Lung mast cells are a source of secreted phospholipases A2

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Background: Secreted phospholipases A2 (sPLA2s) are released in plasma and other biologic fluids of patients with inflammatory, autoimmune, and allergic diseases. Objective: We sought to evaluate sPLA2 activity in the bronchoalveolar lavage fluid (BALF) of asthmatic patients and to examine the expression and release of sPLA2s from primary human lung mast cells (HLMCs). Methods: sPLA2 activity was measured in BALF and supernatants of either unstimulated or anti-IgE-activated HLMCs as hydrolysis of oleic acid from radiolabeled Escherichia coli membranes. Expression of sPLA2s was examined by using RT-PCR. The release of cytoeindyl leukotriene (LT) C4 was measured by means of enzyme immunoassay. Results: Phospholipase A2 (PLA2) activity was higher in the BALF of asthmatic patients than in the control group. BALF PLA2 activity was blocked by the sPLA2 inhibitors dithiothreitol and Me-Indoxam but not by the cytosolic PLA2 inhibitor AZ-1. HLMCs spontaneously released a PLA2 activity that was increased on stimulation with anti-IgE. This PLA2 activity was blocked by dithiothreitol and Me-Indoxam but not by AZ-1. HLMCs constitutively express mRNA for group IB, IIA, IID, IIE, IIF, III, V, X, XIIA, and XIIB sPLA2s. Anti-IgE did not modify the expression of sPLA2s. The cell-impermeable inhibitor Me-Indoxam significantly reduced (up to 40%) the production of LTC4 from anti-IgE–stimulated HLMCs. Conclusions: sPLA2 activity is increased in the airways of asthmatic patients. HLMCs express multiple sPLA2s and release 1 or more of them when activated by anti-IgE. The sPLA2s released by mast cells contribute to LTC4 production by acting in an autocrine fashion. Mast cells can be a source of sPLA2s in the airways of asthmatic patients. (J Allergy Clin Immunol 2009;124:558-65.)

Key words: Lung mast cells, secreted phospholipase A2, leukotriene C4, arachidonic acid

Phospholipases A2 (PLA2s) are key enzymes involved in the mobilization of arachidonic acid from membrane phospholipids. This is the initial step in the metabolic cascade, leading to the synthesis of eicosanoids (prostaglandins, leukotrienes [LTs], and other). PLA2s thought to play a role in arachidonic acid release are currently classified as high-molecular-weight cytosolic phospholipases A2 (cPLA2s) and low-molecular-weight secreted PLA2s (sPLA2s).

Ten isoforms of sPLA2s have been identified in human cells and tissues. sPLA2s are released in extracellular fluids during local or systemic inflammation. In addition, it has been previously shown that sPLA2 activity is detectable in the bronchoalveolar lavage fluid (BALF) of healthy individuals. This activity is increased in the airways of patients with inflammatory lung diseases (pneumonia, adult respiratory distress syndrome, and sarcoidosis). Moreover, sPLA2 activity is also increased in the BALF of patients with bronchial asthma and in the nasal fluid of patients with allergic rhinitis after local allergen challenge. These observations indicate that sPLA2 enzymes can be released during allergic reactions in both the upper and lower airways. However, these studies did not provide information on the cellular sources of these enzymes.

The role of sPLA2 isoforms in airway inflammation has been investigated in rodents. For example, several sPLA2s, such as GIIA, GIID, GIIIE, GV, and GX, are overexpressed in lung biopsy specimens in experimentally induced pulmonary inflammation.

In particular, GX, the isoform with the highest phospholipolytic activity in mammalian cells in vitro, is constitutively expressed in the lung. Interestingly, GX expression did not change on LPS- or carrageenin-induced lung inflammation, whereas it was significantly increased in the airways of mice with ovalbumin-induced asthma. Moreover, knocking out GX reduced all the histologic and functional features associated with the inflammatory response and airway remodeling in this model of asthma. These studies demonstrated that certain sPLA2s play an important role in the pathogenesis of inflammatory and allergic diseases of the lung.

The expression of sPLA2 isoforms in the upper and lower airways has been examined in patients with chronic rhinosinusitis or pneumonia. Immunohistochemistry revealed that low levels of human GIIA (hGIIA) were expressed in the nasal epithelium and submucosal glands of healthy donors, whereas the expression of this sPLA2 was increased in patients with rhinosinusitis. In the lung only human GX (hGX) was detected in bronchial epithelial cells and subepithelial interstitium of both healthy donors and patients with pneumonia. In inflamed, but not normal, lung tissue hGIIA was found in vascular smooth muscle cells and bronchial chondrocytes, whereas human GIID, GV, and GX were found in epithelial cells and macrophages.
Immunostaining analysis of cells from induced sputum demonstrated that hGX was expressed by bronchial epithelial cells and macrophages in healthy donors and patients with asthma. However, in asthmatic patients the expression of hGX was significantly higher than in healthy subjects and was further increased during exercise-induced bronchoconstriction. These observations indicate that sPLA2 expression is upregulated in human airways during inflammatory and allergic disorders and suggest that cells resident in the lung might produce distinct sPLA2s.

Mast cells play a primary role in the pathogenesis of bronchial asthma and rhinitis. These cells can be activated by IgE- and non-IgE-mediated stimuli to release a variety of preformed and de novo synthesized proinflammatory mediators. Mast cells are particularly abundant at the body’s interface with the external environment, such as the mucosa of the respiratory and gastrointestinal tracts and the skin. This unique location justifies the important role of mast cells in allergic inflammation, as well as innate immunity and host defense against infections.

Studies on the expression of sPLA2s in mast cells have been primarily carried out in mice. Enomoto et al showed that bone marrow–derived mast cells (BMMCs) from BALB/C and C57BL/6J mice express all members of the group II subfamily of sPLA2s, including GIIC, GIID, GIIE, GIIF, and GV. GIIA is expressed in BALB/CJ but not in C57BL/6J mast cells because the latter strain has a natural disruption of the gene encoding for GIIA. BMMCs from either strains do not express GIB and GX.

METHODS

Reagents

Percoll, dimethyl sulfoxide, L-glutamine, antibiotic-antimycotic solution (10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 µg/mL amphotericin B), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St Louis, Mo). Dithiothreitol (DTT) was from MP Biomedicals (Solon, Calif). Me-Indoxam and AZ-1 were prepared as previously described. Tritated oleic acid (OA)–labeled Escherichia coli membranes were kindly provided by Dr Gianfrancesco Goracci (University of Perugia, Perugia, Italy). The rabbit anti-human Fcε antibody was donated by Drs T. Ishizaka and K. Ishizaka (La Jolla Institute for Allergy and Immunology, La Jolla, Calif).

Study population

Bronchoalveolar lavage was performed in 14 patients with mild persistent asthma and 19 nonasthmatic subjects (see the Methods section in this article’s Online Repository at www.jacionline.org). The study protocol was approved by the Ethical Committee of the University of Naples Federico II, and informed consent was obtained from each subject before bronchoscopy.

Bronchoalveolar lavage procedure

Bronchoscopy and bronchoalveolar lavage were performed according to a standardized protocol based on current National Heart, Lung, and Blood Institute guidelines (see also the Methods section in this article’s Online Repository).

Cell isolation

The study protocol involving the use of human lung tissue was approved by the Ethical Committee of the University of Naples Federico II, and informed consent was obtained from patients undergoing thoracic surgery. Human mast cells were isolated from the lungs of patients undergoing thoracic surgery and were purified (>98%) by means of immunomagnetic selection, as previously described (see also the Methods section in this article’s Online Repository).

Cell incubation

Mast cells suspended in PCG buffer (10⁶/mL) were incubated at 37°C for 15-120 minutes with anti-IgE (0.03-1 µg/mL). For LTC₄ production, the cells (10⁵/mL) were preincubated at 37°C for 15 minutes with increasing concentrations (0.01-10 µmol/L) of Me-Indoxam or AZ-1 before stimulation at 37°C for 30 minutes. The reactions were stopped by means of centrifugation (at 800g for 5 minutes at 4°C), and the cell-free supernatant was stored at −80°C for determination of PLA2 activity, histamine release, LTC₄ production, or β-hexosaminidase release. The cell pellets were lysed with freeze-thaw cycles in distilled water, and aliquots were stored at −80°C for determination of total content of histamine or β-hexosaminidase.

PLA₂ assay

PLA₂ activity in BALF and HLMC supernatants was measured as previously described by using tritiated OA–labeled E coli membranes. PLA₂ activity was determined in 50 mmol/L Tris HCl (pH 7.5) and 10 mmol/L CaCl₂ in a total volume of 1.0 mL. The reaction was initiated by the addition of 0.1 µCi of tritiated OA–labeled E coli membranes. At the end of incubation (90 minutes at 37°C), the reaction was stopped by adding 2 mL of methanol, 1 mL of chloroform, and 50 µL of 9% formic acid, and lipids were extracted and separated by means of thin-layer chromatography. Tritiated OA was

Abbreviations used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
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<tr>
<td>BMMC</td>
<td>Bone marrow--derived mast cell</td>
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<tr>
<td>cPLA₂</td>
<td>Cytosolic phospholipase A₂</td>
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<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Human GX</td>
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<td>HLMC</td>
<td>Human lung mast cell</td>
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<tr>
<td>IC₅₀</td>
<td>Inhibitory concentration of 50%</td>
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<tr>
<td>LTC₄</td>
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<td>Phospholipase A₂</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<tr>
<td>qPCR</td>
<td>Real-time quantitative PCR</td>
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<tr>
<td>SPLA₂</td>
<td>Secreted phospholipase A₂</td>
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*The Roman numeral after the letter G indicates the group, and the uppercase letter after the numeral indicates the subgroup (eg, GIB indicates group IB PLA₂).*
measured by means of liquid scintillation counting (Tri-Carb 2800 TR; PerkinElmer, Waltham, Mass), and PLAg activity was expressed as picomoles of tritiated OA released per minute per milliliter of BALF or HLMC supernatants. Aliquots of BALF or HLMC supernatants were incubated (for 1 hour at 37°C) with 10 mmol/L DTT, 10 μmol/L Me-Indoxam, 10 μmol/L AZ-1, or 2 mmol/L PMSF before PLAg assay to examine the effect of various inhibitors on PLAg activity.

Mediator release assays

Histamine was measured in duplicate determinations by using a commercially available enzyme immunoassay (Immunotech, Praha, Czech Republic). β-Hexosaminidase was measured in duplicate determinations by using a colorimetric assay.28 LTC4 was measured in mast cell supernatants in duplicate determinations with a commercially available enzyme immunoassay (GE Healthcare, Fairfield, Conn). The linearity range of this assay was 15 to 1,000 pg/mL. Inhibition of LTC4 production was expressed as a percentage of maximum response calculated as follows: \( (R - R_b)/(R_{\text{max}} - R_b) \times 100 \), where \( R \) is the release in samples treated with the inhibitor, \( R_b \) is the release in unstimulated samples, and \( R_{\text{max}} \) is the release in samples stimulated in the absence of the inhibitor.

RT-PCR for sPLAgS

Total RNA from HLMCs was extracted by using the SV total RNA isolation system (Promega, Madison, Wis), treated with RNase-free DNase I, and suspended in diethylpyrocarbonate-treated (DEPC) water. RNA concentration and quality were assessed by means of spectrophotometry. One microgram of total RNA was reverse transcribed with 25 mmol/L MgCl2, 50 μmol/L oligo(dT), and 200 U of Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif). Semi-quantitative and real-time quantitative PCR (qPCR) were performed as previously described (see the Methods section and Table E1 in this article’s Online Repository at www.jacionline.org).29,30

Statistical analysis

Data are expressed as means ± SEs of the indicated number of experiments. \( P \) values were determined with the Student unpaired or paired \( t \) tests. Correlation was assessed by using the linear regression function of Microsoft (Redmond, Wash) Excel software.

RESULTS

Characterization of PLAg activity in BALF

Initial experiments were performed to verify the presence of PLAg activity in the airways of patients with bronchial asthma. PLAg enzymatic activity was determined in the cell-free unconcentrated BALF of 14 asthmatic patients and 19 control subjects. Fig 1, A, shows that low levels of PLAg activity were found in the BALF of control subjects (9.3 ± 0.9 pmol of tritiated OA/min/mL of BALF), whereas Me-Indoxam, whereas it was not affected by AZ-1 or 2 mmol/L PMSF before PLAg activity.

Release of sPLAgS from HLMCs

The detection of sPLAg activity in the airways of patients with asthma led us to investigate the role of lung mast cells as a potential source of these enzymes. In this group of experiments, we explored the capacity of primary human mast cells purified from the lung parenchyma (HLMCs) to release sPLAg activity on immunologic activation. Fig 2, A, shows that HLMCs spontaneously
released PLA2 activity (23.6 ± 2.4 pmol of tritiated OA/min/mL of HLMC supernatant). Stimulation of HLMCs with anti-IgE (0.03–1 µg/mL) concentration-dependently increased the release of PLA2 activity, with a maximum at 1 µg/mL (75.6 ± 5.7 pmol of tritiated OA/min/mL of HLMC supernatant). To understand whether the PLA2 activity released by lung mast cells was from an sPLA2 and had the same biochemical properties as those present in the BALF of asthmatic patients, supernatants of anti-IgE–stimulated HLMCs were preincubated with the PLA2 inhibitors described in the previous section. Fig 2, B, shows that the PLA2 activity released by HLMCs was completely blocked by the reducing agent DTT and by the site-directed sPLA2 inhibitor Me-Indoxam at concentrations known to inhibit most of the human sPLA2s in vitro.11 In contrast, AZ-1 and PMSF had no effect on PLA2 activity released from mast cells. These data indicate that immunologically activated HLMCs released a PLA2 activity with biochemical and pharmacologic properties of an sPLA2.

In the next group of experiments, we examined the kinetics of release of sPLA2 activity and histamine from anti-IgE–stimulated HLMCs. In 3 different preparations of HLMCs, the release of the sPLA2 activity was detectable already after 15 minutes of stimulation and peaked at 30 minutes (Fig 3, A). The kinetics of sPLA2 release were similar to those of histamine (half-time of sPLA2 release, 15.3 ± 3.2 minutes; half-time of histamine release, 12.8 ± 2.6 minutes). Data obtained with HLMCs from 9 different donors indicated that there was a significant correlation between maximal release of sPLA2s (expressed as biologic activity) and of histamine (expressed as the percentage of the total cellular content) when mast cells were stimulated with an optimal concentration of anti-IgE (1 µg/mL; Fig 3, B). These data suggest that the sPLA2 is stored as a preformed mediator within mast cells and is rapidly released on immunologic activation.

Expression of mRNA for sPLA2s in HLMCs

The results obtained thus far have indicated that human mast cells release 1 or more isoforms of sPLA2s. We therefore examined constitutive gene expression of the known human isoforms of sPLA2s in resting HLMCs by means of RT-PCR by using target-specific primers for the various sPLA2s (see Table E1 in this article’s Online Repository). Fig 4 shows the PCR amplification signals of 2 experiments representative of 4 different preparations of HLMCs. PCR fragments of the expected size encoding for hGIB (341 bp), hGIIA (434 bp), hGIID (294 bp), hGIIE (120 bp), hGIIF (211 bp), hGIII (500 bp), hGV (358 bp), hGX (370 bp), hGXIIA (105 bp), and hGXIB (141 bp) were amplified in all HLMC preparations at subsaturating cycle numbers (35 cycles). As previously reported in human neutrophils, the primers used to evaluate hGV mRNA expression in HLMCs also generated a 251-bp PCR product that was identical to the
hGV mRNA from nucleotides 24 to 381 but lacked the untranslated region corresponding to exon 4. These data indicate that primary HLMCs constitutively express mRNA for most human sPLA2s.

To investigate whether anti-IgE challenge of HLMCs modifies the expression of sPLA2s, we next examined mRNA for the major sPLA2 isoforms (hGIIA, hGIID, hGIIE, hGIIF, hGIII, hGV, and hGX) in both resting and anti-IgE–activated HLMCs. To this end, we carried out qPCR in 3 different preparations of HLMCs incubated (at 37°C for 3 hours) in the absence (unstimulated) or presence of anti-IgE (1 μg/mL). These experiments allowed an accurate quantification of the sPLA2s constitutively expressed by HLMCs. Table I shows that human mast cells express high levels of hGIII, hGV, and hGX; intermediate levels of hGIID and hGIIF; and low levels of hGIIA and hGIIE. Stimulation with anti-IgE did not enhance the expression of any sPLA2 examined. We rather observed a tendency toward a reduction in the expression of all sPLA2s, but these results did not reach statistical significance.

Role of endogenous sPLA2s in the generation of LTC4 from HLMCs

sPLA2s contribute to the generation of eicosanoids in murine mast cells and macrophages. However, it is still debated whether this contribution is due to intracellular or extracellular actions of sPLA2s. Our experiments demonstrated that human mast cells secrete 1 or more sPLA2s based on results obtained with Me-Indoxam. It is worth noting that, being cell impermeable, Me-Indoxam is able to inhibit the activity of sPLA2s only when they are secreted in the extracellular space. Thus we were able to evaluate the role of endogenous PLA2s on LTC4 production once these enzymes have been released by immunologically activated mast cells. In these experiments mast cells were stimulated with anti-IgE in the presence of increasing concentrations (0.1-10 μmol/L) of Me-Indoxam or AZ-1, a potent and cell-permeable inhibitor of GIV-cPLA2. At the end of incubation, LTC4 production was determined in the supernatants. Fig 5 shows that the GIV inhibitor AZ-1 caused a complete suppression of LTC4 synthesis (IC50, 40.3 ± 7.9 nmol/L). However, Me-Indoxam also inhibited, in a concentration-dependent fashion, up to 40% of anti-IgE–induced LTC4 release. Neither Me-Indoxam nor AZ-1 significantly influenced anti-IgE–induced degranulation of mast cells, as assessed by means of β-hexosaminidase release (data not shown). These results indicate that LTC4 synthesis

<table>
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<th>sPLA2s</th>
<th>Unstimulated</th>
<th>Anti-IgE</th>
<th>P value</th>
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<tr>
<td>GIIA</td>
<td>15.73 ± 1.11</td>
<td>16.34 ± 1.17</td>
<td>.179</td>
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<tr>
<td>GIID</td>
<td>9.36 ± 1.03</td>
<td>9.90 ± 0.47</td>
<td>.225</td>
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<td>GIIE</td>
<td>12.06 ± 0.94</td>
<td>13.46 ± 0.64</td>
<td>.155</td>
</tr>
<tr>
<td>GIIF</td>
<td>9.09 ± 0.96</td>
<td>9.36 ± 0.54</td>
<td>.322</td>
</tr>
<tr>
<td>GIII</td>
<td>4.84 ± 0.38</td>
<td>5.29 ± 0.29</td>
<td>.222</td>
</tr>
<tr>
<td>GV</td>
<td>6.81 ± 0.78</td>
<td>7.54 ± 0.99</td>
<td>.211</td>
</tr>
<tr>
<td>GX</td>
<td>7.43 ± 1.03</td>
<td>7.99 ± 1.28</td>
<td>.119</td>
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</table>

*mRNA expression is based on qPCR, and data are expressed as ΔCt (see the Methods section in this article’s Online Repository). A ΔCt of less than 10 means high to medium expression, a ΔCt of 10 to 15 means medium to low expression, and a ΔCt of greater than 15 means low expression. Data are presented as the means ± SEs of 3 different donors. P values were determined by using the Student paired t test.

**The cells were incubated (at 37°C for 3 hours) in the absence (unstimulated) or presence of anti-IgE (1 μg/mL). At the end of incubation, mRNA expression of sPLA2 was evaluated as described above.
DISCUSSION

Patients with mild persistent asthma have higher levels of sPLA₂ activity in BALF than nonasthmatic control subjects. Primary lung mast cells constitutively express mRNA for several sPLA₂s and release, upon immunologic activation, sPLA₂ activity with biochemical characteristics similar to those of the sPLA₂s detected in the BALF of asthmatic patients. Endogenous sPLA₂s released by mast cells significantly contribute to IgE-mediated production of cysteinyl LTs.

Our results indicate that low levels of sPLA₂s are detectable in the airways of control subjects and that this activity is increased in patients with mild asthma. A significant correlation between sPLA₂ activity and protein content exists in the BALF of control subjects. This correlation is lost in asthmatic patients, suggesting that they might be a source of these proinflammatory molecules.

Another unique feature of human mast cells is the expression of mRNA for a number of sPLA₂s. This is at variance with most human inflammatory cells, which express a restricted profile of sPLA₂ isoforms. The amount of mRNA for the various sPLA₂s is rather different because HLMCs constitutively express high levels of hGIIA, hGV, and hGX; medium levels of hGIID and hGIIF; and low levels of hGIIA and hGIIIE. These results suggest that these cells might synthesize different quantities of the various sPLA₂s. The detection of sPLA₂ proteins in mast cells by using conventional techniques (eg, Western blotting) is limited by the low number of cells retrieved from specimens of lung tissue. Thus although our data indicate that mast cells produce messages for all sPLA₂s, they do not define which isoforms are translated into proteins, secreted, or both. However, information on the sPLA₂s secreted by stimulated mast cells can be inferred from the data obtained with Me-Indoxam. This compound inhibits hGIB, hGIIA, hGIIIE, hGV, and hGX with an IC₅₀ of less than 600 nmol/L and hGIID, hGIIF, hGIIIE, hGXIIA, and hGXIIIB with an IC₅₀ of greater than 2 μmol/L. Thus it is conceivable that HLMC supernatants contain those sPLA₂s that can be blocked by Me-Indoxam (hGIB, hGIIA, hGIIIE, hGV, and hGX) rather than those sPLA₂s that are poorly sensitive or nonsensitive to the inhibitory effect of this compound (hGIID, hGIIF, hGIIE, hGXIIA, and hGXIIIB). Further studies with more sensitive and specific techniques for sPLA₂ detection will define which sPLA₂ proteins are synthesized and released by human mast cells.

The role of sPLA₂s in asthma is still under investigation. Some of these molecules, such as hGX, can participate in airway inflammation and remodeling through at least 3 mechanisms.

First, sPLA₂s can contribute to prostaglandin and LT biosynthesis by potentiating the effect of cPLA₂. The results shown in Fig 5 indicate that LTC₄ production in stimulated HLMCs is primarily dependent on GIV-cPLA₂. Nevertheless, the observation that the cell-impermeable sPLA₂ inhibitor Me-Indoxam reduces LTC₄ production by 40% indicates that the sPLA₂s released by HLMCs contribute to LT production by cross-talking with GIV-cPLA₂. These data are reminiscent of those obtained with murine mast cells and macrophages showing that sPLA₂s alone do not initiate LTC₄ production but potentiate the eicosanoid-forming capacity of GIV-cPLA₂. The mechanisms of the cross-talk between cPLA₂ and sPLA₂s are still unclear, but it is currently believed that sPLA₂-induced intracellular signals might increase the activation of the GIV-cPLA₂. Several isoforms of sPLA₂s (GIB, GIIA, GV, and GX) bind to a specific M-type receptor, which generates intracellular signals leading to proinflammatory responses in target cells. Me-Indoxam
blocks not only the catalytic activity of sPLA₂ but also the receptor-mediated activation of inflammatory cells by preventing the binding of sPLA₂ to the M-type receptor. Of relevance to the present study, GIB, GIIA, and GV activate the M-type receptor expressed on these cells, thereby activating G-protein-coupled receptors and promoting arachidonate mobilization and eicosanoid production. Therefore the enhancement of LT production by sPLA₂ in HLMCs might be due to an autocrine effect on the M-type receptor expressed on these cells.

A second mechanism by which sPLA₂ might promote inflammation in asthma is through their nonenzymatic, receptor-mediated activation of inflammatory cells. We and others demonstrated that several sPLA₂ (GIB, GIIA, and GX) activate cytokine and chemokine production by interacting with the M-type or other receptors expressed on human inflammatory cells.

Finally, sPLA₂ might contribute to the pathogenesis of asthma in vivo through the degradation of surfactant phospholipids. Alterations of the physicochemical properties of surfactant occur in asthma and are associated with airway obstruction and hyperreactivity. sPLA₂ hydrolyze surfactant phospholipids, generating lysophospholipids that, in turn, alter surfactant properties and induce proinflammatory effects. Together, these observations help explain why knocking out just 1 sPLA₂ isoform (GX) dramatically reduces allergic inflammation.

The capacity of mast cells to secrete sPLA₂ might also be relevant to the role of these cells in innate immunity. Several sPLA₂ have potent bactericidal activity. In addition, GIIH sPLA₂ inhibits HIV replication by blocking viral entry into the cells. Our results raise the interesting hypothesis that sPLA₂ are mediators supporting the role of mast cells in innate immunity.

In conclusion, sPLA₂ released by immunologically activated mast cells have biochemical properties similar to those of the enzymes secreted in the airways of asthmatic patients, indicating that mast cells might be a major source of sPLA₂ in asthma. The demonstration that sPLA₂ are released by mast cells further reinforces the concept that these molecules have an important role in inflammation and tissue remodeling in asthma.

We thank Dr. Vincenzo Nardi (University of Perugia, Perugia, Italy) who prepared the tritiated OA-labeled E coli membranes.

**Clinical implications:** HLMCs can be a source of sPLA₂ in the airways of asthmatic patients. PLAS secreted by mast cells are implicated in LT synthesis and might provide a novel therapeutic target in asthma.

**REFERENCES**


METHODS
Study population
Fourteen patients (8 male and 6 female patients; age range, 20-54 years) were classified as having mild persistent asthma on the basis of clinical history and pulmonary function studies. All patients were atopic as documented by a positive skin test result for at least 1 aeroallergen. Inhaled corticosteroids were discontinued at least 4 weeks before bronchoscopy, and only short-acting \( \beta_2 \)-agonists on demand were allowed. As a control group, we enrolled patients (14 male and 5 female patients; age range, 18-60 years) with a single lung lesion in whom the BALF used for the experiments was obtained from the contralateral side. They had no history of atopic diseases and at the time of bronchoscopy were free of respiratory symptoms and had forced vital capacity and FEV\(_1\) values of greater than 90% of the predicted value.

Bronchoalveolar lavage procedure
A flexible fiberoptic bronchoscope (Olympus BF type P20; Olympus, Center Valley, Pa) was wedged into a segmental or subsegmental bronchus, and 3 fractions (50 mL each) of saline preheated at 37°C were introduced. Recovered fluid was pooled and filtered through 2 layers of sterile gauze. The fluid was then centrifuged twice at 800 \( \times \)g for 10 minutes at 4°C and stored at −80°C for determination of PLA\(_2\) activity and total protein content by a Bradford-based assay (Bio-Rad, Hercules, Calif).

Purification of human lung mast cells
Lung fragments chopped with scissors were dispersed into their cellular elements by means of enzymatic digestion with pronase (Calbiochem, San Diego, Calif), chymopapain (Sigma), collagenase, and elastase (Calbiochem). The mast cell suspension was enriched (>80%) by means of flotation over Percoll (Sigma) density gradients and then purified by using positive immunomagnetic selection with the CD117 Microbead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Mast cell numbers and purity were determined by means of toluidine blue staining. The purity of mast cells used in the experiments was always greater than 98%.

RT-PCR
For semiquantitative PCR, equivalent templates of cDNAs were amplified by using target-specific primers for hGIB, hGIIB, hGIID, hGIIE, hGIIF, hGIIF, hGV, hGX, hGXIIA, hGXIIIB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; see Table E1). The PCR products were separated on 2% agarose gel, stained with ethidium bromide, and visualized by using the image analysis system ChemidocXRS (Bio-Rad).

Real-time quantitative PCR (qPCR) was performed on the iCycler (Bio-Rad) with the Platinum SYBR Green qPCR kit (Invitrogen). Target-specific primers for hGIIB, hGIID, hGIIE, hGIIF, hGIIF, hGV, hGX, and GAPDH suitable for qPCR were designed by using the Beacon Designer 3.0 (Bio-Rad; see Table E1). After an initial denaturation step at 94°C for 30 seconds, amplification was performed with 40 cycles of denaturation (94°C) for 30 seconds, annealing (55°C) for 30 seconds, and primer extension (72°C). The data were analyzed with iCycler iQ analysis software (Bio-Rad), and the mRNA signals in each sample were normalized to that of the GAPDH mRNA. The quantification of the mRNA target was obtained by calculating the relative expression of the reference gene compared with GAPDH. Data were expressed as \( \Delta \) cycle threshold (Ct), which was calculated as the number of PCR cycles for liftoff for the target mRNA of interest minus the number of PCR cycles for liftoff for GAPDH mRNA: \( \Delta \text{Ct} = \text{Ct (gene of interest)} - \text{Ct (GAPDH)} \). The Ct values for GAPDH were typically around 23. A \( \Delta \text{Ct} \) of less than 10 means high to medium expression, a \( \Delta \text{Ct} \) of 10 to 15 means medium to low expression, and a \( \Delta \text{Ct} \) of greater than 15 means low expression.

REFERENCE
FIG E1. Correlation between PLA2 activity and protein content in the BALF. Cell-free BALF from patients with bronchial asthma (A; n = 14) and control subjects (B; n = 19) was assayed for PLA2 activity by using tritiated OA-labeled E coli membranes, as described in the Methods section, and for total protein content by using a Bradford-based assay. Data are plotted as a function of PLA2 activity versus protein content. Correlation was assessed by using the linear regression function of Microsoft Excel software.
### TABLE E1. Primer sequences and conditions for RT-PCR and qPCR

<table>
<thead>
<tr>
<th>Target*</th>
<th>Product length (bp)</th>
<th>Ta</th>
<th>Primer (5'-3')</th>
<th>GenBank accession no. or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGIB (RT-PCR)</td>
<td>341</td>
<td>60°C</td>
<td>Forward: TCCTTTGTGCTAGCTGTGCTG Degousee et al(^{24})</td>
<td></td>
</tr>
<tr>
<td>hGIIA (RT-PCR)</td>
<td>434</td>
<td>57°C</td>
<td>Forward: ATGAAAGACCCCTCCTACTGTT Degousee et al(^{24})</td>
<td></td>
</tr>
<tr>
<td>hGIIA (qPCR)</td>
<td>117</td>
<td>55°C</td>
<td>Reverse: TCACAAAGAGGAGGTGCTCC NNM_000300</td>
<td></td>
</tr>
<tr>
<td>hGIID (RT-PCR)</td>
<td>294</td>
<td>60°C</td>
<td>Reverse: AAACGCAGTGCTCTTCTTGGTA Degousee et al(^{24})</td>
<td></td>
</tr>
<tr>
<td>hGIID (qPCR)</td>
<td>85</td>
<td>55°C</td>
<td>Forward: CCGACTAGTGTCGAGAG Degousee et al(^{24})</td>
<td></td>
</tr>
<tr>
<td>hGIIIE (qPCR)</td>
<td>120</td>
<td>53°C</td>
<td>Reverse: CCGCAGTACACCCTCTCTCAG Degousee et al(^{24})</td>
<td></td>
</tr>
<tr>
<td>hGIIF (qPCR)</td>
<td>211</td>
<td>55°C</td>
<td>Forward: GACCCCAACCCTCCTCTCC Degousee et al(^{24})</td>
<td></td>
</tr>
<tr>
<td>hGII (RT-PCR)</td>
<td>500</td>
<td>60°C</td>
<td>Forward: TGCTTCAGAGATCAGCAGA Degousee et al(^{24})</td>
<td></td>
</tr>
<tr>
<td>hGV (RT-PCR)</td>
<td>358</td>
<td>60°C</td>
<td>Reverse: GGCTCTCTTCCTCCTGCNTGC Degousee et al(^{24})</td>
<td></td>
</tr>
<tr>
<td>hGV (qPCR)</td>
<td>100</td>
<td>55°C</td>
<td>Forward: GCGAGCAGCGGCGAGAGNM_015715</td>
<td></td>
</tr>
<tr>
<td>hGX (RT-PCR)</td>
<td>370</td>
<td>60°C</td>
<td>Reverse: AGAATCTCACAACCTCACCACACC</td>
<td></td>
</tr>
<tr>
<td>hGX (qPCR)</td>
<td>94</td>
<td>55°C</td>
<td>Forward: GATGTCGCTGCTCTCTCAGNM_003561</td>
<td></td>
</tr>
<tr>
<td>hGXIIA (RT-PCR)</td>
<td>105</td>
<td>55°C</td>
<td>Reverse: CACAGTCTCCTGCAGTCC BC_017218</td>
<td></td>
</tr>
<tr>
<td>hGXIB (RT-PCR)</td>
<td>115</td>
<td>55°C</td>
<td>Forward: GCCTGTAGATTCCCCTGTTGACNM_032562</td>
<td></td>
</tr>
<tr>
<td>GAPDH (qPCR)</td>
<td>141</td>
<td>55°C</td>
<td>Reverse: GTTCACTCTGGCTTCTCAGCACTC CACAGTTCCTGCTCTGTCNM_032562</td>
<td></td>
</tr>
</tbody>
</table>

*For PLA\(_{2}\), \(h\) stands for human, the Roman number after the letter \(G\) indicates the group, and the letter in caps after the number indicates the subgroup (e.g., hGIB indicates human group IB PLA\(_{2}\)).

\(Ta\), Annealing temperature.