Leukotriene A_4 Hydrolase in Human Bronchoalveolar Lavage Fluid

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Abstract

We examined cell-free human bronchoalveolar lavage fluid (BALF) for enzymes of the 5-lipoxygenase pathway. BALF was obtained from six patients who were active smokers and six nonsmokers. Enzymatic activity in cell-free BALF was assessed by specific assays for leukotriene (LT) A_4 hydrolase, 5-lipoxygenase, and LTC_4 synthase using HPLC. Only LTA_4 hydrolase enzymatic activity was found. This activity ranged from 101 to 667 when expressed as picomoles of LTB_4 produced per milliliter BALF. Enzymatic activity in smokers vs nonsmokers was 484±120 vs 129±32 pmol LTB_4/ml BALF (mean±SD, $P < 0.0001$). There were no leukotrienes found in BALF before assay. Immunoblot analysis revealed an immunoreactive band at a relative molecular mass of 69,000 D in all samples, consistent with LTA_4 hydrolase, but no evidence of 5-lipoxygenase. BALF had greater LTA_4 hydrolase activity per milligram of protein than neutrophil cytosol, epithelial cell cytosol, plasma, or serum. The synthesis of LTB_4 was significantly increased when neutrophils were stimulated in BALF. These data indicate the selective presence of LTA_4 hydrolase in BALF which is significantly increased in smokers. This enzyme in BALF may contribute to the inflammatory response in tobacco-related lung disease. (*J. Clin. Invest.* 1994. 93:1042–1050.) Key words: arachidonic acid • inflammatory mediators • leukotriene B_4 • lipoxygenase • smoking

Introduction

Leukotrienes are a family of oxygenated metabolites derived from the 5-lipoxygenase pathway of arachidonic acid metabolism. The enzyme 5-lipoxygenase catalyzes the first two steps of the 5-lipoxygenase pathway (1). The first step results in the conversion of free arachidonic acid to 5(*S*)-hydroperoxy-6,8-*trans*-11,14-*cis*-eicosatetraenoic acid (5-HPETE).[1] The second converts 5-HPETE to 5,6-*trans*-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, leukotriene A_4 (LTA_4). LTA_4 is the pivotal intermediate from which all other leukotrienes are synthesized. LTA_4 can be further metabolized by the enzyme LTA_4 hydrolase to form LTB_4 (2) or may be conjugated with glutathione to form LTC_4 by the enzyme LTC_4 synthase (1). In humans, the distribution of the 5-lipoxygenase enzyme appears to be limited to inflammatory cells (1). However, other enzymes of this pathway are more widely distributed. LTC_4 synthase has been identified not only in mast cells (3) and eosinophils (4), but also in endothelial cells (5) and platelets (6). LTA_4 hydrolase has been found in human plasma (7), human erythrocytes (8), inflammatory cells (1), whole human liver (9), whole human lung (10), and airway epithelial cells (11). Neutrophil LTA_4 hydrolase is now recognized as a zinc-containing metalloproteinase (12) with intrinsic aminopeptidase activity (13). In fact, LTA_4 hydrolase shows considerable structural homology to the aminopeptidase N family of enzymes (14). Recent investigations have demonstrated that various inflammatory cells, in concert with noninflammatory cells such as platelets (15), endothelial cells (5), and epithelial cells (11), can participate in the transcellular synthesis of leukotrienes.

The leukotriene products of the 5-lipoxygenase pathway have wide-ranging effects. LTB_4 is one of the most potent chemotactic agents described, active at nanomolar concentrations or less (1). The known effects of LTB_4 on airway function include increased airway responsiveness to bronchial challenge, weak bronchoconstriction, recruitment of inflammatory cells to the airway, and stimulation of inflammatory cells to adhere and secrete a variety of proinflammatory products (16). Additionally, LTB_4 has been shown to stimulate neutrophils to release elastase (17) as well as to modulate multiple aspects of the immune response (18). LTC_4 and LTD_4 are now known to be the constituents of slow-reacting substance of anaphylaxis. These cysteinyl leukotrienes induce smooth muscle contraction, bronchoconstriction, increased systemic vascular permeability, and stimulation of airway mucus secretion (16).

There have been several studies demonstrating the presence of small quantities of leukotrienes in the cell-free portion of bronchoalveolar lavage fluid (BALF) of normal subjects (19) as well as from those with pulmonary disease (19–23). Further, BALF cells have been shown to contain mRNA encoding for a variety of enzymes of the lipoxygenase pathways, including 15-lipoxygenase (24). Cell-free BALF, however, has not previously been examined for the presence of enzymes of the lipoxygenase pathway. These enzymes, if present in lung lining fluid, could participate in the synthesis of leukotrienes and, in turn, in the pathogenesis of respiratory disease. The purpose of this investigation was to determine if enzymes of the 5-lipoxygenase pathway were present in the cell-free portion of human BALF and to determine if they were enzymatically active.

Methods

Studies examining BALF. The protocol for bronchoscopy was reviewed and approved by the Committee on Investigations Involving

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1. *Abbreviations used in this paper:* ACD, acid-citrate-dextrose; BALF, bronchoalveolar lavage fluid; CMF-HBSS, calcium- and magnesium-free HBSS; FEV_1, forced expiratory volume in 1 s; 5-HETE, 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 5-HPETE, 5(*S*)-hydroperoxy-6,8-*trans*-11,14-*cis*-eicosatetraenoic acid; RP-HPLC, reversed phase HPLC; TBS, Tris-buffered saline; Δ^6-*trans*-12-epi-LTB_4, 5(*S*),12(*S*)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid; Δ^6-*trans*-LTB_4, 5(*S*),12(*R*)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid.

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Human Subjects of the University of California and the Department of Veteran Affairs Medical Center, San Diego. Patients undergoing bronchoscopy for clinical indications were recruited from the bronchoscopy service of the Department of Veterans Affairs Medical Center. Patients were excluded if: (*a*) their forced expiratory volume in 1 s (FEV_1) was < 1.5 liters; (*b*) there was evidence of active pulmonary infection; or (*c*) they did not give informed consent. Pulmonary function data (FEV_1 and FEV_1 percentage predicted) were obtained from the patients' medical records. Topical anesthesia was accomplished with nebulized and topical lidocaine. Sedation with meperidine and/or midazolam was also used. The bronchoscope (BF P10 or BF 1T20D; Olympus) was introduced through the nares. Lavage was performed in the anterior portion of the right middle lobe. Four 25-ml aliquots of normal saline, warmed to 37°C, were instilled and removed by gentle suction. The lavage fluid was immediately placed on ice. Cell counts were performed on unprocessed BALF using a hemocytometer, and viability was determined by exclusion of a 0.04% solution of trypan blue. Under microscopic examination very few disrupted cells were seen. Cells were prepared by cytocentrifugation, and stained with May-Grünwald Giemsa. Differential cell counts were performed by counting 500 cells. Lavage fluid was initially centrifuged at 1,000 *g* at 4°C to produce a cell-free supernatant without disrupting the collected cells. Control experiments did not reveal any LTA_4 hydrolase activity in supernatants when suspensions of neutrophils were centrifuged in an identical fashion or that this enzyme was released from neutrophils incubated at 37°C for extended periods. The cell-free supernatants of BALF were collected and again were centrifuged at 15,000 *g* at 4°C to remove any residual particulate material. Total protein was determined by the Bradford method (25). The fluid was placed in a polypropylene tube under argon and then was flash frozen in liquid nitrogen and stored at −70°C until further analysis.

Studies on peripheral blood. Peripheral blood was obtained from normal healthy subjects without known allergy and who had not taken any medication for 2 wk before blood donation. All subjects and patients were volunteers and had given informed consent to a protocol approved by the Committee on Investigations Involving Human Subjects of the University of California and the Department of Veteran Affairs Medical Center.

To prepare plasma, blood was collected into a syringe containing acid-citrate-dextrose (ACD), pH 4.5, (1:6, ACD/blood, vol/vol), and was initially centrifuged at 300 *g* for 10 min at 4°C. The supernatant was then removed and centrifuged twice at 1,000 *g* for 15 min at 4°C. Serum was obtained by allowing blood, collected without an anticoagulant, to clot for 1 h at room temperature in a sterile glass tube. The blood was then spun twice at 1,000 *g* for 10 min at room temperature. Both plasma and serum were then aliquoted and either were immediately assayed or flash frozen in liquid nitrogen and stored at −70°C until further analyzed.

Neutrophils were obtained from peripheral blood by modifications of a previously described technique (26). Briefly, blood was drawn through a 19-gauge needle and was anticoagulated with ACD, pH 4.5. Red blood cells were passively sedimented for 45 min by adding 30 ml of whole blood to 10 ml of calcium- and magnesium-free HBSS (CMF-HBSS) with 0.35% BSA and 10 ml of 3% dextran 500 in CMF-HBSS. The leukocyte-rich plasma was then removed and centrifuged at room temperature for 7 min at 250 *g*. The supernatant was removed, and the cells were washed once with 50 ml of CMF-HBSS containing 0.35% BSA. The cells were then resuspended in 25 ml of CMF-HBSS with 0.35% BSA and were layered over 15 ml of Ficoll-paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The suspension was centrifuged for 30 min at room temperature at 400 *g*. Cells pelleting through the gradient were resuspended in 10 ml of cold NH_4Cl lysis solution and were placed on ice for 10 min to lyse any remaining red blood cells. The tube was then immediately centrifuged at 300 *g* for 7 min at 4°C. The cell pellet was resuspended in 50 ml of CMF-HBSS with 0.35% BSA. A small aliquot was then removed and was counted by hemocytometer. On differential cell counts these preparations were > 95% neutrophils.

Neutrophil cytosol was obtained by spinning isolated neutrophils at 300 *g* for 10 min and then resuspending them in 1 ml of sonication media (100 mM Tris, 1 mM EDTA, pH 7.8). The cells were then sonicated (model W140; Heat Systems-Ultrasonics, Inc., Plainview, NY) at power level seven for 30-s pulses three times on ice. PMSF was added to a final concentration of 1.0 mM. An aliquot of sonicated cells was removed and was examined microscopically to assess the degree of disruption. Sonication was repeated if the cells were not > 95% disrupted. The disrupted cells were transferred to a microfuge tube and were centrifuged at 13,000 *g* for 30 min at 4°C. The supernatant was removed, was placed in another microfuge tube, and the centrifugation was repeated. This supernatant was termed cytosol (previous studies have not shown a significant difference in enzymatic activity with additional centrifugation steps to remove microsomal membranes).[2] Total protein in the cytosol was assessed by the Bradford technique (25), and the protein concentration was adjusted to 2.0–3.0 mg/ml by the addition of 100 mM Tris, pH 7.8. Aliquots were flash frozen in liquid nitrogen and were stored at −70°C until needed.

To prepare epithelial cell cytosol a transformed human tracheal epithelial cell line, designated $9HTEo^-$ (gift of Dr. D. C. Gruenert, University of California, San Francisco), was used. These cells have stable LTA_4 hydrolase activity which closely approximates that of primary cultures of human tracheal epithelial cells (11). The cells were grown to confluence on 100-mm tissue culture plates coated with human fibronectin, bovine dermal collagen type I, and BSA in MEM supplemented with 10% heat-inactivated calf serum, penicillin 100 U/ml, gentamicin 40 μg/ml, and amphotericin B 2.5 μg/ml. When confluent, the cells were trypsinized, were washed in HBSS, and then were pelleted at 300 *g* for 10 min. The cells were resuspended in 1 ml of sonication media, and the remainder of the preparation was as described for neutrophil cytosol.

Enzyme assays. LTA_4 was prepared by hydrolysis of its ethyl ester using a previously described technique (27). LTA_4 hydrolase activity was measured in 450-μl aliquots of cell-free BALF, human plasma, human serum, and neutrophil and airway epithelial cell cytosol. These aliquots were added to assay buffer to achieve a final concentration of 100 mM Tris and 0.35% BSA at pH 7.8. Samples were warmed to 37°C for 5 min and then were incubated for 15 min in the presence of 15 μM LTA_4 at 37°C in a circulating water bath.

5-lipoxygenase activity was measured by a modification of a previously described technique (28). Cell-free BALF (450 μl) was added to 50 μl of 10× assay buffer to achieve a final concentration of 100 mM Tris, 2 mM $CaCl_2$, and 1.6 mM EDTA. Samples were warmed to 37°C for 5 min and then were incubated for 15 min in the presence of 100 μM arachidonic acid and 2 mM ATP at 37°C in a circulating water bath.

LTC_4 synthase activity was assessed in cell-free BALF (450 μl) that was added to 50 μl of 10× assay buffer to achieve a final concentration of 100 mM Tris, 0.35% BSA, and 1 mM reduced glutathione at pH 7.8. Samples were warmed to 37°C for 5 min and then were incubated for 15 min in the presence of 15 μM LTA_4 at 37°C in a circulating water bath.

Aminopeptidase activity was assessed by mixing 50-μl aliquots of BALF 1:1 with 2× assay buffer (final concentration 15 mM KH_2PO_4 and 15 mM K_2HPO_4, pH 7.0) and by prewarming to 37°C. L-alanine-*p*-nitroanilide, dissolved in DMSO, was added to samples at a final concentration of 1 mM. The DMSO concentration did not exceed 0.1%, and this concentration had no effect on enzymatic activity. Samples were incubated at 37°C for 10 min in a temperature-controlled microplate reader (Thermomax; Molecular Devices Corp., Menlo Park, CA). Enzymatic activity was assessed by monitoring the samples for release of the chromogenic product, *p*-nitroaniline, at 405 nm and by quantitating the product using its molar extinction coefficient (ϵ9620 at pH 7.0).

2. Bigby, T. D., D. M. Lee, M. Minami, N. Ohishi, T. Shimizu, and J. R. Baker, manuscript submitted for publication.

Identification and quantification of metabolites. After incubation of samples to be assessed for LTA_4 hydrolase, LTC_4 synthase, and 5-lipoxygenase enzymatic activity as described above, the reaction was quenched by the addition of an equal volume of methanol, and the samples were immediately placed on ice. PGB_2 (150 ng) was added as an internal standard to correct for recovery of metabolites. Samples were acidified to pH 4.0–4.5 with 0.1 N hydrochloric acid (or 0.1 N phosphoric acid for the LTC_4 synthase assay). The samples were placed at −20°C for 2 h and were then centrifuged at 13,000 *g* to remove precipitated protein. The supernatants were placed in fresh tubes, and an equal volume of chloroform was added. The vortexed samples were again centrifuged at 13,000 *g*. The chloroform layer was removed, was placed in a fresh tube, and was evaporated to dryness under a stream of nitrogen. The percentage of recovery of the internal standard PGB_2 using this extraction technique ranged from 67 to 97% (81.1±8.7%, mean±SD, $n = 12$).

Extracts were reconstituted in chromatography solvent for analysis by reversed phase HPLC (RP-HPLC). The analysis was performed using a liquid chromatograph (Gradient HPLC System 34; Rainin Instrument Co. Inc., Woburn, MA) fitted with a 4.6 mm × 10 cm column with 3-μm spherical octadecyldimethylsilane packing (Microsorb, Short-one; Rainin Instrument Co. Inc.) that was developed at a flow rate of 1.0 ml/min using a gradient program. Solvent A consisted of methanol/water/phosphoric acid/ammonium hydroxide (50:50:0.01:0.013, vol/vol), apparent pH 4.5. Solvent B consisted of methanol/water/phosphoric acid/ammonium hydroxide (90:10:0.01:0.013,vol/vol), apparent pH 5.8. For detection of LTB_4 and LTC_4, the gradient was developed with 44% solvent B from 0 to 12.5 min, and 100% B from 13 to 18 min. 5-lipoxygenase activity was assessed by the presence of 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HETE) and 5-HPETE. The gradient for 5-HETE and 5-HPETE detection was developed with 60% B from 0 to 20 min and 100% B from 20.5 to 27 min. LTB_4, 5(*S*),12(*R*)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid (Δ^6-*trans*, LTB_4), and 5(*S*),12(*S*)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid (Δ^6-*trans*-12-epi-LTB_4), were detected by their absorbance at 270 nm, using a UV detector (Knauer, Berlin, Germany) interfaced with a computer (Macintosh SE/30; Apple Computers, Cupertino, CA) with real-time data acquisition and analysis software (Dynamax HPLC Method Manager; Rainin Instrument Co. Inc.). 5-HETE and LTC_4 were detected by their absorbance at 235 and 280 nm, respectively. Metabolites were quantitated by UV absorbance using molar extinction coefficients of 50,000 at 270 nm for LTB_4, Δ^6-*trans*-LTB_4, and Δ^6-*trans*-12-epi-LTB_4. Molar extinction coefficients of 27,500 at 235 nm and 49,400 at 280 nm were used for quantitating 5-HETE and LTC_4, respectively. The lower limit of detection of this system was 5 pmol of product. Enzymatic activity was expressed as picomoles of product formed over 15 min either per milliliter of BALF or per milligram protein.

Immunoblots. BALF samples were concentrated using a 10,000-mol wt cutoff membrane (Centricon; Amicon Corp., Danvers, MA), and portions of the concentrated retentates, representing identical starting volumes of BALF, were added to sample buffer and were loaded onto an SDS-polyacrylamide gel (4% stacking, 10% resolving). The samples were subjected to electrophoresis at 150 V under reducing and denaturing conditions. Membrane transfer to a polyvinylidenedifluoride membrane (Immobilon P; Millipore Corp., Bedford, MA) was then performed at 100 V for 1 h in chilled transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol [vol/vol], pH 8.3). After transfer, the membranes were washed in Tris-buffered saline (TBS) (20 mM Tris base, 500 mM NaCl, and 0.02% NaN_3, pH 7.5) and then were blocked by soaking for 1 h at room temperature in a 5% solution of nonfat dry milk in TBS with 0.02% NaN_3. The membranes were probed for 12 h at 4°C using an affinity-purified polyclonal rabbit anti-human antibody raised to recombinant LTA_4 hydrolase (gift of T. Shimizu, University of Tokyo, Japan) or a polyclonal rabbit antibody raised to purified native 5-lipoxygenase (LO-32, gift of Dr. J. Evans, Merck-Frosst Center for Therapeutic Research, Point Claire-Dorval, Quebec, Canada). The membranes were then washed three times in TBS and were sequentially incubated in the secondary antibody (biotinylated goat anti-rabbit in TBS) for 1 h at room temperature, in streptavidin–alkaline phosphatase for 1 h, and then in a developing solution of nitrol blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate containing $MgCl_2$ (29). Development was quenched by washing the membrane in TBS with 20 mM EDTA.

Potential biological role of BALF LTA_4 hydrolase. To determine whether the finding of LTA_4 hydrolase in BALF had biological relevance, two types of experiments were performed. First, these studies examined the synthesis of LTB_4 when neutrophils were stimulated in BALF, as opposed to buffer. Thus, 5–6 × 10^6 neutrophils/ml were resuspended in BALF or HBSS. The cell suspensions were prewarmed to 37°C and then were stimulated with 100 μg/ml of opsonized zymosan for 30 min. Opsonized zymosan was prepared as reported previously (30). A23187 was not used as a stimulus because it displays significant binding to proteins. This limits its effectiveness as a stimulus under conditions where high concentrations of proteins exist, such as BALF.

Second, additional studies examined the amount of LTA_4 released by inflammatory cells, indirectly. This was accomplished by measuring the principal nonenzymatic breakdown products of LTA_4 in aqueous buffers, Δ^6-*trans*-LTB_4 and Δ^6-*trans*-12-epi-LTB_4, in supernatants from neutrophil suspensions stimulated with the calcium ionophore, A23187, or in opsonized zymosan. Although these nonenzymatic products may also be generated intracellularly, prior studies have demonstrated that virtually all of these nonenzymatic products present in supernatants under these conditions result from extracellular release of LTA_4 (Bigby, T., unpublished observations). These data correspond to more direct measures of extracellular release of LTA_4 (31). Previous studies have also shown that human neutrophils, under these conditions, take up little if any LTA_4 from the extracellular milieu (Shindo, K., J. R. Baker, D. A. Munafo, and T. D. Bigby, manuscript submitted for publication). For the experiments, 5–6 × 10^6 neutrophils/ml of HBSS were stimulated with 10 μM A23187 for 15 min or 100 μg/ml of opsonized zymosan for 30 min. Supernatants from these incubations were extracted and analyzed for Δ^6-*trans*-LTB_4 and Δ^6-*trans*-12-epi-LTB_4 by HPLC as noted above.

Materials. Arachidonic acid was obtained from Nu Check Prep, Inc. (Elysian, MN). Ficoll-paque was from Pharmacia LKB Biotechnology Inc. Leukotrienes A_4 (ethyl ester), B_4, and C_4 were gifts from Merck-Frosst Canada (Montreal, Canada). PGB_2 was obtained from Upjohn (Kalamazoo, MI). 5-HETE was obtained from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Immobilon P membrane was obtained from Millipore Corp. Redistilled-in-glass HPLC grade solvents were obtained from Burdick & Jackson (Muskegon, MI). May-Grünwald Giemsa stains were obtained from Baxter Scientific Products (Irvine, CA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO) and were of the finest grade available.

Statistical analysis. Data are expressed as means±SD or SEM, and differences between means are analyzed by a two-tailed unpaired *t* test. A value of $P < 0.05$ is considered significant. Correlations between variables are analyzed by linear regression.

Results

BALF study patients. There were 12 BALF study patients ranging in age from 36 to 76 yr (Table I). One patient had a history of stable mild asthma (current FEV_1 − 111% predicted) and was using theophylline and metered dose inhalers of β_2-agonists on an occasional basis. None of the remaining patients were using any pulmonary medications. The indications for bronchoscopy included chronic cough in two patients, solitary pulmonary nodule in eight patients, and hemoptysis (inactive) in two patients. Of the 12 patients, 6 were smokers and 6 were nonsmokers. All of the smokers had smoked within 24 h of the bronchoscopy and lavage. Of the six nonsmokers, two were life

Table I. Clinical Characteristics of Study Subjects

Patient	Indic	Age	Sex	Smokers	FEV_1	FEV_1
					liters	*percent predicted*
1	Nodule	58	M	Yes	2.68	104
2	Nodule	56	M	Yes	3.17	97
3	Nodule	72	M	No	2.47	81
4	Cough	36	F	No	3.20	119
5	Cough	50	F	No	2.79	111
6	Nodule	68	M	Yes	1.84	67
7	Nodule	76	M	No	3.45	121
8	Nodule	73	M	No	2.45	86
9	Nodule	68	M	No	3.15	112
10	Hemoptysis	38	M	Yes	3.95	100
11	Hemoptysis	48	M	Yes	2.37	70
12	Nodule	40	M	Yes	4.22	108

Indic, indications for bronchoscopy.

long nonsmokers, two had been pipe smokers, one had a 120 pack a year history, and one had a 15 pack a year history. None of the exsmokers had smoked for at least 10 yr.

BALF characterization. Bronchoalveolar lavage was well tolerated in all patients. Lavage return ranged from 22 to 67% of the instilled fluid. Total protein concentrations ranged from 18.0 to 332.1 μg/ml BALF (Table II). Total white cell count in BALF was 20.6±21.5 × 10^6 per lavage (mean±SD) and ranged from 3.2 to 66 × 10^6 cells per lavage. Differential cell counts were similar in all patients. Lavage samples obtained from four patients had > 10^5 red blood cells present per milliliter BALF (Table II).

LTA_4 hydrolase activity. All samples of cell-free BALF supernatant showed LTA_4 hydrolase activity. This activity was detected by LTB_4 production when samples were incubated with the substrate LTA_4 (Figs. 1 and 2*A* and Table III). When BALF samples were directly extracted and analyzed in the absence of added LTA_4, no LTB_4, Δ^6-*trans*-LTB_4, or Δ^6-*trans*-12-epi-LTB_4 were detected in BALF samples from any subject. The mean enzymatic activity in the presence of LTA_4 for all patients' BALF was 306.3±203.3 pmol LTB_4 produced/ml BALF (mean±SD) and ranged from 101.0 to 666.8 pmol/ml BALF. When enzymatic activity was examined per milligram protein in BALF, the specific activity ranged from 1.2 to 9.8 nmol LTB_4/mg protein (3.9±2.5, mean±SD). Active smokers demonstrated significantly more enzymatic activity than nonsmokers: 483.8±119.5 vs 128.8±31.7 pmol LTB_4/ml BALF (mean±SD) ($P < 0.0001$) (Fig. 2). The percentage of conversion of LTA_4 to LTB_4 by BALF for all patients was 1.8±1.2% (mean±SD, $n = 12$). When data were grouped by smoking status, smokers' BALF converted a significantly greater proportion of added LTA_4 to LTB_4 than nonsmokers' BALF (2.9±0.7% vs 0.8±0.2%, means±SD, $n = 6$ in each group, $P < 0.001$).

5-Lipoxygenase and LTC_4 synthase activity. There was no evidence of 5-lipoxygenase activity, as determined by the absence of 5-HPETE and 5-HETE, in any of the BALF supernatants when they were incubated with arachidonic acid in the presence of calcium and ATP (Table III). Similarly, none of the 12 BALF samples showed evidence of LTC_4 synthase activity, as determined by LTC_4 production, when incubated with LTA_4. No 15-lipoxygenase products were detected in the lipoxygenase assays. The limit of detection of the HPLC system used was ~ 5 pmol leukotriene per sample analyzed.

Aminopeptidase activity. Modest amounts of aminopeptidase activity were found in all but two patients' BALF (Table III). These two patients had no detectable aminopeptidase activity in their BALF. There were no significant differences in aminopeptidase activity when patients were grouped according to smoking status, regardless of whether the data were analyzed on a per milliliter of BALF or per milligram of protein basis (Table IV). Furthermore, there was no correlation between aminopeptidase activity and LTA_4 hydrolase activity in BALF samples ($r^2 = 0.111$ for activity per milliliter of BALF, $r^2 = 0.109$ for activity per milligram protein).

Immunoblots. Immunoblots performed on each BALF sample demonstrated a band at a relative molecular mass of

Table II. BALF Characteristics

Patient	Protein	Smokers	WBC × 10^5	Differential cell counts*						RBC × 10^5
				Mø	LYM	PMN	EO	MST	EPI	
	μg/ml		*per ml*			%				*per ml*
1	68.4	Yes	5.2	97.6	0.2	0.4	0.2	0.6	1.0	0.2
2	332.1	Yes	11.0	90.7	4.4	1.4	3.2	0.2	0.2	0.0
3	150.6	No	1.8	93.8	1.9	2.5	0.0	0.2	1.6	78.0
4	18.0	No	1.4	90.3	4.5	1.4	0.0	2.0	1.8	0.3
5	45.0	No	1.3	93.6	0.4	0.4	0.2	0.0	5.4	0.3
6	116.7	Yes	3.7	97.0	0.4	1.0	0.4	0.4	0.8	1.4
7	50.2	No	1.5	95.3	2.4	2.0	0.0	0.0	0.4	26.0
8	48.5	No	0.8	93.3	3.3	2.9	0.0	0.0	0.6	0.4
9	28.9	No	1.1	88.0	1.0	7.4	0.0	0.2	3.4	0.4
10	98.8	Yes	9.9	96.1	2.2	1.2	0.4	0.2	0.0	0.9
11	84.1	Yes	9.6	95.1	1.2	3.0	0.8	0.0	0.0	3.6
12	141.2	Yes	1.3	97.8	0.0	1.0	1.2	0.0	0.2	0.1

WBC, white blood cells; Mø, macrophage; LYM, lymphocyte; PMN, neutrophil; EO, eosinophil; MST, mast cell; EPI, epithelial cell; RBC, red blood cells. * Differentials were obtained on 500 cells.

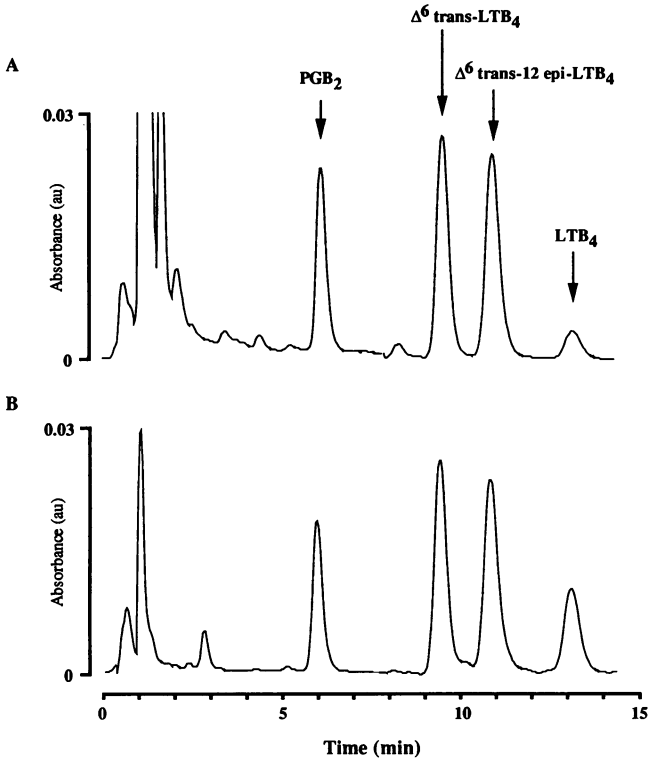

Figure 1. RP-HPLC analysis of bronchoalveolar lavage fluid incubated with LTA_4. (*A*) Nonsmoker. (*B*) Smoker. 450-μl samples of BALF were incubated at 37°C with 15 μM LTA_4 for 15 min to assay for LTA_4 hydrolase activity. Samples were then extracted and subjected to RP-HPLC. Products are identified by their retention times when compared with known authentic standards and quantitated by their absorbance of UV light at 270 nm. The peak for PGB_2 (6.2 min) is an internal standard added to correct for product recovery. The peaks corresponding to the retention times of Δ^6-*trans*-12-epi-LTB_4 (9.5 min) and Δ^6-*trans*-12-epi-LTB_4 (11.0 min) represent nonenzymatic breakdown products of LTA_4. The peak corresponding to the retention time of LTB_4 (13.1 min) is significantly larger in the BALF sample from a smoker than from a nonsmoker. No peaks corresponding to Δ^6-*trans*-LTB_4 (9.5 min), Δ^6-*trans*-12-epi-LTB_4, or LTB_4 were found in BALF extracted without incubation with LTA_4 (data not shown).

69,000 D when probed with an affinity-purified polyclonal antibody to recombinant LTA_4 hydrolase (Fig. 2*B*). This finding is consistent with the previously characterized neutrophil LTA_4 hydrolase (32). Neutrophil cytosol, used as a positive control, also showed a band at a relative molecular mass of 69,000 D. In general, there was broad correspondence between activity in BALF assayed enzymatically and the amount of LTA_4 hydrolase immunoreactivity on immunoblots. None of the 12 patients' BALF samples showed evidence of 5-lipoxygenase immunoreactive protein using an affinity-purified polyclonal antibody to the 5-lipoxygenase (Fig. 2 *B*). Neutrophil cytosol used as a positive control demonstrated a band at a relative molecular mass of ~ 78,000 D, characteristic of 5-lipoxygenase (33).

Cytosolic, serum, and plasma LTA_4 hydrolase activity. Neutrophil and epithelial cell cytosolic fractions were analyzed for LTA_4 hydrolase activity (Fig. 3). Neutrophil cytosol produced 1.7±0.2 nmol LTB_4 per milligram of protein (mean±SD), and epithelial cell cytosol produced 0.33±0.05 nmol LTB_4 per milligram of protein (mean±SD). Samples of human serum and plasma were also analyzed for the presence of LTA_4 hydrolase activity (Fig. 3). Serum samples contained a small amount of activity corresponding to 0.0016±0.00025 nmol LTB_4/mg protein (mean±SD, n = 4 experiments). Plasma samples contained less activity than serum, generating 0.00087±0.00068 nmol LTB_4/mg protein (mean±SD, n = 3 experiments). Immunoblots performed on serum and plasma using the anti-LTA_4 hydrolase antibody did not detect any enzyme in these fluids (data not shown).

Comparisons and correlations. Because BALF from smokers contained significantly more LTA_4 hydrolase activity than from nonsmokers, all data were compared according to smoking status (Table IV). Of those compared, only two additional variables were significantly different between smokers and nonsmokers. Total white cell count per milliliter was significantly higher and was on average fivefold greater in smokers. Macrophages, examined per milliliter of BALF, were also significantly increased in BALF obtained from smokers. Total protein concentrations tended to be higher in smokers' BALF, although this was not statistically significant. Also of note, there was no difference in the specific activity for LTA_4 hydrolase in BALF between smokers and nonsmokers when these data were examined on a per milligram of protein basis.

The data were also correlated with LTA_4 hydrolase activity per milliliter of lavage by simple linear regression (Fig. 4). Of the data analyzed, only total white cells per milliliter and macrophages per milliliter of BALF correlated significantly with LTA_4 hydrolase activity ($P < 0.05$, both correlations). Specifically, no correlation was observed with total protein, red blood cells, epithelial cells, or neutrophils when examined per milliliter of BALF (Fig. 4). All other regression analyses were unrevealing.

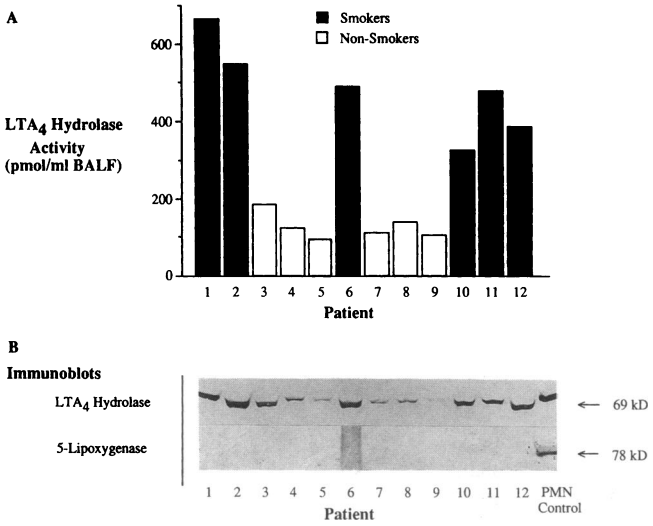

Figure 2. LTA_4 hydrolase activity and immunoreactivity in BALF. (*A*) A 450-μl sample was incubated at 37°C with 15 μM LTA_4 for 15 min. Samples were then extracted, and conversion to LTB_4 was measured by RP-HPLC. (*B*) Immunoblots with antibodies to LTA_4 hydrolase and 5-lipoxygenase were performed in concentrates of all lavage samples. The LTA_4 hydrolase band corresponds to a relative molecular mass of 69,000 D, and the 5-lipoxygenase band corresponds to a relative molecular mass of 78,000 D. *PMN Control;* neutrophil cytosol examined as a positive control for both enzymes.

Table III. Enzymatic Activity of BALF

Patient	Smokers	Enzymatic activity*					
		LTB_4		5-HETE	LTC_4	Aminopeptidase activity	
		pmol/ml	*nmol/mg protein*	*pmol/ml*		*nmol/min per ml*	*nmol/min per mg protein*
1	Yes	667	9.8	ND	ND	0.12	1.76
2	Yes	549	1.7	ND	ND	0.77	2.31
3	No	187	1.2	ND	ND	0.55	3.68
4	No	125	7.0	ND	ND	0.30	17.0
5	No	101	2.3	ND	ND	ND	ND
6	Yes	491	4.2	ND	ND	0.06	0.55
7	No	112	2.2	ND	ND	ND	ND
8	No	140	2.9	ND	ND	0.32	6.66
9	No	108	3.7	ND	ND	0.10	3.52
10	Yes	328	3.3	ND	ND	0.89	8.98
11	Yes	480	5.7	ND	ND	0.72	8.57
12	Yes	388	2.8	ND	ND	0.61	4.32

* Enzymatic activity of LTA_4 hydrolase, 5-lipoxygenase, and LTC_4 synthase as measured by formation of LTB_4, 5-HETE, and LTC_4, respectively. ND, not detected.

Potential biological role of BALF LTA_4 hydrolase. When neutrophils were resuspended in BALF and were stimulated with 100 μg/ml opsonized zymosan for 30 min, they synthesized significantly more LTB_4 than identical samples resuspended in HBSS (Fig. 5).

When neutrophils were stimulated with 10 μM A23187 for 15 min, their supernatants contained 20.3±5.0 pmol of Δ^6-*trans*-LTB_4 plus Δ^6-*trans*-12-epi-LTB_4/10^6 neutrophils (n = 9), indirectly reflecting the release of this amount of intact LTA_4. These same cells released 38.1±9.2 pmol of LTB_4/10^6 cells, suggesting that ~ 35% of the leukotriene released by neutrophils under these conditions is released as intact LTA_4.

Table IV. Characteristics of Smokers and Nonsmokers

Characteristic	Smokers*	Nonsmokers*
Age	51.3±11.5	62.5±16.0
FEV_1 (*liters*)	3.04±0.9	2.92±0.4
FEV_1 (*percentage predicted*)	91.0±18	105.0±17
Protein (*μg/ml*)	140.2±97.4	56.9±47.6
RBC per milliliter (×10^5)	1.0±1.4	17.6±31.3
EPI per milliliter (×10^4)	1.7±1.3	2.0±2.1
Total WBC per milliliter (×10^5)	6.8±3.9‡	1.3±0.3
Macrophages per milliliter (×10^5)	6.4±3.6‡	1.2±0.3
Lymphocytes per milliliter (×10^4)	1.4±1.9	0.3±0.2
Neutrophils per milliliter (×10^4)	1.1±1.1	0.3±0.3
Mast cells per milliliter (×10^3)	1.5±1.3	0.6±1.1
Eosinophils per milliliter (×10^4)	0.9±1.3	0.004±0.001
LTB_4 (*pmol/ml*)	483.8±119.4§	128.8±31.7
LTB_4 (*nmol/mg protein*)	4.6±2.9	3.2±2.0
Aminopeptidase activity (*nmol/min per ml*)	0.53±0.35	0.21±0.22
Aminopeptidase activity (*nmol/min per mg protein*)	4.4±3.6	5.1±6.3

WBC, white blood cell; RBC, red blood cell; EPI, epithelial cell.
* Mean±SD. ‡ $P < 0.01$. § $P < 0.001$.

When neutrophils were stimulated with 100 μg/ml of opsonized zymosan for 30 min, their supernatants contained 2.3±0.4 pmol of Δ^6-*trans*-LTB_4 plus Δ^6-*trans*-12-epi-LTB_4/10^6 neutrophils (n = 3). These same cells released 2.4±0.6 pmol of LTB_4/10^6 neutrophils, suggesting that ~ 50% of the leukotriene released by neutrophils when stimulated by opsonized zymosan is released as intact LTA_4. These data are consistent with a previously published work demonstrating the release of LTA_4 by more direct methods, such as alcohol trapping of liposome-stabilized LTA_4 (31).

Discussion

We sought to determine if enzymes of the 5-lipoxygenase pathway, including LTA_4 hydrolase, 5-lipoxygenase, and LTC_4 synthase, were present in human BALF and to determine if they

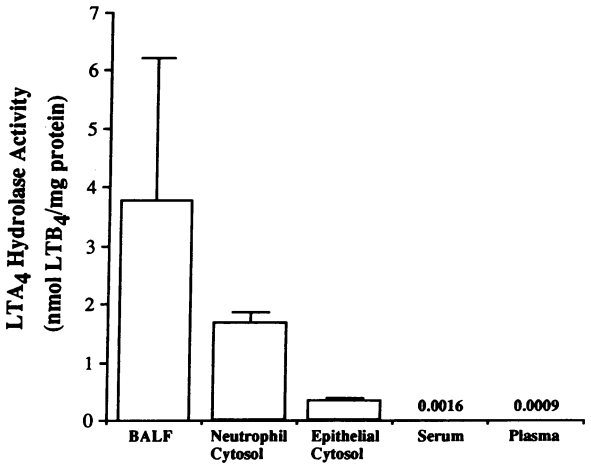

Figure 3. Comparison of LTA_4 hydrolase specific activity in various samples. LTA_4 hydrolase activity was assayed in BALF, neutrophil cytosol, epithelial cell cytosol, serum, and plasma as detailed in Methods. Products were detected by RP-HPLC. Data are expressed as the mean±SD of at least three different determinations, except for BALF which represents all 12 patients studied.

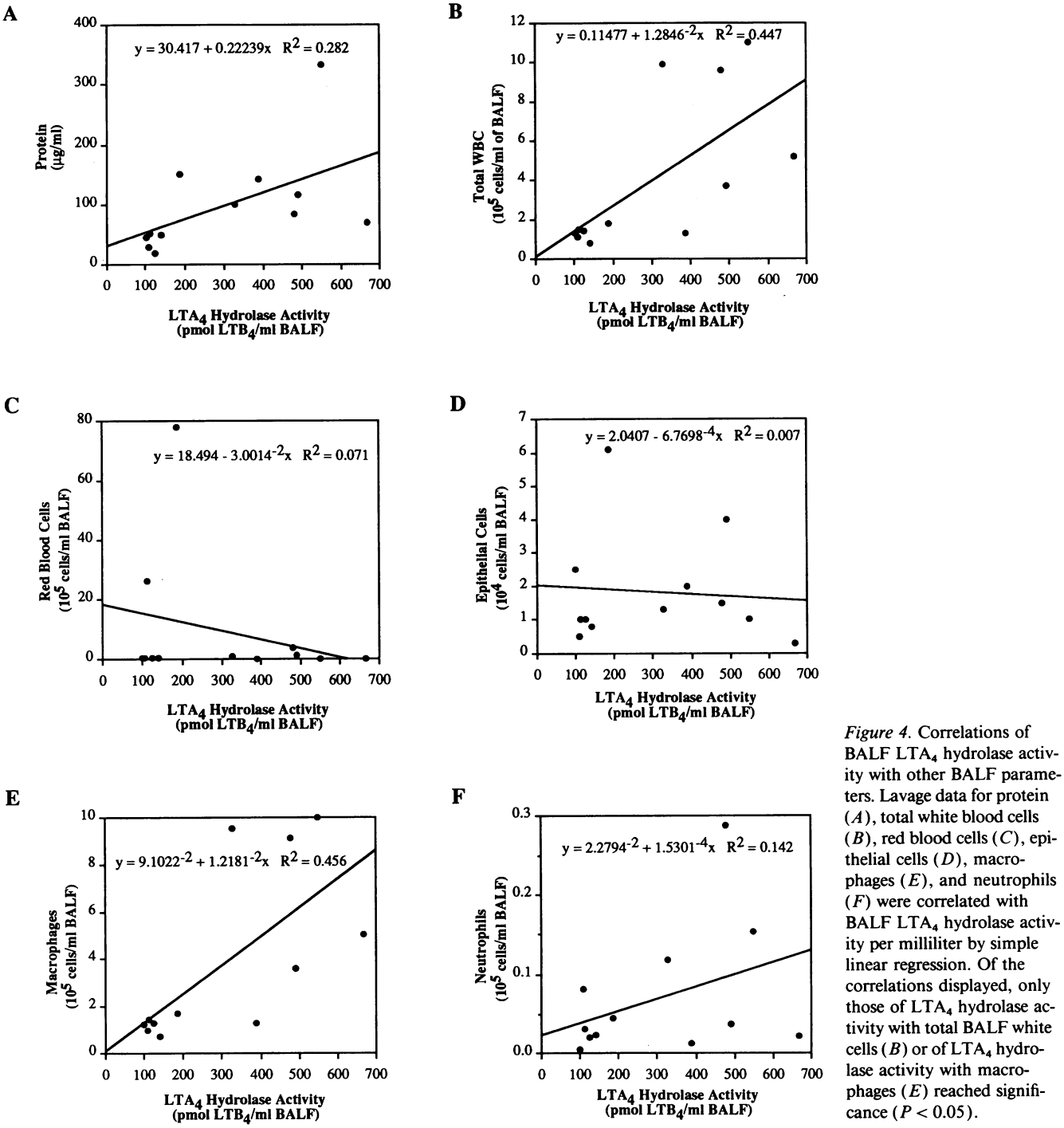

Figure 4. Correlations of BALF LTA_4 hydrolase activity with other BALF parameters. Lavage data for protein (*A*), total white blood cells (*B*), red blood cells (*C*), epithelial cells (*D*), macrophages (*E*), and neutrophils (*F*) were correlated with BALF LTA_4 hydrolase activity per milliliter by simple linear regression. Of the correlations displayed, only those of LTA_4 hydrolase activity with total BALF white cells (*B*) or of LTA_4 hydrolase activity with macrophages (*E*) reached significance ($P < 0.05$).

were enzymatically active. This study documents the selective presence of LTA_4 hydrolase in BALF. Its presence has been demonstrated in two ways. First, LTA_4 hydrolase activity was found in unconcentrated BALF. Second, immunoreactive protein of the appropriate size was found by immunoblot analysis of BALF using a polyclonal antibody raised to recombinant LTA_4 hydrolase. BALF that was not incubated with LTA_4 contained no measurable LTB_4. This indicates that LTB_4 detected in specific enzymatic assays was the product of LTA_4 hydrolase. The selective presence of LTA_4 hydrolase is supported by the fact that functional assays for both 5-lipoxygenase and LTC_4 synthase in BALF showed neither enzymatic activity. Moreover, there was no evidence of 5-lipoxygenase immunoreactive protein in the lavage fluid.

We have also provided indirect evidence that inflammatory cells release significant quantities of intact LTA_4 and that this released LTA_4 can be converted to LTB_4 by extracellular LTA_4 hydrolase present in BALF. This has been demonstrated by showing increased synthesis of LTB_4 when neutrophils are stimulated in BALF as compared with buffers such as HBSS. Further, we have demonstrated the presence of only modest aminopeptidase activity in most BALF samples with no activity in some. There was no correlation between BALF aminopeptidase activity and BALF LTA_4 hydrolase activity.

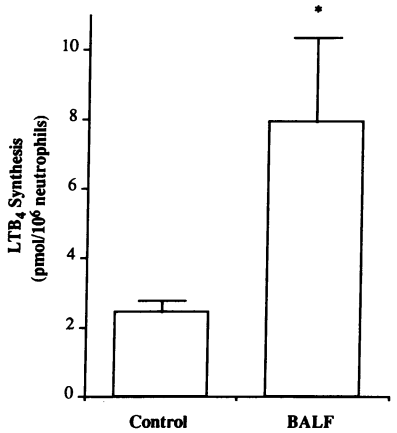

Figure 5. Effects of cell-free BALF on the synthesis of LTB_4 by neutrophils. Neutrophils (6×10^6/ml) were suspended in HBSS or an equal volume of BALF. Neutrophils were then stimulated by incubation with 100 μg/ml of opsonized zymosan for 30 min at 37°C. Samples were extracted and analyzed for LTB_4 by RP-HPLC as noted above. The data represent the mean±SEM of three experiments. Significantly greater amounts of LTB_4 were synthesized by neutrophil cell suspensions when neutrophils were stimulated in BALF rather than in HBSS (* $P < 0.05$).

Previous studies have documented the presence of very small amounts of leukotrienes, including LTB_4, LTC_4, LTD_4, and LTE_4, in human BALF (19–23). The amounts of these mediators previously reported have been < 3 pmol/ml BALF. Patients with adult respiratory distress syndrome, as well as those at risk for developing the adult respiratory distress syndrome, have been found to have increased levels of LTC_4 and LTD_4 in their BALF (34, 35). Asthmatic patients have also been found to have higher levels of leukotrienes in their BALF that were further increased after antigen exposure (20). However, studies have not been reported examining cell-free BALF for the presence of enzymes of the 5-lipoxygenase pathway.

In demonstrating the selective presence of LTA_4 hydrolase in BALF in the absence of other enzymes of the 5-lipoxygenase pathway, we did not attempt to definitively determine the source of this enzyme. Based on the available data, however, we can speculate upon the cellular origin of LTA_4 hydrolase. Other investigations in humans or human tissues have demonstrated that LTA_4 hydrolase is present in inflammatory cells (1), airway epithelial cells (11),[2] erythrocytes (8), whole human lung (10), and liver (9). Previous work by Fitzpatrick and colleagues (7) has also demonstrated the presence of LTA_4 hydrolase in plasma. We have found substantially less activity in plasma but somewhat greater activity in serum when compared to plasma, suggesting the enzyme might be released from blood cells during coagulation. Our data imply that LTA_4 hydrolase is not a major constituent of circulating plasma in humans. Regardless of whether LTA_4 hydrolase is present in plasma, at best it would be present in small quantities, and thus capillary leak of this enzyme is not an adequate explanation for the large quantities found in lung lining fluid. Red blood cells, inflammatory cells, and airway epithelial cells are all potential sources of the LTA_4 hydrolase detected in BALF. The numbers of red blood cells did not correlate with enzyme activity in BALF, making these cells a less likely source. The significant increase in white cells in BALF from smokers is accounted for by increases in macrophages, and this increase correlates with increased LTA_4 hydrolase activity. These observations may, however, be unrelated. Furthermore, increased LTA_4 hydrolase in lung lining fluid may be responsible for synthesis of increased quantities of LTB_4, and, in turn, the recruitment of additional inflammatory cells. Thus, the relationship between this enzyme and inflammatory cells in the lungs of smokers remains to be determined. The absence of enzymatically active or immunoreactive 5-lipoxygenase in BALF suggests that inflammatory cells may not be the source of LTA_4 hydrolase in lung lining fluid. Epithelial cells lining the airspaces of the lung could provide a selective source of LTA_4 hydrolase in lung lining fluid in the absence of 5-lipoxygenase (an enzyme which is not present in the epithelium (36). Possible mechanisms of release of LTA_4 hydrolase from epithelial cells include specific secretion, release after cell injury, or senescence.

Regardless of the cellular source of LTA_4 hydrolase, simple release of this enzyme from the cell cytosol cannot adequately explain the high concentrations of the enzyme found in lung lining fluid. When examined on a per milligram of protein basis, the amounts of LTA_4 hydrolase in BALF were higher than those found in epithelial cell or neutrophil cytosol. The mean specific activity of LTA_4 hydrolase in BALF was on average more than twice, and in some cases more than five times, that observed in neutrophil cytosol. Compared with epithelial cell cytosol, BALF contained more than 12 times the LTA_4 hydrolase activity per milligram of protein. Although these findings would favor active secretion of this enzyme, there is as yet no evidence that this enzyme is specifically secreted, based on its subcellular location (cytoplasm as opposed to secretory vesicles) or on its molecular structure (LTA_4 hydrolase does not have a signal peptide, the usual trafficking signal of most proteins destined ultimately for secretion). A series of investigations by Matthay and colleagues (37) have shown that proteins instilled into the alveolar space are significantly concentrated over time. Their findings may in part explain the high concentrations of LTA_4 hydrolase observed in BALF.

Enzymatic activity, per milliliter of BALF, was on average more than three and a half times greater in actively smoking patients than in nonsmokers. This increase did not result simply from an increase in total protein in smokers' BALF. The dramatic increase in LTA_4 hydrolase found in smokers could be explained by either the toxic effects of tobacco smoke, which could induce cell injury, or a specific constituent of tobacco smoke that could stimulate enzyme release.

The molecular identity of LTA_4 hydrolase in BALF has not been established by this study. However, the modest aminopeptidase activity present in BALF and the absence of a correlation between the hydrolase and aminopeptidase activities in BALF suggest that BALF LTA_4 hydrolase may not be identical to the previously characterized neutrophil LTA_4 hydrolase which clearly has aminopeptidase activity (13). Further, previous work has suggested, although not established, that more than a single LTA_4 hydrolase enzyme may exist in humans (11, 38, 39).[2] Because multiple isoforms of LTA_4 hydrolase may share immunoreactive epitopes, polyclonal antibodies raised to the recombinant neutrophil LTA_4 hydrolase, as used in this study, may not distinguish these isoforms. Therefore, the immunoblot data reported on BALF does not conclusively identify BALF LTA_4 hydrolase as identical to neutrophil LTA_4 hydrolase.

The potential importance of the presence of LTA_4 hydrolase in BALF stems from the finding that within inflammatory cells LTA_4 hydrolase is the rate-limiting step in the synthesis of LTB_4, and thus LTA_4 is synthesized in excess (40). As a result, some of the excess LTA_4 in the extracellular milieu can be metabolized to biologically active leukotrienes independent of inflammatory cells. Our study provides indirect data that large quantities of LTA_4 are released by inflammatory cells upon

stimulation, and these data are consistent with more direct measures of extracellular release of LTA_4 (31). Earlier studies have shown that inflammatory cells can provide LTA_4 that airway epithelial cells metabolize to LTB_4 (11). The presence of LTA_4 hydrolase in lung lining fluid may similarly contribute to the synthesis of LTB_4, thus serving to amplify the inflammatory response in the lung. Our study also provides direct data that BALF significantly enhances the synthesis of LTB_4 by neutrophils. The known effects of LTB_4 in the lungs and the airways (16, 18) suggest that it may play an important role in inflammatory lung disorders including acute lung injury, asthma, and chronic bronchitis. Airways inflammation is also thought to play a role in tobacco-related lung diseases. The increase in LTA_4 hydrolase in the lungs of smokers suggests that this enzyme may play a role in the pathogenesis of tobacco-related lung diseases via the generation of the potent proinflammatory lipid LTB_4.

Acknowledgments

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