

Leukotriene D₄ upregulates eosinophil adhesion via the cysteinyl leukotriene 1 receptor

Makoto Nagata, MD, Keiko Saito, MD, Koji Tsuchiya, PhD, and
Yoshio Sakamoto, MD *Saitama, Japan*

Background: Eosinophils (EOS) are one of the cellular sources of cysteinyl leukotrienes (cysLTs) in allergic inflammation. There is evidence that cysLT₁ receptor antagonists possess anti-inflammatory properties *in vivo* in asthmatic airways. Although the exact mechanism of action remains unknown, cysLTs might regulate the cellular responses involved in allergic inflammation.

Objective: The present study was undertaken to examine whether LTD₄ modifies the adhesive property of EOS.

Methods: EOS were isolated from the blood of healthy subjects. Their adhesion to tissue culture plates or recombinant human (rh) adhesion proteins was then examined in the presence or absence of LTD₄.

Results: LTD₄ significantly augmented EOS adhesion to tissue culture plates (adhesion: 5.0% ± 0.5% by medium control vs 9.1% ± 1.2% by 1 μmol/L; *P* < .01; *n* = 10). The enhanced adhesion induced by LTD₄ was blocked by pranlukast, a cysLT₁ receptor antagonist, or an anti-β₂ integrin antibody. Flow cytometry analysis revealed that LTD₄ significantly enhanced the expression of CD11b and CD18 on the EOS surface. Finally, LTD₄ augmented EOS adhesion to rh intercellular cell adhesion molecule 1 but not to rh vascular cell adhesion molecule 1 or fibronectin.

Conclusions: The results suggest that LTD₄ directly upregulates the adhesive property of EOS via the cysLT₁ receptor and β₂ integrin. LTD₄ generated from EOS or cells of some other type might contribute to the development of phenotypic change in airway EOS. (*J Allergy Clin Immunol* 2002;109:676-80.)

Key words: Eosinophils, leukotriene D₄, cell adhesion, adhesion molecules, leukotriene receptor antagonist

Cysteinyl leukotrienes (cysLTs), including LTC₄, LTD₄, and LTE₄, are potent biological mediators generated from the arachidonic acid of inflammatory cells, including eosinophils (EOS), via the 5-lipoxygenase pathway.^{1,2} cysLTs have been implicated in the pathogenesis of inflammatory diseases, including bronchial asthma, arthritis, and rhinitis.³ The contribution of cysLTs to inflammatory responses depends on the presence of cysLT-producing cells and the cells that respond to these bioactive lipids in inflammation sites. Over the

Abbreviations used

EOS: Eosinophil(s)
cysLT: Cysteinyl leukotriene
ICAM-1: Intercellular cell adhesion molecule 1
VCAM-1: Vascular cell adhesion molecule 1

past decade, increasing evidences has suggested that cysLTs might contribute to the tissue accumulation of EOS, which are the predominant cells in allergic inflammation and one of the cellular sources of cysLTs. For example, Spada et al⁴ reported that LTD₄ induces an *in vitro* chemotactic response by EOS. More recently, LTC₄ and LTD₄ were shown to promote an *in vitro* survival of human EOS.⁵ In human asthmatic airways, inhalation of LTE₄ increases the accumulation of EOS and, to a lesser extent, neutrophils.⁶ Likewise, inhalation of LTD₄ increases the number of EOS in sputum.⁷ Finally, cysLT receptor antagonists have been shown to reduce the number of EOS in sputum or bronchial tissue.⁸⁻¹⁰

EOS recruited to the airways probably play roles in the pathogenesis of asthma.^{11,12} The mechanism by which EOS accumulate in the airways is a complex process that might be regulated by chemical mediators, cytokines, and adhesion molecules. Accumulating evidence has established that EOS integrin adhesion molecules, such as β₂ integrins—including αLβ₂ (LFA-1, CD11a/CD18) and αMβ₂ (Mac-1, CD11b/CD18)—and α₄ integrins—including α₄β₁ (VLA-4, CD49d/CD29)—play important roles in the adhesion to and transmigration through endothelium or airway epithelium.¹³ We previously reported that EOS adhesion via β₂ integrins to intercellular cell adhesion molecule 1 (ICAM-1) augments LTC₄ generation from EOS,¹⁴ suggesting that the interaction between these adhesion molecules is an important step in providing cysLTs to the inflammation sites. One of the possible biological consequences of the enhanced LTC₄ generation would be modification of the functional status of EOS, which might further regulate the interaction between EOS and adhesion proteins. Here we report that LTD₄ upregulates the adhesive property of EOS, partly through the enhanced expression of β₂ integrin, and augments the adhesion of the cells to ICAM-1.

METHODS

Reagents

Percoll was purchased from Pharmacia (Uppsala, Sweden). HBSS, newborn calf serum, FCS, trypsin-EDTA, L-glutamine, and

From the Pulmonary Division, Second Department of Internal Medicine, Saitama Medical School.

Received for publication August 3, 2001; revised December 31, 2001; accepted for publication January 7, 2002.

Reprint requests: Makoto Nagata, MD, Pulmonary Division, Second Department of Internal Medicine, 38 Morohongou, Moroyama-cho, Iruma-gun, Saitama, 350-0495, Japan.

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0091-6749/2002 \$35.00 + 0 1/84/122841

doi:10.1067/mai.2002.122841

penicillin-streptomycin were obtained from Life Technologies (Grand Island, NY). Anti-CD16 mAb-coated magnetic microbeads were obtained from Miltenyi Biotec (Auburn, Calif). LTC₄, LTD₄, and LTE₄ were obtained from Cayman Chemical (Ann Arbor, Mich). Pranlukast hydrate was provided by Ono Pharmaceutical (Osaka, Japan). Recombinant human (rh) ICAM-1 and rh vascular cell adhesion molecule 1 (VCAM-1) were obtained from R&D Systems (Minneapolis, Minn). Except as noted, all other reagents were purchased from Sigma (St Louis, Mo).

EOS separation

EOS were isolated from the peripheral blood specimens of 52 normal volunteers and 4 donors with mild asthma treated only by inhaled β_2 agonists, containing less than 6% EOS of the total leukocytes. Subjects ranged in age from 22 to 37 years, and sex distribution was equal. EOS isolation was performed by negative immunomagnetic bead selection, as previously described.¹⁵ Briefly, heparinized blood was diluted with HBSS without Ca²⁺ and centrifuged for 20 minutes at 700g over 1.090 g/mL Percoll. The plasma, mononuclear cell band, and Percoll were removed, and the red blood cells in the pellet were lysed by hypotonic shock. The resulting granulocytes were washed with 4°C HBSS supplemented with 2% newborn calf serum (HBSS/newborn calf serum) and then incubated with anti-CD16 antibody-coated magnetic beads for 40 minutes at 4°C. The cells were filtered through a steel wool column in a magnetic field (Miltenyi Biotec) to remove neutrophils bound to magnetic beads. CD16⁺ EOS (>98% purity and >99% viability) were collected, washed, and resuspended in HBSS supplemented with 5% FCS (HBSS/FCS). For each of the subsequent experiments, EOS from a single individual were used.

Preparation of adhesion protein-coated microassay plates

rhVCAM-1, ICAM-1, or fibronectin was dissolved in 0.05 mol/L NaHCO₃ coating buffer (15 mmol/L NaHCO₃, 35 mmol/L Na₂CO₃, pH 9.2), added to 96-well tissue culture plates (Corning Incorporated, Corning, NY), and incubated at 4°C overnight.¹⁶ Residual fluid was decanted, and 200 μ L/well FCS was added to reduce nonspecific adhesion. After a 2-hour incubation at ambient temperature, the wells were decanted; they were then ready for the addition of EOS.¹⁶

EOS adhesion assay

EOS adhesion was assessed as residual EOS peroxidase activity of adherent EOS, as previously described.¹⁴⁻¹⁶ Briefly, EOS (100 μ L of 1×10^5 cells/mL in HBSS/FCS) were placed onto tissue culture plates or adhesion protein-coated plates in the presence or absence of an activator and incubated for 30 minutes or 20 minutes (rhICAM-1 only) at 37°C. After 5 washes with 37°C HBSS, 100 μ L HBSS/FCS was added to the reaction wells. In the experiments designed to determine kinetics, the reaction was serially stopped by the partial aspiration and washing of the corresponding wells every 5 minutes for the first 10 minutes and every 10 minutes for the next 50 minutes. As standards, 100 μ L of serially diluted cell suspensions (1×10^3 , 3×10^3 , 1×10^4 , 3×10^4 , and 1×10^5 cells/mL) were added to empty wells. EOS peroxidase substrate (1 mmol/L o-phenylenediamine, 1 mmol/L H₂O₂, and 0.1% Triton X-100 in Tris buffer, pH 8.0) was then added to all wells. After a 30-minute incubation at room temperature, 50 μ L of 4 mol/L H₂SO₄ was added to stop the reaction, and the absorbance at 490 nm was measured. Each experiment was performed in quadruplicate through use of EOS from a single donor, and the mean value was used for the calculation of percent EOS adhesion, which was calculated from the log dose-response curve. EOS viability after the incubation exceeded 98% by trypan blue dye exclusion.

Expression of CD11b and CD18 on EOS

The expression of CD11b and CD18 on EOS was examined by flow cytometric analysis. EOS were exposed to either 1 μ mol/L LTD₄ or buffer (HBSS/5%FCS) alone for 30 minutes at 37°C. The cells were washed 3 times and resuspended in 4°C PBS supplemented with 2% BSA and 0.2% sodium azide (FACS buffer). Cells (1×10^5 /100 μ L) were incubated with FITC-conjugated mouse anti-human CD18 (clone: MHM23, IgG1, Dako A/S, Glostrup, Denmark), FITC-conjugated mouse antihuman CD11a (clone: MHM24, IgG1, Dako A/S), phycoerythrin (PE)-conjugated mouse antihuman C3bi (CD11b) (clone: 2LPM19c, IgG1, Dako A/S), PE-conjugated mouse antihuman CD49d (clone: 9F10, IgG1, PharMingen, San Diego, Calif.), or PE-conjugated mouse antihuman CD29 (clone: MAR4, IgG1, BD PharMingen) on ice for 30 minutes. Similarly PE-conjugated mouse IgG1 and FITC-conjugated mouse IgG1 (Dako A/S) were used as isotype-matched controls. Cells were then washed and resuspended in FACS buffer. Mean fluorescence was measured for at least 10,000 events through use of a FACScan (Becton Dickinson, Franklin Lakes, NJ). Relative mean fluorescence was determined by subtraction of values for the IgG1 isotype-matched controls.

Statistics

Data are presented as means $\pm$ SEMs. ANOVA with repeated measures was used for comparison of more than 2 variables. When the initial *P* value was less than .05, Scheffé's post hoc test was used to determine significance between groups. For paired comparison, the Student *t* test was used. Statistical significance was established at the *P* < .05 level.

RESULTS

Effect of LTD₄ on EOS adhesion

Each leukotriene was originally dissolved in ethanol at a concentration of 200 μ mol/L. To evaluate the effect of ethanol on EOS function, preliminary experiments were performed to determine whether ethanol affects the spontaneous or N-formyl-methionyl-leucyl-phenylalanine-induced EOS adhesion. Ethanol exceeding 1% attenuated EOS adhesion, whereas the highest concentration that did not affect EOS adhesion was 0.5% (data not shown). Consequently, the highest concentration of LTD₄ tested in this study was 1 μ mol/L, which contained 0.5% ethanol. An initial series of experiments was conducted to determine whether LTD₄ modifies EOS adhesion to tissue culture plates. After 30 minutes of incubation, 0.1 to 1 μ mol/L LTD₄ significantly enhanced the EOS adhesion (adhesion: $5.0\% \pm 0.5\%$ by control; $9.0\% \pm 0.7\%$ by 0.1 μ mol/L [*P* < .01]; $9.1\% \pm 1.2\%$ by 1 μ mol/L [*P* < .01], *n* = 10; Fig 1). After incubation with 0.1 or 1 μ mol/L LTD₄, the cell viability exceeded 98%. The effect of 1 μ mol/L LTD₄ did not differ from the maximal effect of LTB₄, which was also obtained at 1 μ mol/L (adhesion: $10.8\% \pm 2.2\%$ by LTD₄ vs $10.6\% \pm 1.8\%$ by LTB₄ [*P* = not significant], *n* = 5). In selected experiments, the effect of LTD₄ on EOS adhesion was examined through use of EOS from donors with mild asthma with similar results—ie, adhesion was augmented (adhesion: $5.0\% \pm 0.9\%$ by control vs $9.1\% \pm 0.8\%$ by 0.1 μ mol/L [*P* = .01] vs $9.6\% \pm 1.6\%$ by 1 μ mol/L [*P* < .01], *n* = 4).

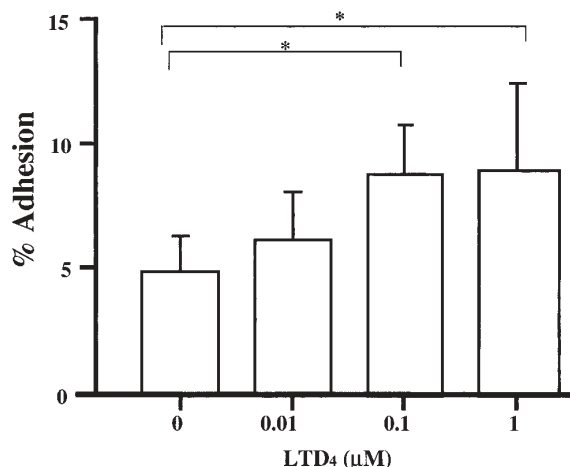


FIG 1. Effect of LTD₄ on EOS adhesion to tissue culture plates. EOS were incubated in the presence or absence of LTD₄ for 30 minutes at 37°C. **P* < .01 versus control; *n* = 10.

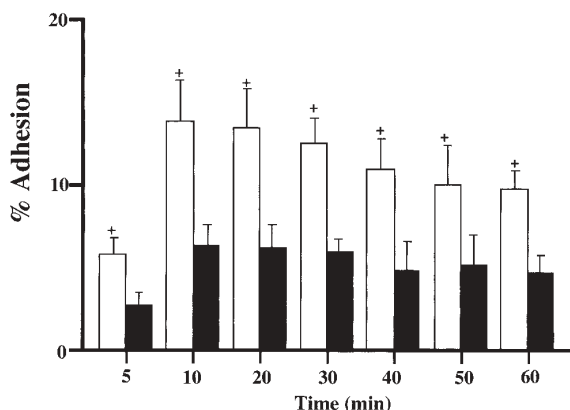


FIG 2. Kinetics of LTD₄-induced EOS adhesion. Open bars represent the adhesion in the presence of 1 μmol/L LTD₄; closed bars represent the adhesion in the absence of LTD₄. **P* < .05; *n* = 4.

The kinetic study revealed that the increase in LTD₄-induced EOS adhesion was significant at 5 minutes and lasted for at least 60 minutes (*P* < .05 at each time point examined; *n* = 4; Fig 2). To confirm whether cysLT1 receptor expressed on the EOS surface is involved in LTD₄-induced EOS adhesion, EOS were preincubated in the presence or absence of pranlukast hydrate, a specific cysLT1 antagonist, and EOS adhesion was then determined. Augmented EOS adhesion by 1 μmol/L LTD₄ was almost completely blocked by pranlukast (inhibition: 99.6% ± 0.4%, *n* = 5; Fig 3). The effect of LTD₄ on EOS adhesion was not modified by pranlukast (data not shown).

Effect of anti-integrin mAbs on LTD₄-induced EOS adhesion

To identify the EOS integrin(s) involved in LTD₄-induced EOS adhesion, EOS were pretreated with anti-β₂ integrin mAb (clone: L130, mouse IgG1 [Becton Dickinson], 3 μg/mL), anti-α₄ integrin mAb (clone: HP1/2, mouse IgG1 [Cosmo Bio], 3 μg/mL), or an isotype-matched control mouse IgG1 (Cappel; 3 μg/mL) at ambi-

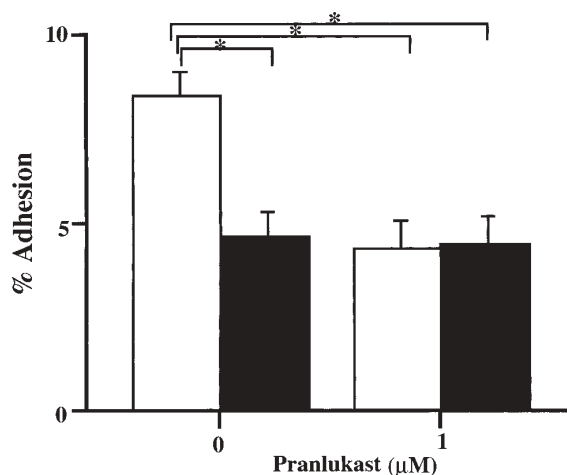


FIG 3. Effect of 1 μmol/L pranlukast hydrate on EOS adhesion. Open bars represent the adhesion in the presence of 1 μmol/L LTD₄; closed bars represent the adhesion in the absence of LTD₄. **P* < .01; *n* = 5.

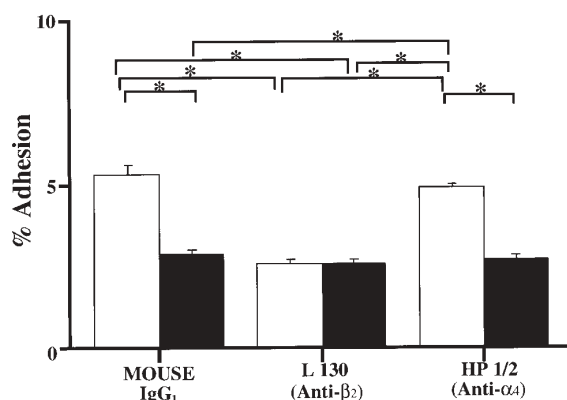


FIG 4. Effects of anti-β₂ integrin mAb (L130) and anti-α₄ integrin mAb (HP1/2) on EOS adhesion. Mouse IgG1 is an isotype-matched control. Open bars represent the adhesion in the presence of 1 μmol/L LTD₄; closed bars represent the adhesion in the absence of LTD₄. **P* < .01; *n* = 5.

ent temperature for 15 minutes; EOS adhesion was then examined. A concentration of 1 μmol/L LTD₄ significantly enhanced the EOS adhesion to tissue culture plates in the presence of mouse IgG1 or anti-α₄ Ab (*P* < .01; *n* = 5; Fig 4). In contrast, the LTD₄ effect was blocked by anti-β₂ mAb (Fig 4).

Effect of LTD₄ on EOS β₂ integrin

To examine whether LTD₄ modifies the number of β₂ integrins on EOS, the expression of β₂ integrins was determined by flow cytometric analysis. After stimulation with 1 μmol/L LTD₄, the expressions of both CD11b and CD18 were enhanced (mean fluorescence index: CD11b, 835.4 ± 144.4 by control vs 987.0 ± 120.4 by LTD₄ [*P* < .01]; CD18, 123.6 ± 14.9 by control vs 141.7 ± 17.8 by LTD₄ [*P* < .01]; *n* = 7; Fig 5, A). On the other hand, the expression of CD11a, CD29, or CD49d was not significantly modified by LTD₄ (data not shown).

To further confirm whether LTD₄ enhanced adhesion via β_2 integrin, EOS adhesion to immobilized rhICAM-1, a ligand for β_2 integrin, was examined. LTD₄ enhanced EOS adhesion to ICAM-1 (adhesion: 3.8% $\pm$ 0.5% by medium alone vs 10.3% $\pm$ 1.8% by 0.1 μ mol/L vs 11.3% $\pm$ 1.6% by 1 μ mol/L; P < .01; n = 8; Fig 5, B). In addition, EOS adhesion to immobilized fibronectin and rhVCAM-1, ligands for α_4 integrin, or to rh P-selectin, a ligand for P-selectin glycoprotein ligand 1, was not modified by 1 μ mol/L LTD₄ (fibronectin, 5.0% $\pm$ 0.9% by medium alone vs 4.6% $\pm$ 0.6% by LTD₄ [P = not significant], n = 8; rhVCAM-1, 23.8% $\pm$ 4.6% by medium alone vs 23.4% $\pm$ 3.8% by LTD₄ [P = not significant], n = 3; rh P-selectin, 9.5% $\pm$ 4.3% by medium alone vs 8.7% $\pm$ 4.2% by LTD₄ [P = not significant], n = 3), indicating that the LTD₄-induced adhesion was exclusively dependent on β_2 integrin.

DISCUSSION

In this investigation, we observed that LTD₄ augments the EOS adhesive property, which was blocked by a specific cysLT1 antagonist, pranlukast hydrate. The LTD₄ effect appears to involve EOS β_2 integrins. First, the enhanced adhesion by LTD₄ was blocked by anti- β_2 integrin but not anti- α_4 integrin antibody. Second, LTD₄ modestly but significantly augmented EOS surface expression of CD11b and CD18, suggesting that the increased expressions of these molecules are, at least in part, involved in the enhancing of EOS adhesion. Finally, we have provided evidence that LTD₄ augments EOS adhesion to ICAM-1, which is constitutively expressed on endothelial or epithelial cells and is a representative counter-ligand for CD11b/CD18.¹³ Although EOS α_4 integrin(s) could also interact with tissue matrix proteins such as fibronectin or endothelial adhesion proteins such as VCAM-1, the anti- α_4 mAb did not affect the LTD₄-induced EOS adhesion. Furthermore, LTD₄ did not enhance the EOS adhesion to fibronectin or rhVCAM-1, indicating that α_4 integrin is not involved in the LTD₄-induced adhesion. Collectively, our results suggest a potentially important biological effect of LTD₄ in the development of adhesive interaction between EOS and the ligand(s) specific for β_2 integrins, such as ICAM-1. Although the LTD₄-induced enhancement in the expression of β_2 integrin was less than 20%, the adhesion increased nearly 2-fold, suggesting another mechanism for the latter effect. In the β_2 integrin-dependent adhesion of leukocytes, the activation states of this integrin might be more important than its levels of surface expression. A variety of stimuli, including cytokines or chemokines, have been shown to induce a conformational change of β_2 integrin that directly increases the avidity for ligand.^{17,18} We therefore speculate that LTD₄ activates both the enhanced expression and the conformational change of β_2 integrin and that the latter effect also confers the adhesiveness of EOS.

The precise concentration of cysLTs in the milieu at allergic inflammatory sites remains unknown. Bron-

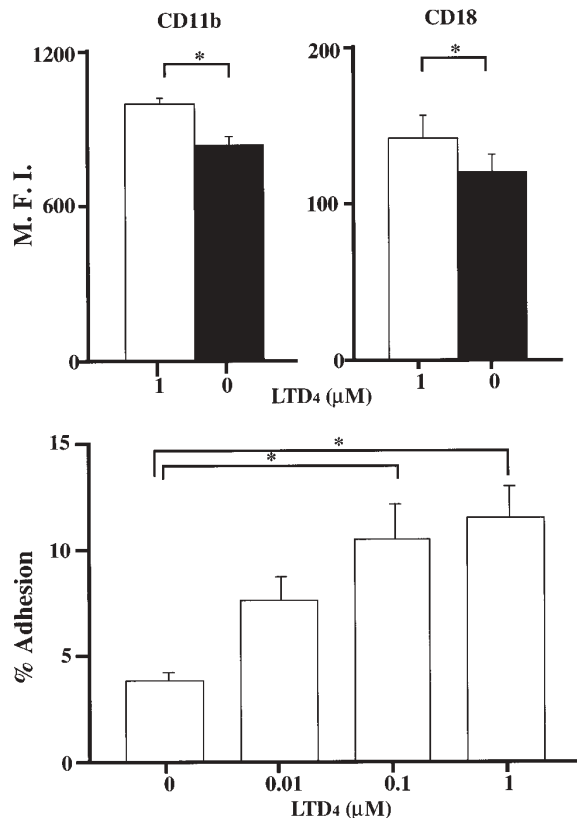


FIG 5. A, Effect of LTD₄ on the EOS expression of CD11b (left) and CD18 (right). Open bars represent the expression after incubation with 1 μ mol/L LTD₄; closed bars represent expression in the absence of LTD₄. * P < .01; n = 7. MFI, Mean fluorescence index. B, Effect of 1 μ mol/L LTD₄ on EOS adhesion to rhICAM-1. EOS were incubated in the presence or absence of LTD₄ for 20 minutes at 37°C. * P < .01 versus control; n = 8.

choalveolar lavage fluid or nasal wash fluid obtained after allergen challenge has been shown to contain a nanomolar amount of cysLT.^{19,20} Sputa obtained from asthmatic individuals during acute asthma and after allergen challenge have contained mean cysLT levels of more than 20 nmol/L.^{21,22} In one report,²² the authors also demonstrated that the increase in the number of sputum EOS correlated with the increase in the cysLT level, suggesting a role for cysLT in the EOS recruitment in response to specific allergen.²² Inside the tissue of allergic inflammation, a variety of inflammatory cells, including EOS, mast cells, and basophils, would generate cysLTs, and it is therefore likely that EOS are exposed to a higher concentration of cysLTs than is seen in sputa or lavage fluids. Thus we speculate that it is possible to reach an LTD₄ concentration in vivo that is similar to that used in this in vitro study.

Our observations are in agreement with earlier works on the interaction between cysLTs and EOS. Spada et al⁴ reported that LTD₄ induces the chemotactic response of EOS. Lee et al⁵ reported that both LTD₄ and LTC₄ cause the inhibition of EOS apoptosis and thereby prolonged the survival of the cells. Recently, Figueroa et al²³ reported

that most peripheral blood EOS express both the mRNA and the protein of the cysLT₁ receptor. These reports and our present observations suggest that cysLTs not only contribute to the development of airway smooth muscle constriction but also directly modify the functional properties of EOS via the cysLT₁ receptor.

These findings also have relevance to the benefit of leukotriene antagonists in the treatment of asthma. The clinical efficacy of these drugs might be attributed, at least in part, to the inhibition of EOS adhesion, chemotaxis, or prolonged survival in the airways.

Our results provide new insights into the mechanisms that regulate the β_2 integrins and the activation of EOS in the airways. When activated, a variety of inflammatory cells is capable of generating cysLTs at sites of allergic inflammation, and therefore EOS are probably exposed to LTD₄. Meanwhile, LTD₄ enhances the interaction between EOS and ICAM-1, which is constitutively expressed on airway epithelium. Interaction with ICAM-1 enhances the effector functions of EOS, including the release of specific granule proteins, respiratory burst, and generation of cysLTs,¹⁴ and the exposure of EOS to these products in autocrine and/or paracrine fashion probably modifies the functions of the cells. Major basic protein, a specific granule protein of EOS, induces the degranulation and IL-8 production of EOS.²⁴ An oxygen metabolite, hydrogen peroxide, augments both the expression of ICAM-1 on endothelial cells²⁵ and EOS adhesion to ICAM-1.¹⁵ Finally, the chemotactic response,⁴ the enhanced survival,⁵ and the interaction between ICAM-1 and the EOS would be augmented by the newly generated cysLTs. LTD₄ can thus modify the adhesion and other β_2 integrin-dependent effector functions of EOS via its interaction with ICAM-1, and it might thereby contribute to the phenotypic change of EOS in the airways. These effects would contribute to the eventual manifestation of allergic diseases such as allergic rhinitis and asthma.

We thank Ms Akemi Yokote for her excellent technical assistance.

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