann-Whitney). Paired analysis (Wilcoxon) showed no significant changes in IL-3, IL-5, or GM-CSF expression after lys-aspirin challenge compared with placebo. *$P < 0.05$ vs. ATA. ‡$P < 0.002$ vs. ATA.

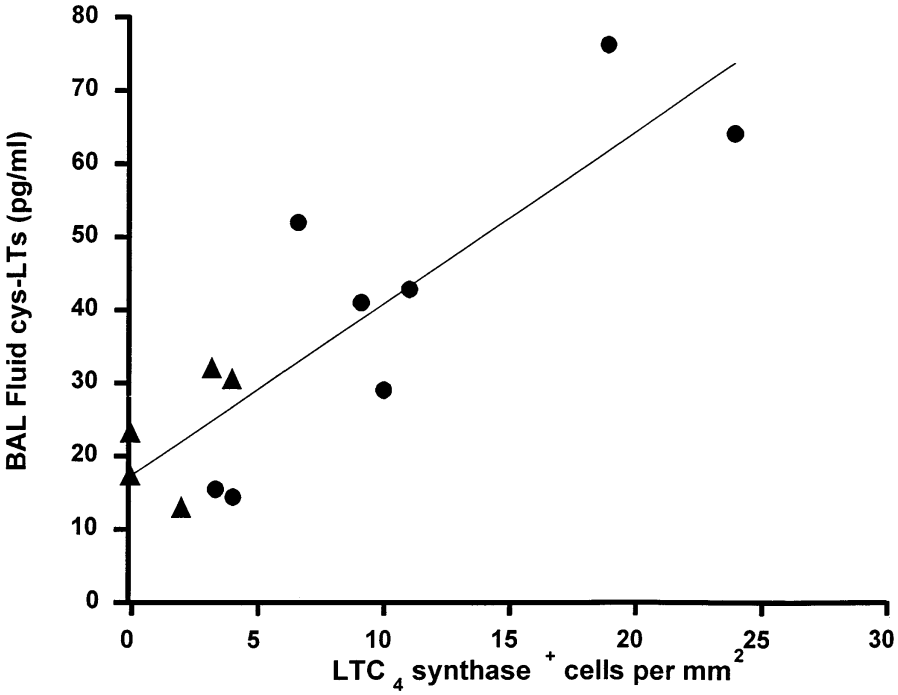

Figure 5. Relationship of baseline concentrations of total cys-LTs measured by EIA in BAL fluid of AIA (●) and ATA (▲) patients (in picograms per milliliter) to counts of cells immunostaining for LTC_4 synthase in the bronchial mucosa (cells per square millimeter) ($\rho = 0.86$, $P < 0.001$, $n = 13$).

two groups were combined ($\rho = 0.71$, $P < 0.001$, $n = 20$). There were no meaningful correlations in either group between eosinophil counts and the numbers of cells expressing IL-3 or GM-CSF.

Effect of local lys-aspirin challenge on leukocyte counts in bronchial biopsies and BAL fluid. BAL fluid and bronchial mucosal biopsies were obtained 15 and 20 min, respectively, after segmental challenge with 10 mg lys-aspirin in 10 AIA and 6 ATA patients. As after placebo challenge, there were fourfold higher counts of $EG1^+$ eosinophils ($P = 0.0029$) and fivefold higher counts of $EG2^+$ eosinophils ($P = 0.0057$) in the AIA compared with ATA biopsies (Table V). There were also significantly more macrophages ($CD68^+$) ($P = 0.034$) and significantly more pan-T lymphocytes ($CD3^+$) ($P = 0.026$) in the AIA than in the ATA biopsies (Table II). However, paired analysis of results after lys-aspirin and placebo challenges in the 10 AIA and 6 ATA subjects who underwent both procedures showed that aspirin challenge did not significantly alter submucosal counts of eosinophils, mast cells, macrophages, or T cell subsets in either subject group compared with the corresponding values after placebo challenge. $EG2^+$ eosinophil counts expressed as a percentage of $EG1^+$ eosinophil counts in each patient also did not change significantly from placebo values.

The proportion of eosinophils in BAL cell pellets rose significantly to 6.7±2.2% after lys-aspirin challenge of the AIA patients ($P = 0.037$ compared with baseline values) but not of the ATA patients (0.6±0.2%; $P > 0.05$). The rise in eosinophil percentage occurred in only 5 of 10 AIA subjects and showed no meaningful relationship to biopsy counts of eosinophils or to increases in BAL fluid cys-LTs.

Effect of lys-aspirin challenge on eicosanoid enzyme and cytokine expression. After lys-aspirin challenge, there were no differences between AIA ($n = 10$) and ATA ($n = 6$) patients in cells immunostaining for COX-2, but there were significantly more cells staining for COX-1 in AIA biopsies (Table III). This difference between the groups had not been apparent after placebo challenge; however, when a paired analysis (Wilcoxon) was performed on those AIA ($n = 10$) and ATA ($n = 6$) patients who underwent both challenges, there were no significant differences in $COX\text{-}1^+$ or $COX\text{-}2^+$ cell counts after lys-aspirin challenge compared with corresponding values after placebo challenge ($P > 0.1$).

After lys-aspirin challenge, there were no differences in the biopsies from AIA ($n = 10$) and ATA ($n = 6$) patients in immunostaining for 5-LO, FLAP, or LTA_4 hydrolase (Table V).

Table V. Effect of Lys-aspirin Challenge on Cell Counts and 5-LO Pathway Enzyme Expression in Biopsies of AIA and ATA Patients

	AIA	ATA	*P*
AA1	18.0±6.1	29.1±4.1	0.11
EG1	58.4±7.1	13.5±3.1	0.0029
EG2	40.8±7.4	8.5±3.9	0.0057
CD68	41.6±7.0	16.0±6.2	0.046
5-LO	54.4±10.2	55.9±10.2	0.88
FLAP	47.0±11.7	51.4±16.1	0.63
LTA4H	27.9±5.6	23.9±5.5	0.87
LTC4S	15.5±4.1	2.5±1.1	0.004

Counts of cell markers and of cells expressing 5-LO pathway enzymes in bronchial biopsies from patients with AIA ($n = 10$) and ATA ($n = 6$), taken 20 min after bronchoscopic challenge with lys-aspirin (5 mg aspirin equivalents). Cell markers are tryptase (AA1, mast cells), total ECP (EG1, eosinophils), translocated ECP (EG2, activated eosinophils), and CD68 (macrophages). Enzymes are 5-LO, FLAP, LTA_4 hydrolase (*LTA4H*), and LTC_4 synthase (*LTC4S*). Values are mean±SEM cells/mm^2. *P* values are AIA versus ATA by Mann-Whitney U test. Paired comparison (Wilcoxon) with data from the same patients after placebo challenge (Figs. 1 and 3) showed no significant changes in cell markers or enzyme expression after lys-aspirin challenge.

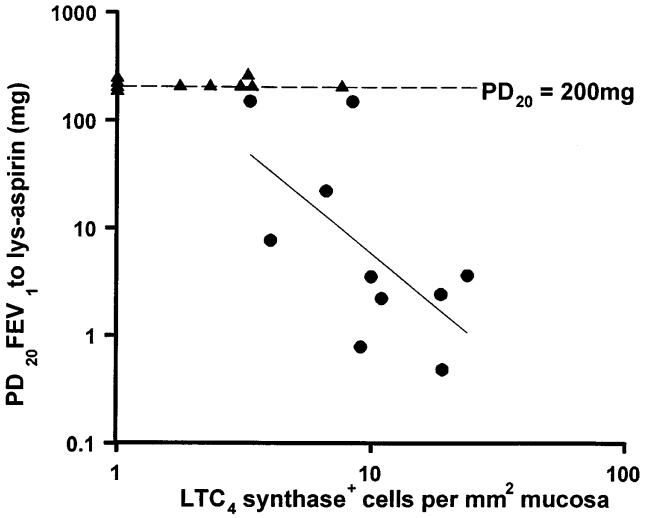

Figure 6. Relationship of bronchial responsiveness to inhaled lys-aspirin, expressed as PD_{20} FEV_1 to aspirin (in milligrams; $\log_{10}$ scale), to the counts of cells immunostaining for LTC_4 synthase (cells per square millimeter; $\log_{10}$ scale) in GMA-embedded bronchial mucosal biopsies of AIA patients (●; $n = 10$). AIA patients had PD_{20} FEV_1 to aspirin values ranging from 199 to 0.48 mg, and correlated significantly with LTC_4 synthase$^+$ cell counts ($\rho = -0.633$, $P = 0.049$, $n = 10$). Equation of regression line: $\log_{10} y = 2.8 - 2.0 \log_{10} x$. Results from biopsies of 10 ATA patients (▲) with PD_{20} FEV_1 to aspirin of ≥ 200 mg are shown as equal to 200 mg for purposes of illustration, but are not included in the linear regression analysis. PD_{20} FEV_1 to aspirin showed no relationship to immunostaining for other eicosanoid enzymes, cytokines, or cell markers, or to clinical characteristics.

In contrast, the mean (±SEM) counts of cells expressing LTC_4 synthase were sixfold higher in AIA biopsies than in ATA biopsies ($P = 0.004$) (Table V), corroborating similar findings after placebo challenge. In both patient groups, paired analysis (Wilcoxon) of results for 5-LO pathway enzyme expression after lys-aspirin challenge showed no changes from corresponding values in the same patients after placebo challenge.

After lys-aspirin challenge, submucosal counts of cells expressing IL-5 were 2.4-fold higher in AIA than in ATA subjects ($P = 0.045$) (Table IV), corroborating similar findings after placebo challenge. There were also significantly more cells immunostaining for IL-3 ($P < 0.002$) (Table IV), a difference which had not been apparent after placebo challenge. There were no group differences in counts of GM-CSF$^+$ cells. When a paired analysis (Wilcoxon) was performed in the 10 AIA and 6 ATA subjects who underwent both challenges, counts of IL-5$^+$, IL-3$^+$, and GM-CSF$^+$ cells were not significantly different after lys-aspirin challenge compared with corresponding values in the same subjects after placebo challenge.

Effect of lys-aspirin challenge on BAL fluid cys-LT levels. BAL fluid cys-LT levels rose sixfold to 240±121 pg/ml 15 min after lys-aspirin challenge in the AIA patients ($n = 8$, $P = 0.014$, Wilcoxon), but did not change significantly in the ATA patients (22.9±4.3 pg/ml, $n = 5$; $P > 0.05$) (Fig. 8). The lys-aspirin–induced changes in BAL fluid cys-LT levels did not correlate with counts of LTC_4 synthase$^+$ cells in the bronchial submucosa in either group (AIA: $\rho = 0.03$, $P = 0.9$, $n = 8$; and ATA: $\rho = 0.45$, $P = 0.4$, $n = 5$), or when the AIA and ATA groups were combined ($\rho = 0.25$, $n = 13$; $P = 0.4$) (data not shown). The lys-aspirin–induced changes in BAL fluid cys-LT levels also showed no significant relationship to counts of cells immunostaining for any other eicosanoid pathway enzyme (5-LO, FLAP, LTA_4 hydrolase, COX-1, or COX-2) or to cytokine expression or cell markers, in either patient group.

Discussion

That adverse respiratory reactions to aspirin and other COX inhibitors (45) are due to a surge in cys-LT production is evident from the assay of cys-LT metabolites (14, 15, 17–19) and the clinical efficacy of agents that block biosynthesis or action of the receptor-active cys-LTs (5, 20–23). Although it is reasonable to attribute the effect to the liberation of the 5-LO pathway from suppression by endogenous PGE_2, it remains unclear why a similar response to NSAIDs is not seen in patients with ATA and in N subjects. We have confirmed previous reports that immunostaining for COX-1, COX-2, and 5-LO in bronchial biopsies from AIA and ATA patients is not different (46, 47), and have extended this finding to FLAP and LTA_4 hydrolase and even to a comparison with N subjects. In contrast, there is a profound overrepresentation in AIA bronchial biopsies of cells expressing LTC_4 synthase, the integral perinuclear membrane enzyme that forms LTC_4 (35, 48, 49), the exported parent of the receptor-active cleavage products LTD_4 and LTE_4 (11), as compared with cells expressing the enzyme in ATA and N biopsies (Fig. 3 and Table V). This unique difference may provide a basis for the chronic overproduction, and for the aspirin-induced increments in cys-LT production in AIA, and for the lack of adverse responses to NSAIDs in ATA patients and N subjects.

LTC_4 synthase$^+$ cell counts in the bronchial submucosa correlated uniquely with basal levels of cys-LTs in BAL fluid (Fig. 5), suggesting that higher LTC_4 synthase$^+$ cell counts may explain chronic cys-LT overproduction and impaired baseline lung function in AIA patients not exposed to NSAIDs (14–16, 22). The synthesis of cys-LTs requires the sequential activity of

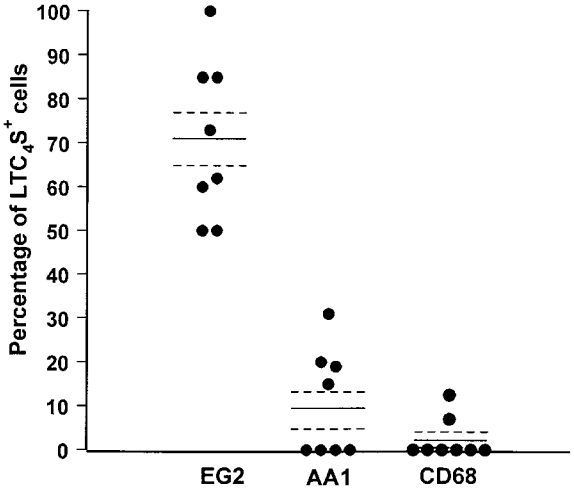

Figure 7. Proportion of LTC_4 synthase$^+$ cells colocalizing to immunostaining for EG2 (eosinophils), AA1 (mast cells), and CD68 (macrophages) in the 8 of 10 AIA biopsies with a density of LTC_4 synthase$^+$ cells sufficient for colocalization on adjacent sections by the camera lucida technique. *Horizontal bars*, mean±SEM.

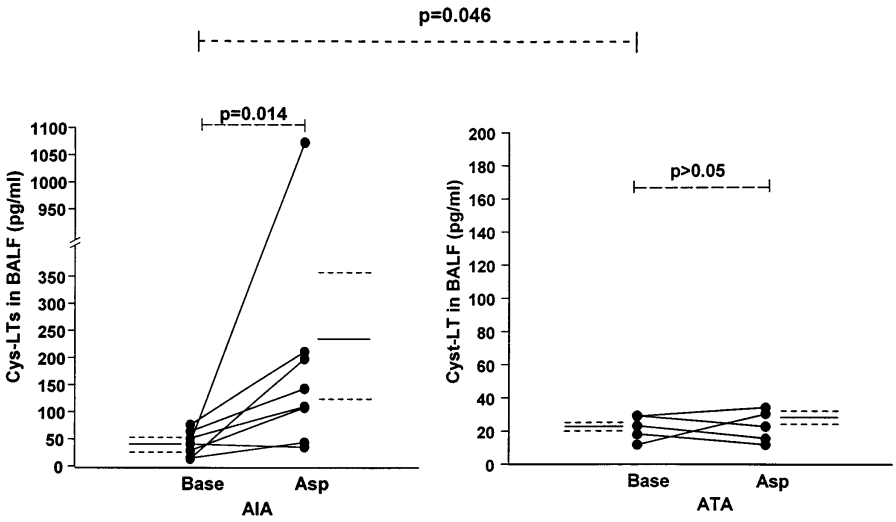

Figure 8. Total concentration of cys-LTs quantified by EIA in BAL fluid (*BALF*) from AIA patients (●, $n = 8$; *left*) and ATA patients (▲, $n = 5$; *right*) immediately before and 15 min after bronchoscopic challenge with lys-aspirin (5 mg aspirin equivalents). Baseline levels are significantly higher in the AIA group (Mann-Whitney), and rise significantly after lys-aspirin challenge only in the AIA group (Wilcoxon).

$cPLA_2$, 5-LO/FLAP, and LTC_4 synthase, and the regulation of cys-LT synthesis may occur at both proximal and distal enzyme sites. In murine bone marrow–derived mast cells, cytokine priming regulates the expression not only of $cPLA_2$, 5-LO, and FLAP, but also of the terminal enzyme LTC_4 synthase (50). Human neutrophils generate large amounts of the 6-trans-diastereoisomers of LTB_4 reflecting the extracellular degradation of excess LTA_4, indicating that the maximal function of the LTA_4 hydrolase to generate LTB_4 is insufficient. Thus, in neutrophils, maximal LTB_4 synthesis is limited by the terminal enzyme LTA_4 hydrolase (51). In the cytokine-induced development of eosinophils from $CD34^+$ cord blood cells, expression of cytosolic PLA_2, 5-LO, and FLAP occurs at an early stage, but the ability of eosinophils to generate cys-LTs emerges only when LTC_4 synthase expression is induced at a later stage (52). Therefore, expression of the terminal enzyme LTC_4 synthase in eosinophils or mast cells may be limiting. In bronchial tissue, then, the production of cys-LTs may be determined predominantly by the number of cells expressing the enzyme. The number of LTC_4 synthase$^+$ cells was fivefold higher in the biopsies of AIA compared with ATA patients, and basal BAL fluid cys-LT levels were also significantly higher in the AIA group. In both groups, BAL fluid cys-LT levels correlated significantly with bronchial biopsy counts of LTC_4 synthase cells$^+$, and this relationship was unique, as baseline BAL fluid cys-LT levels did not correlate with the expression of any other eicosanoid pathway enzyme, including 5-LO, FLAP, COX-1, or COX-2. Our data provide a powerful explanation for chronic overproduction of cys-LTs in AIA patients even in the absence of exposure to NSAIDs (14–16, 22).

Immunohistochemical analysis of bronchial biopsies was also combined with assays of cys-LTs in BAL fluid after challenge with inhaled lys-aspirin. In response to challenge, AIA patients, who had a high density of LTC_4 synthase$^+$ cells (11.5 ± 2.2 cells/mm^2), produced large amounts of cys-LTs in BAL fluid (200 ± 121 pg/ml), whereas the ATA patients, who had one fifth the density of LTC_4 synthase$^+$ cells (2.2 ± 0.7 cells/mm^2), generated minimal quantities of cys-LTs (0.7 ± 5.1 pg/ml) (Fig. 8). That the baseline relationship between LTC_4 synthase$^+$ cell number and cys-LT generation does not hold for lys-aspirin challenge implies an additional variable, possibly due to the incremental loss of 5-LO/LTC_4 synthase regulation. Post-challenge levels of BAL fluid cys-LTs also did not correlate with the expression of other eicosanoid pathway enzymes, including 5-LO, FLAP, COX-1, or COX-2. It is unlikely that only a subgroup of LTC_4 synthase$^+$ cells activated by lys-aspirin, as there were no correlations between BAL fluid cys-LT levels after challenge and biopsy counts of individual cell types. Alternatively, the lack of correlation after inhaled lys-aspirin challenge may be due to conversion of cys-LTs to their sulfoxides and to 6-trans-LTB_4 diastereoisomers by hypochlorous acid, formed by eosinophil peroxidase released by activated eosinophils undergoing the respiratory burst (53). In vivo inhibition of this reaction was not feasible in these clinical studies. Although there was no quantitative correlation, the qualitative association of uniquely higher counts of LTC_4 synthase$^+$ cells in the bronchial biopsies of AIA patients with dramatic increases in BAL fluid cys-LT levels after lys-aspirin challenge suggests nevertheless that LTC_4 synthase overexpression may contribute significantly to aspirin-induced exacerbations in AIA patients.

Indeed, over a 400-fold range of PD_{20} FEV_1 values, bronchial responsiveness to inhaled lys-aspirin challenge correlated exclusively with counts of LTC_4 synthase$^+$ cells in the bronchial mucosa (Fig. 6), and not with counts of cells expressing any other 5-LO or COX pathway enzyme, or with individual cell-types, cytokine expression, or clinical characteristics. That the cell populations in biopsies of AIA and ATA patients did not differ appreciably in the expression of any eicosanoid pathway enzyme other than LTC_4 synthase (Figs, 2, 3, and 4), and did not differ in cell type, as defined by markers, except for eosinophils, mast cells, and macrophages (Fig. 1), which possess the complete 5-LO/LTC_4 synthase pathway, suggests that an enhanced total number of cells expressing LTC_4 synthase in the bronchial wall, irrespective of cell type, is the principal determinant of adverse respiratory reactions to aspirin.

A persistent failure in the PGE_2 braking mechanism with increased sensitivity to inhibition by NSAIDs has been postulated to explain why AIA patients overproduce cys-LTs both chronically and after low doses of NSAIDs. However, baseline BAL fluid levels of PGE_2, PGD_2, $PGF_{2\alpha}$, thromboxane A_2, and other prostanoids are not different in AIA and ATA patients (19), suggesting the absence of any chronic defect in prostanoid metabolism in the AIA lung. Furthermore, inhaled lys-

aspirin can reduce BAL fluid and nasal PGE_2 levels in ATA patients to the same extent as in AIA patients (18, 19, 54), suggesting that there is no fundamental difference in the ability of NSAIDs to inhibit PGE_2 synthesis, but only the AIA patients generate significant cys-LTs as a result. The pharmacokinetics of aspirin are similar in AIA and ATA patients (55). The finding of enhanced LTC_4 synthase expression in AIA may resolve this paradox by making the hypothesis of an anomaly in the prostanoid pathway redundant. Instead, NSAIDs may reduce PGE_2 synthesis equally effectively in all subjects, but in the AIA lung more LTC_4-generating cells are thus liberated from PGE_2 suppression than in the ATA or N lung, leading to detectable cys-LT release and bronchoconstriction only in AIA patients.

That the predominant population of LTC_4 synthase$^+$ cells was eosinophils was recognized by colocalization of LTC_4 synthase to cell markers using the camera lucida technique on adjacent single-stained sections; colocalization or comparisons of two or more different antigens can only be semiquantitative, as equivalent intensity of immunostaining does not necessarily reflect equivalent quantities of each antigen, and the results must therefore be treated with caution. The majority (71%) of LTC_4 synthase$^+$ cells in AIA biopsies were EG2$^+$ eosinophils, with only a small proportion of AA1$^+$ mast cells and CD68$^+$ macrophages (Fig. 7). The proportion of EG2$^+$ eosinophils expressing LTC_4 synthase was significantly higher in AIA biopsies (mean 51%) than in ATA biopsies (21%), suggesting that upregulation is induced by the microenvironment. The substantial fraction of LTC_4 synthase$^+$ eosinophils, together with the three- and tenfold higher counts of EG2$^+$ eosinophils in AIA biopsies compared with the ATA and N biopsies, respectively, relates the association of LTC_4 synthase with airway reactivity to the eosinophil.

In contrast, relatively few mast cells stained positively for LTC_4 synthase in any subject group, and the number of LTC_4 synthase$^+$ cells detected in normal biopsies was minimal (0.6 cells/mm^2) compared with the relatively high number of mast cells (mean 48 cells/mm^2). Although other studies report the presence of more AA1$^+$ mast cells in AIA than in ATA biopsies (46), this may be related to the higher proportion of AIA patients who were atopic (7/12 skin test positive) compared with this study (1/10). In our study, mast cell counts were similar in AIA and ATA biopsies, although both were lower than in N biopsies, possibly indicating ongoing degranulation in both asthmatic groups.

We found that the number of macrophages (CD68$^+$) was nonsignificantly higher in AIA biopsies compared with ATA biopsies ($P = 0.07$) and significantly higher than in N biopsies ($P = 0.03$). A report of fewer macrophages in AIA than in ATA biopsies (46) was based on the mAb EBM11, which recognizes a form of CD68 common to both macrophages and all myeloid cell-types, whereas we used mAb PG-M1, which recognizes a macrophage-specific form of CD68 (43).

The relative contributions of mast cells, macrophages, and eosinophils to overproduction of cys-LTs in AIA may differ with experimental challenge based on the tissues involved. In aspirin-sensitive patients, the increase in cys-LTs and decreases in prostanoid levels that occur in nasal secretions after local or oral aspirin challenge (54, 56) are associated with increments in nasal histamine and tryptase, indicating mast cell activation (56, 57). Tryptase and histamine levels also rise in the serum of patients experiencing systemic reactions to oral aspirin, but not in those challenged with inhaled lys-aspirin (17, 58). In two bronchoscopy studies in which AIA patients were given lys-aspirin by inhalation or via the bronchoscope, a decrease in BAL fluid PGE_2 and an increase in cys-LTs were not accompanied by an increment in BAL fluid tryptase levels (18, 19). Since BAL fluid tryptase rises significantly and rapidly after inhaled allergen challenge of atopic asthmatics (59), it is possible that bronchial mast cells respond differently to lys-aspirin than those at nasal or cutaneous sites, and that eosinophils are the predominant source of cys-LTs in the airways in AIA. Eosinophilia is a prominent feature in the blood, airways, and nasal polyps of aspirin-sensitive patients (2). A remarkable influx of eosinophils into the nasal airways of aspirin-sensitive patients after nasal lys-aspirin challenge has been described recently (60). Oral aspirin causes a decrease in blood eosinophil counts and a trend toward incremental serum ECP levels, suggestive of eosinophil activation and migration, and this is accompanied by an increase in methacholine bronchial responsiveness (17). In BAL fluid, prechallenge eosinophil counts and ECP levels are raised in AIA compared with ATA patients (18). The effect of lys-aspirin challenge is contradictory, with ECP reported either to decrease (18) or to remain unchanged (19). Although eosinophil counts were elevated in bronchial biopsies of AIA relative to ATA patients, lys-aspirin challenge did not elicit a further net eosinophil presentation in either patient group (Table V). Since lys-aspirin–elicited bronchoconstriction is mediated by cys-LT (5, 20–23), and the dose-dependency is inversely related to the numbers of LTC_4 synthase$^+$ cells in the airways (Fig. 6), of which the predominant cells are eosinophils, it seems most likely that the latter are the critical source of the mediator.

Lys-aspirin challenge reduces BAL fluid levels of the eosinophil-associated prostanoids PGE_2 and TXB_2, but it does not diminish levels of the mast cell–distinctive prostanoids PGD_2 or $PGF_{2\alpha}$ in AIA patients (19). Each prostanoid is inhibited in ATA patients (19). A similar phenomenon has been observed in aspirin-induced nasal reactions (54), and a modest increase is seen even in urinary levels of 9α,11β-PGF_2, a metabolite of PGD_2, in AIA patients after aspirin bronchoprovocation (61). The PGD_2 could contribute to the bronchoconstriction in the absence of the bronchodilatory PGE_2. Evidence that AIA patients tolerate the relatively selective COX-2 inhibitor nimesulide (62) suggests that the endogenous PGE_2 brake may be linked to COX-1 activity. In vitro studies using NSAIDs to negate any prostanoid control of cys-LT biosynthesis are limited by the poor ligand response of human peripheral blood eosinophils, the difficulty in obtaining human lung mast cells, and the absence of studies with cells from AIA donors in parallel protocols. However, the minimal cys-LT generation by ligand-activated peripheral blood eosinophils is augmented 10-fold by indomethacin and reversed by exogenous PGE_2 (29). In contrast, an early study suggested there was no effect of NSAIDs on IgE-mediated cys-LT generation by isolated human lung mast cells dispersed from tissue resected for a malignancy (63). This finding requires confirmation in highly purified lung mast cells. Cys-LT and histamine generation in response to allergen or calcium ionophore stimulation of fragments of lung parenchyma or bronchi from passively sensitized normal human lung are not altered by aspirin (64). These data are consistent with the site of action of NSAIDs and the source of aspirin-induced rises in BAL and urinary cys-LTs in AIA subjects (14, 15, 17–19) being infiltrating cells, perhaps eosino-

phils, which are not resident in large numbers in the normal lung.

We found that counts of eosinophils ($EG1^+$ and $EG2^+$), mast cells ($AA1^+$), macrophages ($CD68^+$), and T cell subsets ($CD3^+$, $CD4^+$, and $CD8^+$) were not significantly different in bronchial biopsies 20 min after segmental lys-aspirin challenge compared with placebo challenge in 10 AIA and 6 ATA patients (Fig. 1 and Table V), contradicting a recent report of a decrease in $AA1^+$ mast cell counts and a small mean increase in $EG2^+$ eosinophil counts after lys-aspirin challenge of AIA patients (65). However, the concept that $EG2^+$ eosinophils represent an activated subpopulation (42) has been seriously questioned (66). The time interval is probably too short for significant recruitment of eosinophils or for definitive degranulation of the reduced baseline number of mast cells. The influx of eosinophils into BAL fluid in AIA but not ATA patients 15 min after lys-aspirin challenge confirms other work (19) and suggests that cell infiltration would have been apparent at a later time-point. Cys-LTs are themselves potent human eosinophil chemoattractants in vitro and in vivo (67, 68) and perhaps contribute to the chronic bronchial eosinophilia in AIA biopsies.

15 min after segmental lys-aspirin challenge, an increase in BAL fluid levels of the eosinophilotactic cytokine IL-5 occurs in AIA but not ATA patients (19); the rapidity of the increase suggests release of preformed granule-associated IL-5 from mast cell or eosinophil granules (40). We found fourfold more cells immunostaining for IL-5 in biopsies from AIA (5.0 ± 1.3 cells/mm^2) compared with ATA patients (1.3 ± 0.4 cells/mm^2), and these cells were almost half mast cells; eosinophils were much less frequent as an IL-5 source. The proportion of mast cells expressing IL-5 tended to be higher in AIA (mean 20%) than in ATA biopsies (10%). These findings did not change meaningfully with lys-aspirin challenge assessed at 20 min. The significant correlation between counts of $IL\text{-}5^+$ cells and the eosinophil counts for the combined AIA and ATA groups suggests a role of mast cell–derived IL-5 in inducing submucosal eosinophilia but does not account for their clinical difference.

The immunohistochemical differences at baseline between the AIA and ATA patient groups are highlighted by fourfold and threefold increases in $EG1^+$ and $EG2^+$ eosinophils, respectively, a fivefold increase in LTC_4 synthase$^+$ cells, which are predominantly eosinophils, and a fourfold increase in $IL\text{-}5^+$ cells, which are predominantly mast cells. Since both patient groups differ in these aspects from tissue biopsies of normal lung, it seems reasonable to consider these abnormalities as a continuum, in which their intensity accounts for the twofold chronic and 11-fold aspirin-elicited overproduction of cys-LT in the AIA compared with the ATA group. Since the gene for human LTC_4 synthase (49) resides on the long arm of chromosome 5 distal to the gene cluster which regulates the development and function of Th2 cells, namely IL-4, IL-13, IL-3, IL-5, GM-CSF, and IL-9 (69), a polymorphism directed to the regulation of LTC_4 synthase expression could be a predisposing factor for the AIA group.

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References

1. Spector, S.L., C.H. Wangaard, and R.S. Farr. 1979. Aspirin and concomitant idiosyncrasies in adult asthmatic patients. *J. Allergy Clin. Immunol.* 64: 500–506.

2. Stevenson, D.D., and R.A. Simon. Sensitivity to aspirin and non-steroidal anti-inflammatory drugs. 1993. *In* Allergy: Principles and Practice. F. Middleton, C.E. Reed, E.F. Ellis, N.F. Adkinson, J.W. Yunginger, and W.W. Busse, editors. Mosby-Year Book, Inc., St. Louis, MO. 1747–1765.

3. Austen, K.F. 1995. From slow-reacting substance of anaphylaxis to leukotriene C_4 synthase. *Int. Arch. Allergy Immunol.* 107:19–24.

4. Holgate, S.T., P. Bradding, and A.P. Sampson. 1996. Leukotriene antagonists and synthesis inhibitors: new directions in asthma therapy? *J. Allergy Clin. Immunol.* 98:1–13.

5. Israel, E., A.R. Fischer, M.A. Rosenberg, C.M. Lilly, J.C. Callery, J. Shapiro, J. Cohn, P. Rubin, and J.M. Drazen. 1993. The pivotal role of 5-lipoxygenase products in the reaction of aspirin-sensitive asthmatics to aspirin. *Am. Rev. Respir. Dis.* 148:1447–1451.

6. Clark, J.D., L.-L. Lin, R.W. Kriz, C.S. Ramesha, L.A. Sultzman, A.Y. Lin, M.N. Milona, and J.L Knopf. 1991. A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell.* 65:1043–1051.

7. Mancini, J.A., M. Abramowitz, M.E. Cox, E. Wong, S. Charleson, H. Perrier, Z.Y. Wang, P. Prasit, and P.J. Vickers. 1993. 5-lipoxygenase-activating protein is an arachidonate-binding protein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 318:277–281.

8. Rouzer, C.A., T. Matsumoto, and B. Samuelsson. 1986. Single protein from human leukocytes possesses 5-lipoxygenase and LTA_4 synthase activities. *Proc. Natl. Acad. Sci. USA.* 83:857–861.

9. Radmark, O., T. Shimizu, H. Jornvall, and B. Samuelsson. 1984. LTA_4 hydrolase in human leukocytes: purification and properties. *J. Biol. Chem.* 259: 12339–12345.

10. Yoshimoto, T., R.J. Soberman, B. Spur, and K.F. Austen. 1988. Properties of highly purified leukotriene C_4 synthase of guinea pig lung. *J. Clin. Invest.* 81:866–871.

11. Lam, B.K., W.F.J. Owen, K.F. Austen, and R.J. Soberman. 1989. The identification of a distinct export step following the biosynthesis of LTC_4 by human eosinophils. *J. Biol. Chem.* 264:12885– 12889.

12. Sok, D.E., J.K. Pai, V. Atrache, and C.J. Sih. 1980. Characterization of slow reacting substances (SRSs) of rat basophilic leukemia (RBL-1) cells: effects of cysteine on SRS profile. *Proc. Natl. Acad. Sci. USA.* 77:6481–6485.

13. Bergstrom, K., and S. Hammarstrom. 1981. Metabolism of leukotriene D by porcine kidney. *J. Biol. Chem.* 256:9579–9582.

14. Christie, P.E., P. Tagari, A.W. Ford Hutchinson, S. Charlesson, P. Chee, J.P. Arm, and T.H. Lee. 1991. Urinary LTE_4 concentrations increase after aspirin challenge in aspirin-sensitive asthmatic subjects. *Am. Rev. Respir. Dis.* 143: 1025–1029.

15. Kumlin, M., B. Dahlen, T. Bjorck, O. Zetterstrom, E. Granstrom, and S.E. Dahlen. 1992. Urinary excretion of LTE_4 and 11-dehydro-TXB_2 in response to bronchial provocations with allergen, aspirin, LTD_4, and histamine in asthmatics. *Am. Rev. Respir. Dis.* 146:96–103.

16. Smith, C.M., R.J. Hawksworth, F.C. Thien, P.E. Christie, and T.H. Lee. 1992. Urinary leukotriene E_4 in bronchial asthma. *Eur. Respir. J.* 5:693–699.

17. Sladek, K., and A. Szczeklik. 1993. Cysteinyl leukotriene overproduction and mast cell activation in aspirin-provoked bronchospasm in asthma. *Eur. Respir. J.* 6:391–399.

18. Sladek, K., R. Dworski, J. Soja, J.R. Sheller, E. Nizankowska, J.A. Oates, and A. Szczeklik. 1994. Eicosanoids in bronchoalveolar lavage fluid of aspirin-intolerant patients with asthma after aspirin challenge. *Am. J. Respir. Crit. Care Med.* 149:940–946.

19. Szczeklik, A., K. Sladek, R. Dworski, E. Nizankowska, J. Soja, J.R. Sheller, and J. Oates. 1996. Bronchial aspirin challenge causes specific eicosanoid response in aspirin-sensitive asthmatics. *Am. J. Respir. Crit. Care Med.* 154:1608–1614.

20. Nasser, S.M.S., G.S. Bell, S. Foster, K.E. Spruce, R. MacMillan, A.J. Williams, T.H. Lee, and J.P. Arm. 1994. Effect of the 5-lipoxygenase inhibitor ZD2138 on aspirin-induced asthma. *Thorax.* 49:749–756.

21. Christie, P.E., C.M. Smith, and T.H. Lee. 1991. The potent and selective sulfidopeptide leukotriene antagonist, SK&F 104353, inhibits aspirin-induced asthma. *Am. Rev. Respir. Dis.* 144:957–958.

22. Dahlen, B., M. Kumlin, D.J. Margolskee, C. Larsson, H. Blomqvist, V.C. Williams, O. Zetterstrom, and S.E. Dahlen. 1993. The leukotriene-receptor antagonist MK-0679 blocks airway obstruction induced by inhaled lysine-aspirin in aspirin-sensitive asthmatics. *Eur. Respir. J.* 6:1018–1026.

23. Yamamoto, H., M. Nagata, K. Kuramitsu, K. Tabe, H. Kiuchi, Y. Sakamoto, K. Yamamoto, and Y. Dohi. 1994. Inhibition of analgesic-induced asthma by leukotriene receptor antagonist ONO-1078. *Am. J. Respir. Crit. Care*

Med. 150:254–257.

24. Phillips, G.D., R. Foord, and S.T. Holgate. 1989. Inhaled lysine-aspirin as a bronchoprovocation procedure in aspirin-sensitive asthma: its repeatability, absence of a late-phase reaction, and the role of histamine. *J. Allergy Clin. Immunol.* 84:232–241.

25. DeWitt, D.L., and W.L. Smith. 1988. Primary structure of PGG/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc. Natl. Acad. Sci. USA.* 85:1412–1416.

26. O'Banion, M.K., V.D. Winn, and D.A. Young. 1992. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc. Natl. Acad. Sci. USA.* 89:4888–4892.

27. Vane, J.R. 1994. Towards a better aspirin. *Nature.* 367:215–216.

28. Pavord, I.D., and A.E. Tattersfield. 1995. Bronchoprotective role for endogenous PGE_2. *Lancet.* 345:436–438.

29. Tenor, H., A. Hatzelmann, M.K. Church, C. Schudt, and J.K. Shute. 1996. Effects of theophylline and rolipram on LTC_4 synthesis and chemotaxis of human eosinophils from normal and atopic subjects. *Br. J. Pharmacol.* 118: 1727–1735.

30. Docherty, J.C., and T.W. Wilson. 1987. Indomethacin increases the formation of lipoxygenase products in calcium ionophore stimulated human neutrophils. *Biochem. Biophys. Res. Commun.* 148:534–538.

31. Ham, E.A., D.D. Soderman, M.E. Zanetti, H.W. Dougherty, E. McCauley, and F.A.J. Kuehl. 1983. Inhibition by prostaglandins of LTB_4 release from activated neutrophils. *Proc. Natl. Acad. Sci. USA.* 80:4349–4353.

32. Elliott, G.R., A.P.M. Lauwen, and I.L. Bouta. 1989. PGE_2 inhibits and indomethacin and aspirin enhance A23187-stimulated LTB_4 synthesis by rat peritoneal macrophages. *Br. J. Pharmacol.* 96:265–268.

33. Sestini, P., L. Armetti, G. Gambaro, M.G. Pieroni, R.M. Refini, A. Sala, A. Vaghi, G.C. Folco, S. Bianco, and M. Robuschi. 1996. Inhaled PGE_2 prevents aspirin-induced bronchoconstriction and urinary LTE_4 excretion in aspirin-sensitive asthma. *Am. J. Respir. Crit. Care Med.* 153:572–575.

34. Matsumoto, T., C.D. Funk, O. Radmark, J. Hoog, H. Jornvall, and B. Samuelsson. 1989. Molecular cloning and amino acid sequence of human 5-lipoxygenase. *Adv. Prostaglandin Thromboxane Leukotriene Res.* 19:466–469.

35. Penrose, J.F., J. Spector, B.K. Lam, D.S. Friend, K. Xu, R.M. Jack, and K.F. Austen. 1995. Purification of human lung leukotriene C_4 synthase and preparation of a polyclonal antibody. *Am. J. Respir. Crit. Care Med.* 152:283–289.

36. Holtzman, M.J. 1991. Arachidonic acid metabolism: implications of biological chemistry for lung function and disease. *Am. Rev. Respir. Dis.* 143:188–203.

37. American Thoracic Society. 1985. Summary and recommendations of a workshop on the investigative use of fiberoptic bronchoscopy and bronchoalveolar lavage in asthmatics. *Am. Rev. Respir. Dis.* 132:180–182.

38. Djukanovic, R., J.W. Wilson, C.K.W. Lai, S.T. Holgate, and P.H. Howarth. 1991. The safety aspects of fiberoptic bronchoscopy, bronchoalveolar lavage, and endobronchial biopsy in asthma. *Am. Rev. Respir. Dis.* 143:772–777.

39. Britten, K.M., P.H. Howarth, and W.R. Roche. 1993. Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small bronchial biopsies. *Biotech. Histochem.* 68:271–280.

40. Bradding, P., J.A. Roberts, K.M. Britten, S. Montefort, R. Djukanovic, R. Mueller, C.H. Heusser, P.H. Howarth, and S.T. Holgate. 1994. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am. J. Respir. Cell Mol. Biol.* 10:471–480.

41. Walls, A.F., A.R. Bennett, H.M. McBride, M.J. Glennie, S.T. Holgate, and M.K. Church. 1990. Production and characterization of monoclonal antibodies specific for human mast cell tryptase. *Clin. Exp. Allergy.* 20:581–589.

42. Tai, P.C., C.J.F. Spry, C. Peterson, P. Venge, and I. Olsson. 1984. Monoclonal antibodies distinguish between storage and secretory forms of ECP. *Nature.* 309:182–184.

43. Falini, B., L. Flenghi, S. Pileri, M. Gambacorta, B. Bigerna, H. Durkop, F. Eitelbach, J. Thiele, R. Pacini, A. Cavaliere, et al. 1993. PG-M1: a new monoclonal antibody directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. *Am. J. Pathol.* 142:1359–1372.

44. Szczeklik, A., L. Mastalerz, E. Nizankowska, and A. Cmiel. 1996. Protective and bronchodilator effects of PGE_2 and salbutamol in aspirin-induced asthma. *Am. J. Respir. Crit. Care Med.* 153:567–571.

45. Szczeklik, A., R.J. Gryglewski, and G. Czerniawska-Mysik. 1975. Relationship of inhibition of prostaglandin biosynthesis by analgesics to asthma attacks in aspirin-sensitive patients. *Br. Med. J.* 1:67–69.

46. Nasser, S.M.S., R. Pfister, P.E. Christie, A.R. Sousa, J. Barker, M. Schmitz-Schumann, and T.H. Lee. 1996. Inflammatory cell populations in bronchial biopsies from aspirin-sensitive asthmatic subjects. *Am. J. Respir. Crit. Care Med.* 153:90–96.

47. Sousa, A.R., R. Pfister, P.E. Christie, S.J. Lane, S.M. Nasser, M. Schmitz-Schumann, and T.H. Lee. 1997. Enhanced expression of cyclooxygenase isoenzyme 2 (COX-2) in asthmatic airways and its cellular distribution in aspirin-sensitive asthma. *Thorax.* 52:940–945.

48. Lam, B.K., J.F. Penrose, G.J. Freeman, and K.F. Austen. 1994. Expression cloning of a cDNA for human leukotriene C_4 synthase, an integral membrane protein conjugating reduced glutathione to leukotriene A_4. *Proc. Natl. Acad. Sci. USA.* 91:7663–7667.

49. Penrose, J.F., J. Spector, M. Baldasaro, K. Xu, J. Boyce, J.P. Arm, K.F. Austen, and B.K. Lam. 1996. Molecular cloning of the gene for human leukotriene C_4 synthase: organization, nucleotide sequence, and chromosomal localization to 5q35. *J. Biol. Chem.* 271:11356–11361.

50. Murakami, M., K.F. Austen, C.O. Bingham, D.S. Friend, J.F. Penrose, and J.P. Arm. 1995. Interleukin-3 regulates development of the 5-lipoxygenase/leukotriene C4 synthase pathway in mouse mast cells. *J. Biol. Chem.* 270:22653–22656.

51. McGee, J.E., and F.A. Fitzpatrick. 1986. Erythrocyte-neutrophil interaction: formation of leukotriene B by transcellular biosynthesis. *Proc. Natl. Acad. Sci. USA.* 83:1349–1353.

52. Boyce, J.A., B.K. Lam, J.F. Penrose, D.S. Friend, S. Parsons, W.F. Owen, and K.F. Austen. 1996. Expression of LTC_4 synthase during the development of eosinophils in vitro from cord blood progenitors. *Blood.* 88:4338–4347.

53. Lee, C.W., R.A. Lewis, A.I. Tauber, M. Mehrotra, E.J. Corey, and K.F. Austen. 1983. The myeloperoxidase-dependent metabolism of leukotrienes C_4, D_4, and E_4 to 6-trans-leukotriene B_4 diastereoisomers and the subclass-specific *S*-diastereoisomeric sulfoxides. *J. Biol. Chem.* 258:15004–15010.

54. Picado, C., I. Ramis, J. Rosello, J. Prat, O. Bulbena, V. Plaza, J.M. Montserrat, and E. Gelpi. 1992. Release of peptide leukotriene into nasal secretions after local instillation of aspirin in aspirin-sensitive asthmatic patients. *Am. Rev. Respir. Dis.* 145:65–69.

55. Dahlen, B., L.O. Boreus, P. Anderson, R. Andersson, and O. Zetterstrom. 1994. Plasma acetylsalicylic acid and salicylic acid levels during aspirin provocation in aspirin-sensitive subjects. *Allergy.* 49:43–49.

56. Ferreri, N.R., W.C. Howland, D.D. Stevenson, and H.L. Spiegelberg. 1988. Release of leukotrienes, prostaglandins, and histamine into nasal secretions of aspirin-sensitive asthmatics during reaction to aspirin. *Am. Rev. Respir. Dis.* 137:847–854.

57. Fischer, A.R., M.A. Rosenberg, C.M. Lilly, J.C. Callery, P. Rubin, J. Cohn, M.V. White, Y. Igarashi, M.A. Kaliner, J.M. Drazen, and E. Israel. 1994. Direct evidence for a role of the mast cell in the nasal response to aspirin in aspirin-sensitive asthma. *J. Allergy. Clin. Immunol.* 94:1046–1056.

58. Bosso, J.V., L.B. Schwartz, and D.D. Stevenson. 1991. Tryptase and histamine release during aspirin-induced respiratory reactions. *J. Allergy. Clin. Immunol.* 88:830–837.

59. Wenzel, S.E., A.A. Fowler, and L.B. Schwartz. 1988. Activation of pulmonary mast cells by bronchoalveolar allergen challenge: in vivo release of histamine and tryptase in atopic subjects with and without asthma. *Am. Rev. Respir. Dis.* 137:1002–1008.

60. Kowalski, M.L., J. Grzegorczyk, B. Wojciechowska, and M. Poniatowska. 1996. Intranasal challenge with aspirin induces cell influx and activation of eosinophils and mast cells in nasal secretions of ASA-sensitive patients. *Clin. Exp. Allergy.* 26:807–814.

61. O'Sullivan, S., B. Dahlen, S.-E. Dahlen, and M. Kumlin. 1996. Increased urinary excretion of the PGD_2 metabolite 9α,11β-PGF_2 after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction. *J. Allergy Clin. Immunol.* 98:421–432.

62. Bianco, S., M. Robuschi, G. Petrigni, M. Scuri, R.M. Refini, A. Vaghi, and P.S. Sestini. 1993. Efficacy and tolerability of nimesulide in asthmatic patients intolerant to aspirin. *Drugs.* 46:115–120.

63. Peters, S.P., D.W.J. MacGlashan, R.P. Schleimer, E.C. Hayes, N.F.J. Adkinson, and L.M. Lichtenstein. 1985. The pharmacologic modulation of the release of arachidonic acid metabolites from purified human lung mast cells. *Am. Rev. Respir. Dis.* 132:367–373.

64. Salari, H., P. Borgeat, M. Fournier, J. Hebert, and G. Pelletier. 1985. Studies on the release of leukotrienes and histamine by human lung parenchymal and bronchial fragments upon immunologic and nonimmunologic stimulation. Effects of nordihydroguaiaretic acid, aspirin, and sodium cromoglycate. *J. Exp. Med.* 162:1904–1915.

65. Nasser, S.M.S., P.E. Christie, R. Pfister, A.R. Sousa, A. Walls, M. Schmitz-Schumann, and T.H. Lee. 1996. Effect of endobronchial aspirin challenge on inflammatory cells in bronchial biopsies from aspirin-sensitive asthmatic subjects. *Thorax.* 51:64–70.

66. Jahnsen, F.L., T.S. Halstensen, and P. Brandtzaeg. 1994. Erroneous immunohistochemical application of monoclonal antibody EG2 to detect cellular activation. *Lancet.* 344:1514–1515.

67. Spada, C.S., A. Nieves, A. Krauss, and D.F. Woodward. 1994. Comparison of LTB_4 and D_4 effects on human eosinophil and neutrophil motility in vitro. *J. Leukoc. Biol.* 55:183–191.

68. Laitinen, L.A., A. Laitinen, T. Haahtela, V. Vilkka, B.W. Spur, and T.H. Lee. 1993. Leukotriene E_4 and granulocytic infiltration into asthmatic airways. *Lancet.* 341:989–990.

69. Van Leeuwen, B.H., M.E. Martinson, G.C. Webb, and I.G. Young. 1989. Molecular organisation of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on human chromosome 5. *Blood.* 73: 1142–1148.