

5-Oxo-6,8,11,14-eicosatetraenoic acid induces the infiltration of granulocytes into human skin

Shigeo Muro, MD, PhD,^a Outayba Hamid, MD, PhD,^a Ronald Olivenstein, MD,^a Rame Taha, MD,^a Joshua Rokach, PhD,^b and William S. Powell, PhD^a Montreal, Quebec, Canada, and Melbourne, Fla

Background: 5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) is an arachidonic acid metabolite with potent *in vitro* chemoattractant effects on eosinophils and neutrophils. It has also been shown to induce pulmonary eosinophilia in Brown Norway rats, but it is not known whether it is active in human beings *in vivo*.

Objective: To determine whether 5-oxo-ETE can induce cellular infiltration in patients with atopic asthma and nonatopic control subjects after intradermal administration.

Methods: 5-Oxo-ETE was administered intradermally to 11 patients with atopic asthma and 10 nonatopic control subjects. Skin biopsy specimens were taken 6 or 24 hours later and examined by immunocytochemistry for cells expressing specific markers for eosinophils (major basic protein), neutrophils (elastase), macrophages (CD68), lymphocytes (CD3), and mast cells (tryptase).

Results: 5-Oxo-ETE (1.5 and 5 μ g) elicited the infiltration of both eosinophils and neutrophils into the skin in both control and atopic asthmatic subjects. Increased numbers of eosinophils were observed at 6 and 24 hours after injection, whereas significantly elevated neutrophil numbers were present only after 24 hours. Eosinophils were >3 times higher in patients with atopic asthma compared with control subjects after injection of the highest dose of 5-oxo-ETE. Macrophage numbers were also elevated, but only at the highest dose of 5-oxo-ETE. No effects were observed on the numbers of either lymphocytes or mast cells.

Conclusions: 5-Oxo-ETE elicits the infiltration of eosinophils and neutrophils into the skin of human beings *in vivo* after intradermal administration. Asthmatic subjects are more responsive to this substance than nonallergic control subjects.

These results suggest that 5-oxo-ETE may be an important mediator of inflammation. (*J Allergy Clin Immunol* 2003;112:768-74.)

Key words: Eicosanoids, 5-oxo-ETE, inflammation, asthma, skin, eosinophils, neutrophils, immunocytochemistry

Inflammation is characterized by the infiltration of leukocytes into the involved tissue. Eosinophils are very prominent in the lungs in asthma, whereas high levels of neutrophils occur in chronic obstructive pulmonary disease. Mobilization of these cells is dependent on their release into the circulation from the bone marrow under the influence of cytokines and their accumulation in tissues in response to locally released chemoattractants. There are two main classes of chemoattractants: peptide chemokines and lipid mediators. Among the chemokines, IL-8¹ is a potent chemotactic factor for neutrophils, whereas eotaxin is selective for eosinophils and basophils.² RANTES is also a potent chemoattractant, but it has a broader selectivity.³ In the case of lipid mediators, platelet-activating factor (PAF) and the 5-lipoxygenase product leukotriene B₄ (LTB₄) are potent neutrophil chemoattractants,^{4,5} whereas another 5-lipoxygenase product, 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) has similar effects but is less potent.⁶ In contrast, 5-oxo-ETE is the most active lipid mediator in inducing a chemotactic response in human eosinophils,⁷ followed by PAF and prostaglandin D₂, which acts by the DP₂ receptor/CRTH2.^{8,9}

5-Oxo-ETE is a metabolite of arachidonic acid that is formed by the oxidation of the 5-lipoxygenase product 5S-hydroxy-6,8,11,14-eicosatetraenoic acid (5S-HETE). The enzyme responsible for this reaction, 5-hydroxy-eicosanoid dehydrogenase, is highly selective for 5S-HETE and is found in a variety of inflammatory cells including, among others, neutrophils¹⁰ and eosinophils.⁷ In addition, 5-oxo-ETE can be formed during lipid peroxidation and in mouse macrophages from 5S-hydroperoxy-6,8,11,14-eicosatetraenoic acid.¹¹ 5-Oxo-ETE is a potent activator of eosinophils¹²⁻¹⁵ and neutrophils,⁶ inducing a variety of responses, including cell migration, calcium mobilization, actin polymerization, adhesion molecule expression, and degranulation. These responses are mediated by a G-protein-coupled receptor,^{16,17} which has recently been cloned.^{18,19} This receptor is

From ^aMeakins-Christie Laboratories, Department of Medicine, McGill University, Montreal, Quebec, Canada, and ^bClaude Pepper Institute and Department of Chemistry, Florida Institute of Technology, Melbourne, Fla. Received for publication February 28, 2003; revised June 23, 2003; accepted for publication July 1, 2003.

Reprint requests: Dr William S. Powell, Meakins-Christie Laboratories, Department of Medicine, McGill University, 3626 St Urbain St, Montreal, Quebec, Canada H2X 2P2.

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TABLE I. Profiles of asthmatic and nonasthmatic control subjects

	n	Male /female	Age (y)	Total serum IgE (IU/mL)	Periph eos (cells/ μ L)	Skin test
Nonasthmatic	10	8/2	34 $\pm$ 6.1	54 $\pm$ 30	85 $\pm$ 30	All -ve
Asthmatic	11	8/3	37 $\pm$ 7.2	168 $\pm$ 72	270 $\pm$ 78	All +ve

The skin test was a prick test for 12 common allergens.
Periph eos, Peripheral eosinophils.

Abbreviations used

APAAP: Alkaline phosphatase-antialkaline phosphatase
cys-LT: Cysteinyl-LT
LT: Leukotriene
MBP: Major basic protein
PAF: Platelet-activating factor
5-Oxo-ETE: 5-Oxo-6,8,11,14-eicosatetraenoic acid
5S-HETE: 5S-hydroxy-6,8,11,14-eicosatetraenoic acid

highly selective for 5-oxo-ETE, as its precursor, 5S-HETE, and several of its metabolites have 100 to 1000 times lower potency.¹⁶

In addition to its *in vitro* effects on granulocytes, intratracheal administration of 5-oxo-ETE to Brown Norway rats elicits pulmonary eosinophilia.²⁰ However, the effects of 5-oxo-ETE on the infiltration of other inflammatory cells were not investigated in this study. There is as yet no information on the *in vivo* effects of 5-oxo-ETE on inflammatory cell infiltration in human beings. To address this question, we investigated the effects of intradermal injection of this substance on the accumulation of leukocytes in the skin of human subjects.

METHODS

Subjects

Eleven subjects with mild atopic asthma, recruited from the Montreal Chest Hospital, and 10 healthy control subjects were entered into the study. Subject characteristics are summarized in Table I. Ethical approval was obtained from the Montreal Chest Institute Research Ethics Board. Written informed consent was obtained from all subjects. Asthmatic subjects fulfilled the ATS criteria for asthma.²¹ They had documented airway reversibility to inhaled β_2 -agonists, increased airway responsiveness to methacholine (PC₂₀ <8 mg/mL), and were all atopic, based on skin prick test reactions to 12 common allergens. Asthma symptoms were controlled only by on-demand use of inhaled selective β_2 -agonists. None of the subjects had taken oral or inhaled corticosteroids or leukotriene antagonists during the preceding 12 weeks. Asthmatic patients were nonsmokers without any other serious lung disease. Control subjects were nonsmokers, nonatopic, based on negative skin prick test reactions as described above, and had not taken any corticosteroids during the year preceding the study

Preparation of 5-oxo-ETE

5-Oxo-ETE was synthesized chemically²² and purified before use by high-performance liquid chromatography. Aliquots containing the required amounts were evaporated to dryness, and the 5-oxo-ETE was dissolved in ethanol (5 μ L) and sterile saline (195 μ L) under sterile conditions. The vehicle was prepared in an identical fashion.

Intradermal injections and skin biopsy specimens

Each subject received intradermal injections of vehicle (100 μ L) and vehicle (100 μ L) containing 2 doses of 5-oxo-ETE at 3 separate sites in the forearm. Injection sites were marked with a surgical pen, and punch biopsies (4-mm diameter) were performed either 6 or 24 hours later under local anesthesia with 1% lidocaine without epinephrine. Biopsy specimens were immediately snap-frozen in isopentane and cooled in liquid nitrogen. The specimens were then cut with a cryostat in 5- μ m sections, fixed in acetone/methanol, and stored at -20°C until further processed for immunocytochemistry.

Study design

Asthmatic (n = 8) or control (n = 7) subjects were injected intradermally in the forearm with vehicle and 2 doses of 5-oxo-ETE (either 0.3 and 1.5 μ g or 1.5 and 5 μ g per site). After 24 hours, punch biopsy samples were taken as described above. Alternatively, asthmatic or control subjects (n = 3 for each group) were injected with vehicle and 2 doses (0.3 and 1.5 μ g) of 5-oxo-ETE, and biopsy specimens were obtained after 6 hours.

Antibodies

Sections were immunostained with the following antibodies: anti-human CD3 (T-cells; Becton Dickinson, Mississauga, Ontario, Canada); anti-human CD68 (macrophages; Becton Dickinson); anti-human neutrophil elastase (neutrophils; Dako, Glostrup, Denmark); anti-human major basic protein (MBP) (eosinophils; a generous gift from Dr Redwan Moqbel, University of Alberta); anti-human tryptase (mast cells; Chemicon International Inc, Temecula, Calif).

Immunocytochemistry

To identify the inflammatory cells in the biopsy specimens, immunostaining was performed with the use of the alkaline phosphatase-antialkaline phosphatase (APAAP) technique as previously described.²³ Briefly, sections were incubated overnight at 4°C with the primary monoclonal antibody, followed by treatment with rabbit anti-mouse immunoglobulin as a secondary antibody and then APAAP. The reaction was visualized with fast red. For negative control preparations, the primary antibody was replaced by either nonspecific immunoglobulin or Tris-buffered saline solution.

Quantification and Data Analysis

Slides were coded and counted blindly by using $\times 200$ magnification with an eyepiece graticule. All of the immunoreactive cells were counted in a minimum of 3 fields for each section, and the results were expressed as averages of the numbers of positive cells per mm² in the entire area of each of the fields. All results are expressed as mean $\pm$ SEM. The statistical significance of differences was determined through the use of analysis of variance followed by the Student-Newman-Keuls test. A value of *P* < .05 was considered statistically significant.

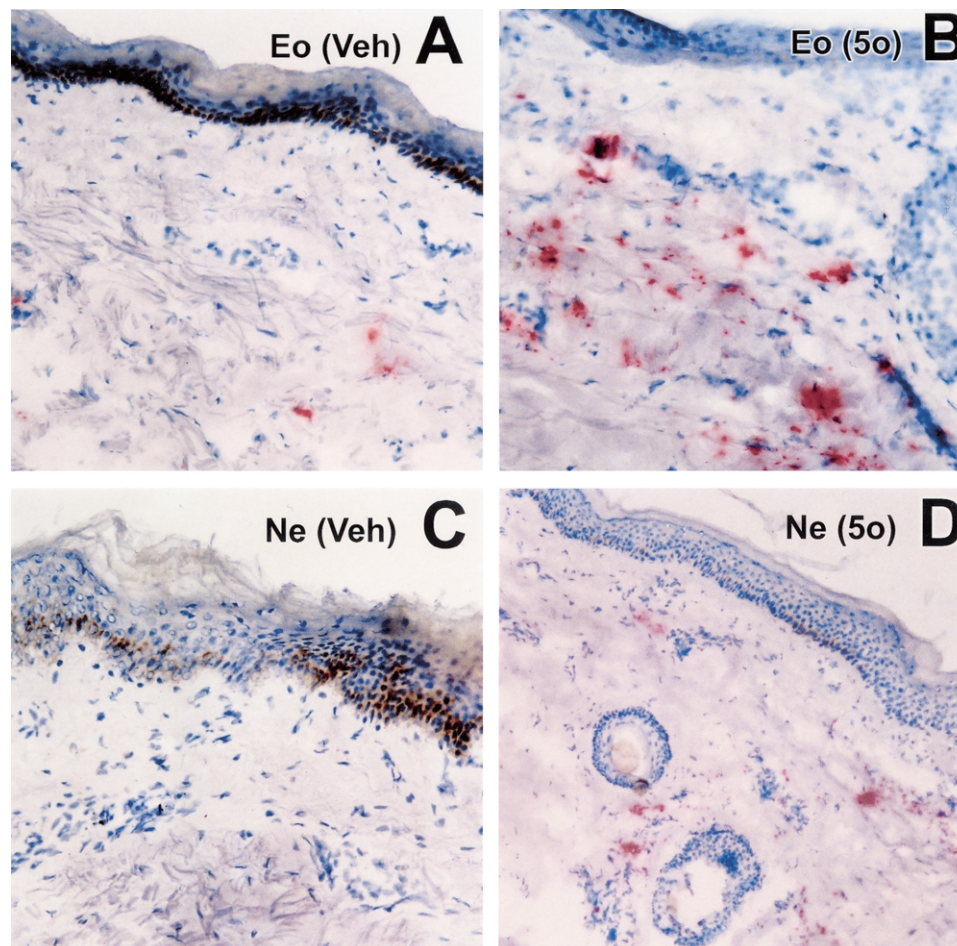


FIG 1. Representative immunocytochemical staining for eosinophil (*Eo*) and neutrophil (*Ne*) markers in skin biopsy sections from an asthmatic subject. Sections from sites treated with vehicle (*Veh*; **A, C**) or 5-oxo-ETE (*5o*; 5 μ g) (**B, D**) were stained with an antibody to either MBP (**A, B**) or neutrophil elastase (**C, D**).

RESULTS

The effects of 5-oxo-ETE on leukocyte infiltration into the skin of atopic asthmatics and nonatopic control subjects, defined as shown in Table I, was investigated. Injection of 5-oxo-ETE did not result in any observable changes to the surface of the skin in any of the subjects in the study. There was no detectable wheal-and-flare response or induration at the injection site. Neither was there any pain or itching during the 24-hour period after injection of either 5-oxo-ETE or saline. Both wounds healed normally and equally well after the punch biopsy, and there was no long-lasting effect of either 5-oxo-ETE or saline.

Inflammatory cells in skin from vehicle-treated control and asthmatic subjects

The numbers of eosinophils, neutrophils, lymphocytes, macrophages, and mast cells in skin biopsy specimens from vehicle-treated nonasthmatic and asthmatic subjects were determined on the basis of immunostaining, as shown in Table II. The numbers of eosinophils, neutrophils, and lymphocytes were similar in control and asthmatic subjects 24 hours after injection of vehicle

(Table II). Although the numbers of macrophages and mast cells tended to be higher in asthmatic subjects, these differences were not significant.

5-Oxo-ETE induces eosinophil infiltration into the skin

Intradermal injection of 5-oxo-ETE (5 μ g) into an asthmatic subject resulted in the infiltration of eosinophils into the skin, as revealed by MBP staining of sections of biopsy specimens obtained 24 hours later (Fig 1, B). Although we did not measure either the percentages of cells that showed extracellular staining or the areas of extracellular MBP immunoreactivity, this was clearly present around some of the positively staining cells, indicative of degranulation. In contrast, very few eosinophils were present after injection of vehicle alone into the same subject (Fig 1, A). This response was highly reproducible among individuals and was greater in asthmatic subjects than in control subjects. Twenty-four hours after vehicle treatment, the density of eosinophils was $0.5 \pm .03$ and 0.3 ± 0.3 cells/mm² in control and asthmatic subjects, respectively. Injection of 5-oxo-ETE (5

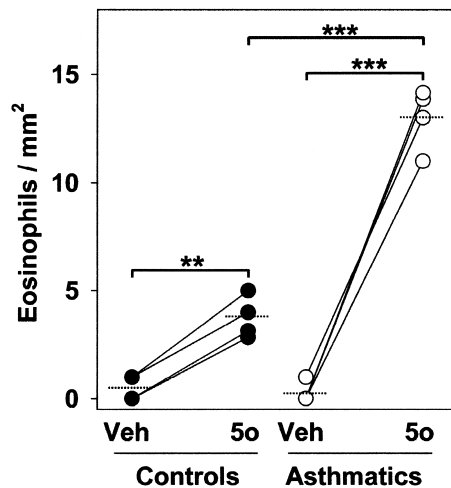


FIG 2. Effects of 5-oxo-ETE on eosinophil numbers in skin of asthmatic and control subjects. Vehicle (Veh) or 5-oxo-ETE (5o; 5 μ g) were injected intradermally into either control (●) or asthmatic (○) subjects, and the numbers of MBP positive cells (ie, eosinophils) in sections from biopsy specimens taken 24 hours later were determined by immunocytochemistry. Mean values are indicated by dashed lines. ** $P < .01$; *** $P < .001$.

μ g) resulted in an increase to 3.8 ± 0.5 cells/mm² in control subjects ($P < .01$) and to 13 ± 0.7 cells/mm² in asthmatic subjects ($P < .001$) (Fig 2).

The effect of 5-oxo-ETE on eosinophil infiltration after 24 hours was dose-dependent (Fig 3, A). Eosinophil numbers tended to rise after injection of the lowest dose (0.3 μ g), but this was not significant, perhaps because of the limited numbers of subjects tested at this dose. At a dose of 1.5 μ g 5-oxo-ETE, the numbers of eosinophils were substantially higher than observed after injection of vehicle alone for both control and asthmatic subjects ($P < .01$ and $P < .001$, respectively). No further increase was observed in control subjects treated with a higher dose (5 μ g) of 5-oxo-ETE, whereas the numbers of eosinophils in asthmatic subjects increased dramatically to approximately 3.5 times the value for control subjects treated with this dose of 5-oxo-ETE ($P < .001$).

Effects of 5-oxo-ETE on neutrophil infiltration

5-Oxo-ETE also induced the infiltration of neutrophils into the skin 24 hours after intradermal injection (Fig 1, D) compared with injection of vehicle alone (Fig 1, C). Neutrophil numbers reached near-maximal levels at the lowest dose of 5-oxo-ETE and then plateaued (Fig 3, B). The numbers of these cells were significantly higher compared with vehicle-treated sites in asthmatic subjects at all three doses tested, whereas in control subjects, neutrophils were significantly higher ($P < .005$) only at the highest dose. However, there were no significant differences in neutrophil numbers between control and asthmatic subjects at any of the doses tested.

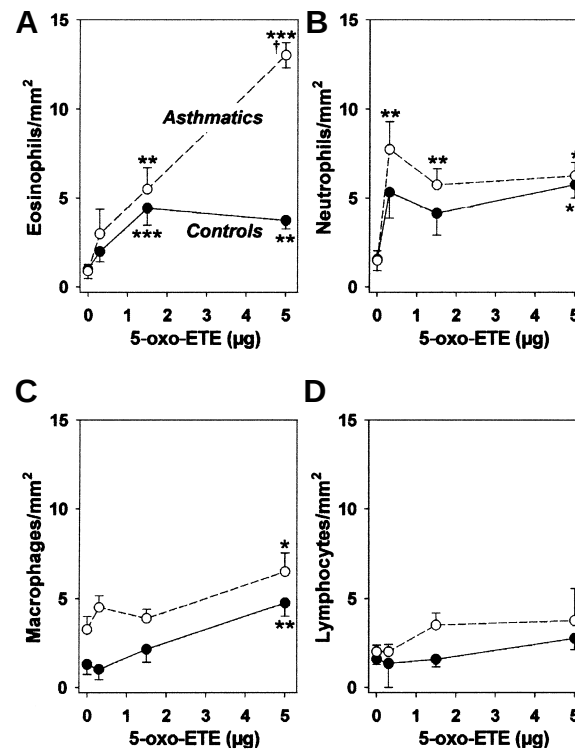


FIG 3. Dose-response curves for effects of 5-oxo-ETE on the numbers of inflammatory cells in biopsy sections obtained 24 hours later from control nonatopic subjects (●) and subjects with atopic asthma (○). Doses of 5-oxo-ETE used were 0.3 μ g ($n = 3$ for control subjects and $n = 4$ for asthmatic subjects); 0 (ie, vehicle) and 1.5 μ g ($n = 7$ for control subjects and $n = 8$ for asthmatic subjects); and 5 μ g ($n = 4$). Significant differences are indicated as follows: * $P < .05$; ** $P < .01$; *** $P < .001$ when compared with vehicle-injected sites; † $P < .001$ when compared with nonatopic control subjects injected with 5-oxo-ETE (5 μ g).

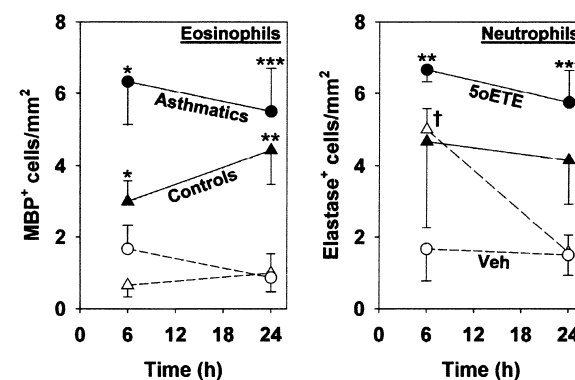


FIG 4. Time courses for effects of 5-oxo-ETE on the numbers of eosinophils and neutrophils in skin biopsy specimens. 5-Oxo-ETE (5oETE; 1.5 μ g; ●, ▲) or vehicle (Veh; ○, △) were injected intradermally in asthmatic (●, ○) or control (▲, △) subjects; numbers of MBP-positive and neutrophil elastase-positive cells were determined by immunocytochemistry in sections obtained either 6 hours ($n = 3$) or 24 hours (control subjects, $n = 7$; asthmatic subjects, $n = 8$) later. Significant differences are indicated as follows: * $P < .05$; ** $P < .01$; *** $P < .005$ when compared with vehicle-injected sites at the same time point; † $P < .005$ when compared with vehicle-treated control subjects at 24 hours.

TABLE II. Numbers of inflammatory cells in sections from punch biopsy specimens 24 hours after injection of vehicle

Cell type	Eosinophils	Neutrophils	Lymphocytes	Macrophages	Mast cells
Marker	MBP	Elastase	CD3	CD68	Tryptase
Nonasthmatic	1.0 ± 0.5	1.6 ± 0.5	1.6 ± 0.3	1.3 ± 0.6	3.3 ± 1.4
Asthmatic	0.9 ± 0.4	1.5 ± 0.6	2.0 ± 0.4	3.3 ± 0.7	7.9 ± 2.5

Cell numbers (shown as cells/mm² ± SEM; n = 7 for control subjects and n = 8 for asthmatic subjects) were determined by immunocytochemistry as described in Methods section.

Effects of 5-oxo-ETE on other inflammatory cells

5-Oxo-ETE also elicited increased numbers of dermal macrophages in both asthmatic and control subjects, but only at the highest dose (Fig 3, C). Overall, the numbers of macrophages were higher in skin from asthmatic subjects. 5-Oxo-ETE did not induce any significant infiltration of lymphocytes into the skin at any of the doses tested, nor were there significant differences between control and asthmatic subjects (Fig 3, D). The numbers of mast cells were also similar in biopsy specimens from vehicle and 5-oxo-ETE-treated sites (data not shown).

Time course for effects of 5-oxo-ETE on granulocyte infiltration

The effects of 5-oxo-ETE (1.5 µg) on leukocyte infiltration were also investigated in a limited number of subjects (n = 3) at an earlier time point (6 hours). The numbers of eosinophils were higher after injection of 5-oxo-ETE than after vehicle in both asthmatic and control subjects at both 6 and 24 hours (Fig 4, A). However, there were no significant differences between the two time points. Neutrophil numbers were higher after injection of 5-oxo-ETE at both time points in asthmatic subjects but not in control subjects and appeared to be elevated at 6 hours compared with 24 hours after injection of vehicle alone (*P* < 0.005) (Fig 4, B).

DISCUSSION

The current study shows that 5-oxo-ETE induces the infiltration of both eosinophils and neutrophils into human skin. This is the first demonstration that 5-oxo-ETE is active in vivo in human beings and suggests that it may also be active at other sites, such as the lungs. This would be consistent with our earlier finding that intratracheal administration of 5-oxo-ETE induces pulmonary eosinophilia in Brown Norway rats.²⁰ The effect of 5-oxo-ETE on the infiltration of neutrophils into the skin of human subjects is in agreement with in vitro experiments demonstrating that it is a potent activator of these cells, inducing chemotaxis and a variety of other responses.^{6,24} Elevated eosinophil numbers in response to 5-oxo-ETE were observed in both control and asthmatic subjects at the earliest time point tested, whereas in the case of neutrophils, this was true only for asthmatic subjects. 5-Oxo-ETE appeared to induce some eosinophil degranulation, as indicated by diffuse staining around some of the MBP⁺

cells (Fig 1, A). It also induces eosinophil degranulation in vitro, particularly in the presence of GM-CSF.¹³

5-Oxo-ETE also had a weak stimulatory effect on the numbers of macrophages in the skin, but this effect was significant only at the highest concentration tested. It has been reported to have a modest chemoattractant effect on human monocytes in vitro and to enhance their response to MCP-1. However, it is clearly a more limited activator of monocytes in vitro, as it fails to induce calcium mobilization²⁵ and CD11b expression (unpublished results), in contrast to its stimulatory effects on these responses in neutrophils and eosinophils.

We did not find any evidence for an effect of 5-oxo-ETE on the numbers of lymphocytes or mast cells in the skin. However, we did not investigate its effects on basophils in the present study. We recently found that 5-oxo-ETE can elicit certain responses in these cells in vitro, but it is not as potent as fMLP and does not induce histamine release (unpublished results). 5-Oxo-ETE did not cause a wheal-and-flare response, as reported after injection of PAF,²⁶ LTD₄,²⁷ and eotaxin.²⁸ Neither was there any induration, as observed for LTB₄.²⁷

The effects of 5-oxo-ETE on eosinophil infiltration contrasts with those of other lipid mediators. LTB₄ clearly elicits a robust influx of neutrophils into the skin of human subjects, but it has little effect on eosinophil infiltration,²⁹ although in some other species, including the guinea pig³⁰ and the rat,³¹ it is a potent eosinophil chemoattractant. PAF is a chemoattractant for human eosinophils in vitro³² but is considerably less active than 5-oxo-ETE.^{7,14} However, PAF was reported to induce little or no influx of these cells following intracutaneous injection in nonatopic subjects, in contrast to atopic subjects, in whom an intense infiltration of eosinophils was observed.³³ Cys-LT₁ receptor antagonists have been shown to reduce airway eosinophils in asthmatic subjects, suggesting that LTD₄ or other cysteinyl-LTs are involved in the recruitment of these cells.³⁴ Inhalation of LTE₄ has been reported to induce eosinophil infiltration into the lungs of asthmatic subjects.³⁵ However, conflicting result have been obtained after inhalation of LTD₄ by human subjects,^{36,37} and LTD₄ did not elicit recruitment of eosinophils in Brown Norway rats after intratracheal administration.²⁰ Moreover, LTD₄ was not noted to induce eosinophil infiltration after intracutaneous injection²⁷ and has only very weak activity in an in vitro chemotaxis assay.⁷ It would seem likely that the effect of cys-LTs on eosinophil infiltration is indirect, perhaps mediated by stimulation of the production of

cytokines such as IL-5.³⁸ The response to intradermal injection of 5-oxo-EETE thus differs from those to LTB₄ and PAF, the two other lipid mediators most often associated with leukocyte migration and infiltration. This is consistent with previous findings indicating that 5-oxo-EETE acts independently of these substances, through its own unique receptor.^{6,17,20}

Among the chemokines, RANTES,³⁹ eotaxin,²⁸ and eotaxin-2²⁸ have all been shown to elicit eosinophil infiltration into the skin. RANTES was selective for eosinophils over neutrophils, but, unlike 5-oxo-EETE, also induced the influx of mononuclear cells, primarily lymphocytes.³⁹ Like 5-oxo-EETE, RANTES did not induce a wheal-and-flare response,³⁹ in contrast to eotaxin, which induced a transitory response.²⁸ As was the case for 5-oxo-EETE, eotaxin stimulated the infiltration of both eosinophils and neutrophils but not lymphocytes.²⁸ The effect of eotaxin on neutrophil infiltration was presumably indirect, possibly mediated by release of IL-8.²⁸ Although we did not directly compare the effects of 5-oxo-EETE with those of any of the above chemokines, the numbers of eosinophils in skin biopsy specimens after chemokine treatment were reported to be higher than the numbers found in the current study. It is possible that this may have been due to methodological differences in the counting procedures, although we cannot exclude the possibility that RANTES and eotaxin are more efficacious chemoattractant molecules *in vivo* compared with 5-oxo-EETE. However, we found that *in vitro*, 5-oxo-EETE has, if anything, greater efficacy than these chemokines in stimulating eosinophil migration.⁴⁰

Although asthmatic and control subjects responded similarly to lower doses of 5-oxo-EETE, eosinophil recruitment was much greater in asthmatic subjects at the highest dose tested. This may have been related to the higher numbers of circulating eosinophils in the asthmatic subjects (Table II) or, alternatively, eosinophils from asthmatic subjects could be more responsive to 5-oxo-EETE, as cytokines such as GM-CSF have been shown to markedly enhance their activation by this lipid mediator.¹³ Similarly, asthmatic subjects are also more responsive than control subjects to PAF³³ and RANTES³⁹ with respect to eosinophil recruitment.

Because there are no 5-oxo-EETE receptor antagonists or selective synthesis inhibitors, it is difficult to evaluate its physiological role in allergic responses. Nevertheless, the stimulatory effects of 5-oxo-EETE on eosinophil migration, both *in vitro* and *in vivo*, suggest that it may be an important physiological mediator in diseases such as asthma that are characterized by tissue eosinophilia. It would seem unlikely that any one chemoattractant is responsible on its own for eosinophil recruitment in these conditions. Whereas various strategies aimed at blocking CCR3 receptor-mediated responses inhibit eosinophil recruitment in allergic models, including the skin,^{41,42} the same is true for blocking the formation of 5-LO products, either by gene disruption⁴³ or by the use of enzyme inhibitors in human beings⁴⁴ and other species.⁴⁵ There is evidence implicating a 5-LO product other than LTB₄

and cys-LTs in pulmonary eosinophilia induced by Sephadex beads⁴⁶ and allergen⁴⁷ in animal models, suggesting that 5-oxo-EETE may play a role in these responses. Furthermore, we have shown that synergistic interactions occur between 5-oxo-EETE and both PAF⁷ and CC chemokines.⁴⁰ Since 5-LO products, PAF, and chemokines are all released after allergen challenge, interactions among these different mediators may be important in eliciting eosinophil infiltration in allergic diseases. Thus, it would appear that both CC chemokines and lipid mediators, in particular 5-oxo-EETE, are important for eosinophil recruitment to inflammatory sites.

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