Leukotrienes mediate part of Ova-induced lung effects in mice via EGFR

B. Boris Vargaftig and Monique Singer

Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur-Institut National de la Santé et de la Recherche Médicale U485, Institut Pasteur, 75015 Paris, France

Submitted 5 November 2002; accepted in final form 28 May 2003

Vargaftig, B. Boris, and Monique Singer. Leukotrienes mediate part of Ova-induced lung effects in mice via EGFR. Am J Physiol Lung Cell Mol Physiol 285: L808–L818, 2003.—Antigen induces murine bronchial hyperreactivity (BHR), inflammation, mucus accumulation, and airway remodeling. To investigate whether leukotrienes (LT) mediate the effects of antigen [ovalbumin (Ova)], we studied 5-lipoxygenase (5-LO) expression in immunized BP2 mice and blocked LT synthesis with the 5-LO inhibitor zileuton or antagonized their effects with receptor antagonists [cysteinyl leukotriene (Cys-LT)-ra MK-571, LY-171883; LTB4-ra PHI-163]. Cys-LT content increased in the bronchoalveolar lavage fluid (BALF) as early as 15 min after the intratracheal instillation of Ova. Zileuton inhibited LT release in the BALF and eosinophil recruitment in the lungs, and dose dependently reduced BHR, mucus accumulation, and remodeling, as did the LT-ra. Thus LT, released just after antigen challenge, might constitute the first step in accounting for the effects of Ova. Because mucus accumulation is regulated via the EGF receptor (EGFR), which is also implicated in the effects of LT and in the asthma-like syndrome triggered by antigen in mice.

LEUKOTRIENES (LT) are important mediators of inflammation and asthma (18, 19, 26, 33, 46–48). They promote bronchoconstriction (18, 19, 47), recruitment of inflammatory cells (18, 19, 46, 47), plasma extravasation, and edema. They also increase mucus release (18, 23, 28, 47) and reduce ciliary function (37), thus promoting bronchial obstruction. Drugs preventing the synthesis or the effects of LT have been developed as a treatment for asthma (1, 12, 20, 21, 30, 38, 42) and have become useful tools to study the role of LT (18, 19, 37, 39, 44, 46, 47). We recently demonstrated that the effects of antigen and of IL-13 may involve secondary mediators and suggested that LT could account for some of these (39, 46, 47). This led us to study the kinetics of LT production following the intratracheal administration of Ova to immunized mice and the role of LT in these responses using specific inhibitors and antagonists. The present results show that LT mediate numerous effects of Ova, such as bronchial hyperreactivity (BHR), inflammation, remodeling, and mucus accumulation. Takayama et al. (40) previously demonstrated that mucus production is regulated by the EGF receptor (EGFR) pathway, and we have shown that EGFR mRNAs are induced in the murine model (46), which has also been shown in asthmatic patients (2, 4). This led us now to investigate the role of the EGFR pathway in the induction of BHR, inflammation, and lung remodeling, using the EGFR tyrosine kinase (EGFR-TK) inhibitor AG-1478. The latter dose-dependently inhibited BHR, inflammation, mucus accumulation, and remodeling that follow challenge with antigen or with the potential mediators we have previously studied, such as LT, recombinant murine (rm) IL-13, and monocyte chemoattractant protein (MCP)-1 (39, 46, 47). Indeed, these molecules induce and amplify LT and their effects, as well as a large part of the effects of Ova (46), and promote a positive loop of regulation that perpetuates the asthma-like syndrome. Accordingly, the downregulation of these molecules by AG-1478 inhibits the amplification process of the inflammatory reaction, suggesting that EGFR is involved in the effects of LT and in the asthma-like syndrome triggered by antigen in mice.

MATERIALS AND METHODS

Animals, immunization, and materials. The experimental design was approved by the Institut Pasteur Review Board, according to the European directives. Male BP2 mice (Centre d’Elevage R. Janvier, Le Genest St. Isle, France), aged 5–6 wk, were immunized subcutaneously at 1-wk intervals with 1 μg of ovalbumin (Ova; ICN Pharmaceuticals, Orsay, France) and 1.6 mg of aluminum hydroxide (Merck, Darmstadt, Germany) in 0.4 ml of 0.9% saline (39). Two weeks later, groups of five mice were challenged intratracheally with Ova (10 or 20 μg) in 50 μl of endotoxin-free 0.9% saline, animals being lightly anesthetized with 12% xylazine (20 mg/kg) and ketamine 500 (45 mg/kg, both from Sigma, St. Louis, MO). Repeated challenges with Ova were also performed (10 μl/...
day for 3 days). We performed challenge with other molecules induced by Ova, i.e., leukotriene (LT) C₄ or LTB₄ (1 μg, evaporated under N₂ and dissolved in NaCl, both from Cayman Chemicals, Ann Arbor, MI), IL-13 receptor antagonist (=ra, 4 μg; Snafu Elf Bioresearches, Liege, France), or MCP-1 (1 μg; Imogene, Los Angeles, CA) (46, 47). Groups of mice were treated separately with different drugs; the 5-lipoxygenase (5-LO) inhibitor zileuton (Zytho; Abbott, Chicago, IL) (30) was first used, because it inhibits the initial step of the transformation of arachidonic acid via this pathway, providing cysteinyl leukotrienes (Cys-LT, by LTC₄ synthase) or LTB₄ (by LTA₄ hydrolase) (1, 26, 33); zileuton was given by oral gavage at 50 mg/kg 1 h before challenge, then three times a day up to 24 h (for BHR and cytokines) or 72 h (for BHR and mucus accumulation). The chemical component of zileuton ICI-230487 (Astra Zeneca), available only in small amounts, was also injected at 35 mg/kg iv (30). Two receptor antagonists specific for the Cys-LT LY-171883, or Tomelukast (12), and MK-571 (21), both from Cayman Chemicals, were instilled at 1, (3 or) 5, 15, or 50 mg/kg it 1 h before challenge, 6 h later, then once a day for 2 days (47). The IL-5 receptor antagonist (20) was inserted under the same conditions, at 1, 3, 10, or 30 mg/kg it as described previously (47).

Dexamethasone (sodium salt, Sigma) was injected at 1.25 mg/kg iv (at 18 and 1 h before and 6, 24, 48 h after challenge) (39).

The EGFR-TK inhibitor AG-1478 (35) (Calbiochem, Schwalbach, Germany) was instilled at 0.5, then 4, and 20 mg/kg it 1 h before the challenge with Ova, 6 h later, then once a day for 2 days. These doses were in the range for specific EGFR inhibition in mice (5). Seventy-two hours after challenge, mice were anesthetized with urethane (35 mg/kg), then bronchoalveolar lavage fluid (BALF), and lungs were collected.

To evaluate cell proliferation in vivo, we injected bromodeoxyuridine (BrdU, Sigma) at 120 mg/kg it (21) 1 h before the intratracheal challenge, then 1 h later at 50 mg/kg, and subsequently twice a day until the animal was killed.

Evaluation of BHR. Basal enhanced pause (Penh) of the airways and BHR were assessed in unrestrained conscious animals by barometric plethysmography (Buxco Electronics, Troy, NY). We evaluated bronchial reactivity using noncumulative methacholine challenges as described (17). Mice were placed in a Buxco chamber, and respiratory parameters were measured after methacholine aerosol inhalation for 90 s at 60 mM (39, 46, 47) (Nebulizor Type LS light; System Assistance Medical, Le Ledat, France). Penh was calculated on the mean of these measurements as Penh = [(expectory time/relaxation time) – 1] × peak expctatory flow/peak inspiratory flow (15, 17, 27).

For graphic representation, BHR (expressed in area under the curve) is measured by the difference between the surface under the curve of the peak of Penh and the surface under the curve of the basal level of Penh, evaluated for 10 min after the start of methacholine inhalation (39, 46, 47).

BDCF. Mice were anesthetized with urethane (45 mg/30 g body wt ip), and the trachea was cannulated. BDCF was collected with 0.5 ml followed by 2 × 1 ml of sterile saline containing EDTA (0.005 M), PMSF (0.005 M), and DTT (0.005 M), all from Sigma, then immediately centrifuged to eliminate the cells, and immersed in liquid nitrogen. The total number of nucleated cells was determined as described previously (39).

Determination of Cys-LT, LTB₄ proteins by enzyme immunoassay and MUC5AC, IL-4, IL-13 proteins in the BALF by ELISA. Fresh cell-free BALF kept at 4°C for <1 h or nitrogen-frozen cell-free BALF kept for <72 h was used, since the stability of LT in these conditions has been verified (47). The quantification (pg/ml) was achieved by enzyme immunoassay according to the manufacturer’s instructions (kit for Cys-LT or for LTB₄, Cayman Chemicals) (47).

MUC5AC, IL-4, and IL-13 by ELISA were determined as described previously (39).

Quantitative RT-PCR. After lung washing, isolation, and dispersion, mRNAs were extracted as described (46, 47). We performed intron-differential RT-PCR for lungs, using specific primers for 5-LO, IL-4, IL-13, MCP-1, MCP-5, KC, MUC5AC, and β-actin (39, 46, 47); for eotaxin, oligos 5′ TGCAACCTGAAGCCATAGCTT and 3′ TTATCTCTGATATTAGGCTC were used. The cDNAs and PCR were obtained as described previously (7, 27, 39). Standards (PCR products or plasmids) were prepared, and the copy number was determined (7, 39, 46). The results are given as a ratio of specific mRNAs/β-actin copies.

Determination of lung myeloperoxidase or eosinoperoxidase activities. After being washed, lungs were homogenized with a Potter for 1 min at 4°C, then centrifuged. Myeloperoxidase (MPO) and eosinoperoxidase (EPO) activities were determined on supernatants of homogenates or for LTB4, Cayman Chemicals) (47).

Histology. The lungs were flushed to remove blood, then inflated with optimum cutting temperature medium (Sakura Finetek, Torrance, CA), diluted 1:1 (vol/vol) in saline, and immersed in 10% formaldehyde in PBS overnight at 4°C, then processed to paraffin wax. Five-micrometer sections were stained with periodic acid-Schiff (PAS)/hematoxylin for mucins. Collagen was visualized by acidic picrofuschine staining of van Gieson (14).

Quantification of mucins and collagen was achieved with the Optilab software, version 2.1 (Grastek, Mirmande, France). In the case of mucins, the labeled area of airway epithelia was measured on longitudinal lung sections, always at the same place of the main bronchiile, by surrounding the epithelium and measuring the total area (labeled plus nonlabeled) minus the nonlabeled area. For each sample, the same total area of epithelium was evaluated [approximately the same length of airways, as previously described (39)]. The sum of the values of five fields/slide for five slides is provided for each animal, and the area in pixels is converted in millimeters squared with a coefficient variable according to the objective, the same one for the whole experiment. Five animals were used for each treatment, and the mean of the five values is given in Table 1, and standard deviation was calculated on the mean of these five values (in three independent experiments). All the data were obtained in a blind fashion at >200 magnification. These results are representative of those obtained by other ways of investigation, such as the measure of the labeled area on the same surface of the airway sections on five random fields for each slide, which was performed for mucus and for collagen.

The effect of the treatment was estimated by the number of labeled airways on the total number of airways for each slide (not shown, since it did not modify the results presented in Table 1 in terms of order of magnitude).

Immunohistochemistry. We determined α-actin from smooth muscle actin (α-SMA) by immunohistochemistry (36), using a mouse anti-α-SMA monoclonal antibody amplified by a biotin-streptavidin-peroxidase antibody system (Dako, as described previously (47), revealed by 3-amin-9-ethylcarbazole (Sigma). Slides were counterstained by hematoxylin Gill-2 (Shandon, Pittsburgh, PA). Quantification was achieved with the Optilab System.

Cell proliferation in vivo. Immunodetection of cell-incorporated BrdU (32, 47) was performed in the lung sections, with the streptavidin-biotin antibody system for BrdU staining...
LEUKOTRIENES MEDIATE ANTIGEN EFFECTS VIA EGFR

Table 1. Drug modulation after ovalbumin challenge

<table>
<thead>
<tr>
<th>Lung airways</th>
<th>Saline</th>
<th>Ova</th>
<th>Ova + Zileuton 50 mg/kg, 3x/day, 3 days</th>
<th>Ova + MK-571 15 mg/kg</th>
<th>Ova + PH-163 10 mg/kg</th>
<th>Ova + AG-1478 25 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucus, mm²</td>
<td>0</td>
<td>32.2 ± 8.3</td>
<td>3 ± 2</td>
<td>6.6 ± 1.7</td>
<td>2.7 ± 1.6</td>
<td>15.1 ± 6.1</td>
</tr>
<tr>
<td>BrdU + nuclei, hand count in % of total cells</td>
<td>&lt;1%</td>
<td>7 ± 3</td>
<td>4 ± 2</td>
<td>3 ± 2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Collagen around airways, mm²</td>
<td>&lt;1</td>
<td>42.3 ± 7.6</td>
<td>15.6 ± 4.2</td>
<td>17.1 ± 3.1</td>
<td>-12.5 ± 1.7</td>
<td>8.0 ± 2.1</td>
</tr>
<tr>
<td>α-SMA ICC, mm²</td>
<td>4 ± 1</td>
<td>9.3 ± 2</td>
<td>5.3 ± 1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SD and are expressed as percentages of labeled nuclei per total nuclei. Quantification was performed by the Optilab system and software of periodic acid-Schiff-stained mucins in the airways (area in mm², see MATERIALS AND METHODS) of collagen deposition after acidic picrofuscin staining of van Gieson (in area) of immunocytochemistry (ICC) of α-smooth muscle actin (SMA, in area). Bromodeoxyuridine (BrdU)-labeled nuclei were counted by eye. Ova, ovalbumin; ND, not determined. *P < 0.05, n = 5.

RESULTS

Induction by Ova challenge of the expression of 5-LO mRNAs in the lungs and of LT release into the BALF: drug modulation. 5-LO mRNAs, which were expressed intensively in the lungs at 15 min after challenge with Ova and persisted up to 72 h (Fig. 1A), were downregulated by zileuton at all time points (Fig. 1B, and not shown).

The products of 5-LO, Cys-LT (composed of LTC4, D4, E4), and LTB4 accumulated in the BALF after challenge with Ova (Fig. 2A). Large amounts of Cys-LT were already released at 15 min, followed by LTB4 between 2 and 24 h and again by Cys-LT at 48–72 h (Fig. 2A), showing a bimodal kinetics of expression. Cys-LT production was reduced by zileuton 15 min after challenge with Ova, and at 6 h, all the LT were reduced (Fig. 2B, other time points not shown).

Dexamethasone inhibited the expression of 5-LO mRNAs at both 6 (Fig. 1B) and 72 h (not shown) after Ova challenge and, as a consequence, the release of LT into the BALF (Fig. 2B).

LT mediate Ova-induced BHR: drug modulation. To investigate whether LT are involved in Ova-induced BHR, we gave zileuton orally at 15, 35, 50, and 70 mg/kg (not shown). Only doses of 70 mg/kg twice a day for 3 days, or 50 mg/kg three times a day for 3 days, reduced BHR (Fig. 3A). ICI-230487, injected at the dose of 35 mg/kg iv, reduced Ova-induced BHR to 55% (Fig. 3A); higher doses failed to improve efficacy (not shown).

The mediator role of the individual LT was confirmed by use of receptor antagonists. The LTD4-ra MK-571 or LY-171883, instilled intratracheally 1 h...
before and 6 h after challenge with Ova, dose dependently inhibited BHR evaluated 24 h later (Fig. 3, B and C, respectively) and was still efficacious 72 h later (not shown) after daily instillation of the drug.

The LTB4-ra PH-163, instilled intratracheally, also dose dependently reduced BHR 24 (or 72) h after Ova (Fig. 3D, and not shown), supporting the involvement of LT in BHR.

LT are involved in Ova-induced cell recruitment, Th2 cytokine, and chemokine expression: drug modulation. Zileuton (50 mg/kg, three times a day for 3 days) inhibited eosinophil recruitment into the lungs and, consequently, their release into the BALF (measured by EPO titers at 72 h, Fig. 4A), as did the LTD4-ra MK-571 and the LTB4-ra PH-163. This reduction was confirmed by hematoxylin-eosin staining of the corresponding lung sections (not shown). Neutrophils were poorly recruited into the lungs after Ova and were reduced by zileuton and by PH-163, but less so by MK-571 (measured by MPO titers at 24 h, Fig. 4B). Moreover, zileuton and the LT-ra reduced the expression of IL-4 and IL-13 mRNAs in the lungs at 6 h (Fig. 5A) and the corresponding proteins in the BALF 6–24 h after antigenic challenge (Fig. 5, C and D). These drugs also reduced the expression of mRNAs for MCP-1, MCP-5, KC, and eotaxin 24 h after challenge with antigen (Fig. 5, C–F).

Involvement of LT in mucus synthesis/accumulation and in mucus secretion after Ova. Zileuton dose dependently inhibited mucus accumulation in the lungs after Ova challenge and completely so at 50 mg/kg three times a day for 3 days (39, 47), as evaluated by RT-PCR for MUC5AC mRNAs (Fig. 6A), by ELISA for MUC5AC proteins (Fig. 6B), and by PAS staining of mucus in the airways (Fig. 7C, and Table 1). The LTD4-ra MK-571 also dose dependently inhibited MUC5AC (Fig. 6, A and B) and mucus accumulation in epithelial cells as verified morphologically (Fig. 7D and Table 1), as did the LTB4-ra PH-163 72 h after challenge with Ova (Figs. 6, A and B, 7E; Table 1).

We have previously shown that LT promote MUC5AC synthesis and production as well as the direct release of mucus into the BALF, without mucus accumulation in the airways (47). We demonstrate now that LT, as well as Ova, are potent secretagogues since they induce, when instilled intratracheally, MUC5AC secretion from epithelial cells previously filled with mucus (i.e., 72 h after a single challenge with Ova) (Fig. 6C). This was confirmed by histology (not shown). These observations indicate that new challenges by Ova or LT on airway epithelial cells full of mucus promote mucus release and bronchial obstruction [as described with rmIL-13 (47)].

Cell proliferation and mucus or remodeling: role of LT. A single instillation of Ova (10 μg it) induced only a very limited BrdU labeling of the nuclei at the basal pole of mucus-producing cells (1 ± 2% in saline vs. 5 ± 2% 72 h after Ova). Three instillations of Ova (10 μg per day for 3 days) were more efficacious, the number of labeled nuclei increasing to 8 ± 3% (n = 4), which was not affected by zileuton (not shown). This agrees with earlier observations, which do not indicate a role of LT in mucus cell proliferation (47).

Finally, alveolar macrophages also showed a marked BrdU labeling, possibly related to their morphological alterations observed in the BALF.

Collagen deposition in the airways and remodeling: role of LT. As early as 72 h after the intratracheal instillation of Ova, collagen deposition was observed around bronchioles, highlighted by acidic picrofuschine staining from van Gieson (Fig. 7, G and H), as well as DNA synthesis at the same site, as evaluated by BrdU labeling (Fig. 7F). Zileuton, as well as the LTB4-ra PH-163 and the Cys-LT-ra (LY-171883 and MK-571), reduced collagen deposition around the airways (Fig. 7, C–E), as confirmed by quantification with the Optitab system (Table 1). Contrary to rmIL-13 (47), Ova did not

---

**Fig. 2.** Kinetics of cysteinyl leukotrienes (Cys-LT, A) or leukotriene (LT) B4 (B) production in the bronchoalveolar lavage fluid (BALF) of BP2 mice after saline or Ova challenge (10 μg). C: interference of zileuton (orally, 50 mg/kg, 1 h before challenge, then 3 times a day for 3 days), with the 5-LO products Cys-LT (high level of expression at 15 min) and LTB4 (at 6 h). mn, Min. *P < 0.05; n = 5.
Fig. 3. Bronchopulmonary hyperreactivity (BHR) to methacholine (MCh) 72 h after the intratracheal challenge of immunized mice with saline (Sal or S) or Ova and drug modulation. A: dose-dependent interference of zileuton (Zil) with BHR to MCh. B, C: dose-dependent interference of LTD₄ receptor antagonists (-ra) with BHR to MCh using MK-571 (MK; B) or LY-171883 (LY; C). D: interference of the LTB₄-ra PH-163 (PH), with BHR to MCh in the same conditions. BHR is expressed as the area under the curve (AUC, in arbitrary units).

*P < 0.05, n = 5.

Fig. 4. Inhibition by zileuton (orally, 50 mg/kg, 1 h before challenge, then 3 times a day for 3 days), by MK-571 (MK, 15 mg/kg), or by PH-163 (PH, 10 mg/kg) of eosinoperoxidase (EPO, A) and myeloperoxidase (MPO, B) in lung tissue, and of the number of eosinophils (A) or of neutrophils (B) in the BALF after Ova (10 µg) compared with saline challenge. OD, optical density. *P < 0.05, n = 5.
induce a marked vascular endothelium remodeling 72 h after challenge (Fig. 7F), but an increased α-SMA protein expression was detected. In this case, three challenges with Ova (once a day for 3 days) were needed to detect a statistically significant cell proliferation (not shown).

Thus LT mediate various and important effects of antigen, in addition to those of IL-13 (47).

EGFR-TK inhibitor AG-1478 reduces BHR, eosinophilic inflammation, and mucus accumulation after challenge with Ova or with its potential mediators. Because the EGFR pathway is involved in mucus accumulation induced by Ova (and by IL-13) (35, 40, 50), we hypothesized that a similar pathway might be implicated in BHR and inflammation induced by Ova or by molecules generated by Ova, such as LT, IL-13, and MCP-1, which we previously studied (39, 46, 47).

Therefore, we used the specific inhibitor of the EGFR-TK AG-1478, which, administered 1 h before challenge, dose dependently inhibited BHR due to the intratracheal instillation of Ova, LT (LTC4 and LTB4), rmIL-13, or MCP-1 (Fig. 8A).

The recruitment of eosinophils into the lungs and consequently their passage into the BALF after challenge with Ova, with LTC4, or with rmIL-13 were also reduced by AG-1478 (Fig. 8B). This was confirmed by hematoxylin-eosin staining of lung sections (not shown).

In addition, no mucus-containing epithelial cells were observed in the lungs of mice under the same conditions (Fig. 8C). Similar results were obtained after challenge with IL-13, rmIL-13, or rmMCP-1 (Fig. 8C). Finally, AG-1478 also reduced collagen deposition around the airways after challenge with antigen (Table 1).

These experiments strongly suggest the involvement of EGFR in BHR, inflammation, and remodeling, in addition to its known involvement in mucus accumulation.

**DISCUSSION**

Our earlier results suggest that LT mediate the major effects of rmIL-13 (47) on murine lungs. Indeed, when instilled intratracheally, LT induce typical sequelae of allergy such as BHR, inflammatory cell recruitment, edema, lung remodeling, and collagen deposition in case of LTB4 challenge (M. Singer, unpublished observation). We show here that Cys-LT and LTB4 also participate in the mediation of the effects of Ova and postulate that Cys-LT, released just after antigenic challenge and before IL-13, probably constitute the initial step of the inflammatory reaction. Indeed, the rapid 5-LO activation and LT synthesis after challenge may occur within several minutes via activation of constitutive enzymes in cells (ATP, Ca, or glutathione dependent) (6). LT synthesis takes place in the nuclear envelope of activated cells, where their local high concentration might favor the direct activation of LT nuclear receptors and transcription factors or of signaling pathways (6) that promote the subse-
quent effects of antigen. This might include the EGFR pathway. LT would also amplify the reaction by inducing cytokine and chemokine expression (46).

Our results suggest that Cys-LT are directly involved in eosinophilic inflammation. Indeed, the LTD4-ra reduced eosinophil counts after Ova, and the intratracheal instillation of LTC4 or of LTD4 promoted their recruitment (47). By contrast, the effects of LTB4 may be indirect.

LTB4, which induced a poor recruitment of eosinophils when instilled intratracheally, has been shown to cooperate with eotaxin for eosinophil recruitment (24). Accordingly, the LTB4-ra PH-163 may have inhibited eosinophilia by suppressing this cooperation.

LT inhibition reduced a variety of parameters of allergic inflammation, such as eosinophil recruitment, Th2 cytokine (IL-4 and IL-13), and chemokine expression (MCP-1, MCP-5, KC, and eotaxin). This agrees with the reduced cell response to Ova observed in 5-LO-deficient mice (13). Globally, our present observations support the concept of a basic cooperation among LT, cytokines, and chemokines in promoting positive loops of regulation that amplify inflammation, as we have previously described (46). In addition, we now show that repeated exposures to some of these molecules (such as Ova, LT, or IL-13) promote mucus secretion favoring bronchial obstruction, as observed in asthma.

LT are potent bronchoconstrictor agents in humans and induce BHR in BP2 mice (47). Our results show that Ova-induced BHR was only halved by LT inhibition or antagonism via oral or intravenous zileuton (Fig. 3) or LT-ra. Other as-yet unidentified eicosanoids may account for these residual effects (10, 38, 43). By contrast, 5-LO inhibition failed to inhibit BHR after multiple antigen challenge (19, 20). LT may also potentiate or cooperate with other agents likely to induce BHR, such as IL-13, cytokines, or chemokines (46, 47).

EGFR has been claimed to be implicated in the regulation of smooth muscle cell functions (22, 49), and EGFR immunoreactivity is increased in bronchial smooth muscle of asthmatics (35, 38, 49). Therefore, we investigated whether EGFR is involved in the cascade of reactions triggered by antigen. The EGFR-TK inhibitor AG-1478 indeed reduced BHR after Ova, at least to the same extent as zileuton or the LT-ra, suggesting EGFR involvement. Cholinergic muscarinic receptors stimulated by methacholine might transduce the signal to EGFR (45). Indeed, the latter is activated by a ligand-dependent mechanism [via EGFR or transforming growth factor (TGF)-α; see Refs. 22, 29], a ligand-independent mechanism (41, 45), or the direct G pro-
tein coupled-mediated NF-κB activation via MAP/ERK kinases (50). Notably, EGF induces airway contractions via the cascade of tyrosine kinase and LT production (31). Finally, these numerous pathways converge on the EGFR and promote the syndrome observed.

Recent genetic linkage studies support our present and previous findings (46, 47). Indeed, the EGF response factor 1 [on human chromosome (chr) 14q1] has been identified as a candidate for asthma (16), and the EGFR region (on chr 7) has been linked to BHR (8).

Fig. 7. Periodic acid-Schiff (PAS) staining of neutral mucins in the airways (bright pink, counterstained with hematoxylin in purple) collected 72 h after the intratracheal instillation of saline (A), Ova (10 μg) (B), zileuton (for dose, see Fig. 1B) + Ova (C), LY-171883 (15 mg/kg) + Ova (D), and PH-163 (10 mg/kg) + Ova (E). Note Ova-induced hypertrophy of epithelial cells, inflammatory infiltrate, and mucus accumulation (B). LT inhibition/antagonism abolished mucous cell metaplasia and reduced the inflammatory infiltrate around the airways (C, D, and arrow a in E) and neighboring vessels (arrow b in E). Immunodetection of bromodeoxyuridine (BrdU)-containing nuclei (of proliferating cells) in the airways (F) 72 h after Ova (10 μg) with hematoxylin counterstain: labeled cells are observed in airway epithelium (arrow a), at the site of collagen deposition (arrow b), and in alveoli (arrow c, alveolar macrophages). G–K: lung tissue remodeling after Ova and role of LT. Collagen deposition around the airways (G, H) was induced by the intratracheal instillation of Ova, identified by specific acidic picrofuschine staining of van Gieson. Collagen (in brown/orange) accounts for hypertrophy of the tissues around the bronchioles and a reduction of the diameter of the lumen of airways (G, H). Effect of zileuton is indicated by arrow a in K; no matrix deposition in the tissue was observed. For quantification after specific staining, see Table 1. I–K: remodeling of airways after Ova identified by α-smooth muscle actin immunocytochemistry: smooth muscle cells after saline (I). Note thickening of α-actin labeling after Ova (J), which was reduced by zileuton (arrow a in K). Vessels (v) were poorly affected after Ova (not shown), and zileuton had no effect (arrow b in K). L: PAS staining of airway section after 72 h of Ova (i.e., on airway epithelial cells full of mucus as in E) and 30 min after a new intratracheal instillation of Ova; the latter induced mucus secretion since no or little mucus was detected in epithelial cells. The bar represents 100 μm.
Other studies identified LTC4 synthase and IL-13 among the key mediators regulating the susceptibility to asthma (26). In mice, quantitative trait loci were also identified for BHR, as well as MUC2, MUC5AC, and MUC6 (and MCP-5) in the central region of chr 11 (2, 9, 11, 22, 26).

We now show that LT mediate MUC5AC expression [which is regulated via EGFR (40, 41)] and that the intense mucous cell metaplasia observed 72 h after challenge with antigen is only marginally due to mucous cell proliferation, suggesting that mucous induction is independent of a growth factor (such as EGF). Indeed, TGF-α is the ligand for EGFR in this model (40, 41). Alternative mechanisms might be involved in mucus production, since MUC genes are induced by numerous cytokines, including IL-1β, TNF-α, IL-4, IL-13, and IL-9 (3) and by chemokines (46).

LT seem involved as well with tissue remodeling and airway obstruction after challenge with antigen (19). Indeed, zileuton, PH-163, and MK-571 reduced collagen deposition in the airways (Table 1). Our previous studies highlighted the fact that LT, when instilled intratracheally, promote cell recruitment in the lungs and airways and fibroblast growth in the vascular endothelium (47). In addition, LT4 induces edema around the airways and vessels, as well as collagen deposition in and around the airways 72 h after challenge (M. Singer, unpublished observation). These observations agree with studies performed on LT-deficient mice, which are protected from fibrosis (34). Moreover, 5-LO and 5-LO-activating protein immunoreactivity has been described in endothelial cells and in (inflammatory) cells around the airways after Ova challenge (6), at the site where we observed remodeling and inflammation. These findings suggest a major role for LT in remodeling of the lungs after challenge with antigen. Furthermore, since AG-1478 reduced collagen deposition and mucous accumulation in the airways.
after antigen challenge (Table 1), EGFR is probably involved as well in airways remodeling. However, although the doses of AG-1478 in this study stay within the dose range recognized for EGFR-TK inhibition (5), we cannot rule out the possibility that this tyrosine kinase inhibitor modulates other kinases in vivo, which might contribute to a reduction of the severity of lung allergy and remodeling.

In conclusion, we demonstrate here that LT, released just after challenge with antigen, are involved in various of its pulmonary effects (BHR, eosinophilic inflammation, mucosal metaplasia, and lung remodeling). Because we previously demonstrated that LT directly promote BHR, inflammation, cytokines, and chemokine expression, as well as lung remodeling (46), we suggest that LT promote the inflammatory reaction and the asthma-like syndrome observed after challenge with antigen. Notably, Ova induces LT before IL-13 or chemokines such as MCP-1, which amplify the effects of Ova (46, 47). Now we suggest that these molecules may converge to the EGFR pathway to promote BHR, eosinophilic inflammation, and remodeling after challenge with antigen. This scenario may also occur in situations where these molecules are generated (25).

As a corollary, a strategy targeting EGFR merits further investigation, since it may prevent most effects of allergens, particularly if associated with LT-ra in asthma as in chronic obstructive diseases.

We thank Drs. J. P. Girard, C. Bonne, and P. Hullet, from the laboratory of Chimie Biomolécule et Physiologie, (Centre National de la Recherche Scientifique Esa 5074, Université de Montpellier I, Faculté de Pharmacie, Paris, France), for providing the LTβ-r4 PH-163 and its isomer, Dr. S. Ho (see Refs. 14, 15 in Ref. 39) for anti-MUC5AC antibody for ELISA, Dr. A. Minty (Sano Faculte e Institut Pasteur, 25, rue du Dr Rous, 75015 Paris, France). The Optilab system was kindly provided by Dr. B. Hurtel (Institut Pasteur, Paris, France). The Optilab system was kindly provided by Dr. B. Hurtel (Institut Pasteur, Paris, France). The Optilab system was kindly provided by Dr. B. Hurtel (Institut Pasteur, Paris, France). The Optilab system was kindly provided by Dr. B. Hurtel (Institut Pasteur, Paris, France). The Optilab system was kindly provided by Dr. B. Hurtel (Institut Pasteur, Paris, France).

DISCLOSURES

We thank Professor P. Sansonetti (Unité de Pathogénie Microbienne Moléculaire, Unité Associée Institut Pasteur-INSERM U389, Institut Pasteur, 25, rue du Dr Rous, 75015 Paris, France.

REFERENCES


45. Tsai W, Morieri AD, and Peralta EG. The m1 muscarinic acetylcholine receptor transactivates the EGFR receptor to modulate ion channel activity. *EMBO J* 15: 1037–1044, 1997.


Downloaded from http://ajplung.physiology.org/ by 10.220.32.247 on July 10, 2017