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Lipid mediators from pollen act as chemoattractants and activators of polymorphonuclear granulocytes

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Background: Under natural exposure conditions, pollen grains function as allergen carriers that release allergens from internal binding sites on contact with the aqueous phase of mucosa membranes. In addition, we recently demonstrated that pollen are a rich source of eicosanoid-like mediators, which are rapidly released on contact with the aqueous phase.
Objective: The current study was designed to characterize the biochemical nature of pollen-derived lipid mediators in more detail and to delineate their biologic activity on polymorphonuclear granulocytes (PMNs).
Methods: Aqueous and lipid extracts from *Phleum pratense* L and *Betula alba* L pollen were analyzed by means of HPLC. PMNs were exposed to aqueous extracts or lipid fractions from pollen or to HPLC-purified lipid mediators identified in pollen extracts. Effects on PMNs were tested with transwell migration, calcium mobilization, and surface expression of CD11b.
Results: Aqueous pollen extracts (APEs) contained predominantly monohydroxylated products derived of linoleic acid and linolenic acid. In chemotaxis assays PMNs displayed significant migration to APEs. Lipid extracts from pollen and the HPLC fraction containing 13-hydroxy-octadecadienoic acid/hydroxy-linoleic acid and 13-hydroxy-octadecatrienoic acid/hydroxy-linolenic acid induced migratory responses, although to a lesser degree than the APEs. In addition, APE, as well as lipid, extracts induced PMN activation, as documented by means of calcium mobilization and upregulation of CD11b.
Conclusion: Pollen grains release mediators that recruit and activate PMNs in vitro. Similar mechanisms may be effective in vivo, suggesting that pollen-derived lipid mediators may act as adjuvants in the elicitation phase of allergic reactions.

Key words: *Chemotaxis, polymorphonuclear granulocyte, pollen, allergy, oxylipins*

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Abbreviations used
APE: Aqueous pollen extract
HIP: Hexan-isopropanol
HODE: Hydroxy-octadecadienoic acid/hydroxy-linoleic acid
HOTE: Hydroxy-octadecatrienoic acid/hydroxy-linolenic acid
LT: Leukotriene
PMN: Polymorphonuclear granulocyte
RP: Reverse phase

The importance of grass pollen as a potent allergen source was established almost 130 years ago, when Blackley[1] performed provocation tests and documented the relationship between pollen exposure and allergic symptoms. Apart from pollen as an allergen carrier,[2] we recently demonstrated that pollen grains, on contact with the aqueous phase, also liberate eicosanoid-like substances, as determined by means of cross-reactivity in an ELISA for leukotriene (LT) B_4 and prostaglandin E_2.[3] Prostaglandins and LTs are metabolites of arachidonic acid, which is not present in birch and grass pollen.[4] Other unsaturated fatty acids, such as linoleic acid and linolenic acid, are major components of membrane fatty acids in plants.[4,5] Recently, oxidized derivatives of linoleic acid have been identified in a variety of cells, and these metabolites have been shown to exhibit a number of biologic functions, including modulation of cell proliferation, apoptosis, and inflammation.[6-8]

Even though the precise role of polymorphonuclear granulocytes (PMNs) in the pathophysiology of allergic diseases is still a matter of debate, there is a general consensus that PMNs contribute to the manifestation of allergic inflammation. As cells of the innate immune system, PMNs play an important role in a number of allergic diseases.[9-11] In asthma PMNs are among the first cell type to enter the lung after allergen challenge,[12] and in allergic rhinitis local inflammation is associated with an accumulation of PMNs in the nasal tissue.[13,14]

The recruitment of PMNs into tissues is a multistep process that involves selectin-mediated tethering, integrin-mediated adhesion, transendothelial migration, and directed migration along chemotactic gradients through the extracellular matrix to the site of inflammation.[15] Directed migration of PMNs is regulated by a number of chemoattractants, of which there are 2 main types: chemokines and lipid mediators. LTB_4, a metabolite of arachidonic acid, is

the most potent chemoattractant lipid mediator for PMNs described thus far,[16,17] binding to the low-affinity (BLT2) and high-affinity (BLT1) receptor.[18,19]

The present study was designed to characterize the biochemical nature of mediators released from pollen grains and to delineate their biologic activity on PMNs. We demonstrate for the first time that aqueous pollen extracts (APEs) induce a significant migration and activation of PMNs. The observed effects were independent of the donor sensitization status (ie, could be induced in PMNs from allergic, as well as nonallergic, individuals) and could not be triggered by means of stimulation with allergen alone. In addition, we provide evidence that pollen-derived lipid mediators, particularly monohydroxylated linoleic acid derivatives, contribute to the chemotactic and stimulating activity of APEs on PMNs. We hypothesize that the rapid release of bioactive mediators from pollen during contact with the aqueous phase of mucosal or respiratory membranes may act as allergen-independent proinflammatory factors contributing to the manifestation or aggravation of allergic inflammation.

METHODS

Donors

Patients included in the study had a history of allergic rhinitis, with a positive skin prick test response for grass (n = 3) or birch pollen (n = 9) and with RAST results from the respective allergen of greater than class 3. Healthy control subjects (n = 11) had no history of allergic diseases. In addition, RAST and skin prick test results for 15 common allergens, including birch and grass pollen, were negative, and total IgE was less than 20 IU/mL. All volunteers were without medication for at least 15 days before blood sampling. The ethical committee of the Technical University of Munich approved the study, and volunteers were enrolled in the study after providing written informed consent.

Antibodies and reagents

The mAb anti-CD11b/Mac-1 (clone ICRF44) and the corresponding FITC-labeled isotype control were purchased from Becton Dickinson (San Jose, Calif). LTB_4 was provided by Cayman Chemicals (Ann Arbor, Mich). The selective LTB_4 receptor antagonist LY293111 was kindly provided by Lilly Pharma (Greenfield, Calif).

Pollen grains and generation of APEs

The pollen grains studied (birch, *Betula alba* L; grass, *Phleum pratense* L) were purchased from commercial producers (Allergon, Ängelholm, Sweden) and maintained under dry conditions at 4°C until use. APEs were prepared from incubations of 30 mg/mL pollen for 30 minutes at 37°C in RPMI-1640 medium by means of centrifugation (500*g*) and sterile filtration (0.2 nm). This concentration was chosen because APEs of 34 mg/mL resulted, in the LTB_4 ELISA, in an average concentration of 3.9×10^{-10} mol/L LTB_4, which is known to be the concentration to induce migration in PMNs.

Hexan-isopropanol extraction of pollen and HPLC analysis

Oxidized fatty acids (oxylipins) were extracted according to the method of Weichert et al.[20] In case of the structural analysis of oxylipins, 0.5 g of pollen and, for preparative isolation of oxylipins, 2 g of pollen were added to 30 mL of extraction medium (isohexane-isopropanol 3:2 by volume with 0.0025% wt/vol butyl hydroxy toluol), and 150 µL of 1 mol/L HCl was added and immediately homogenized with an Ultra Turrax for 30 seconds. The extract was centrifuged for 10 minutes at 4500*g* at 4°C. The supernatant was collected, and the pellet was extracted an additional 3 times with 3 mL of extraction medium. To the combined organic phases, a 6.7% (wt/vol) solution of potassium sulfate was added to a total volume of 47 mL, and the upper hexane-rich layer was removed. The upper organic phase containing the oxidized fatty acid derivatives was dried, redissolved in 1 mL of methanol/water/acetic acid (85:15:0.1 by volume), and stored in argon at –80°C until analysis.

HPLC analysis was carried out on an Agilent 1100 HPLC system coupled to a diode array detector. At first, oxylipins were purified on reversed-phase (RP) HPLC. This was carried out either on an ET250/2 Nucleosil 120-5 C18 column (Macherey-Nagel, 2.1 × 250 mm, 5-µm particle size) for analytic purposes or on an ET250/2 Nucleosil 250-5 C18 column (Macherey-Nagel, 4.6 × 250 mm, 5-µm particle size) for preparative purposes with a solvent system of methanol/water/acetic acid (85:15:0.1) and a flow rate of 0.18 mL · min^{-1}. Straight-phase HPLC of the hydro(pero)xy fatty acids was carried out on a Zorbax Rx-SIL column (Agilent, 150 × 2.1 mm, 5-µm particle size), with a solvent system of *n*-hexane/isopropanol/acetic acid (100:1:0.1) and a flow rate of 0.1 mL · min^{-1}. Chiral-phase HPLC of the hydro(pero)xy fatty acids was carried out on a Chiralcel OD-H column (Daicel, 150 × 2.1 mm, 5-µm particle size) with a solvent system of *n*-hexane/isopropanol/acetic acid (100:5:0.1) and a flow rate of 0.1 mL · min^{-1}. Absorbance of conjugated diene systems at 234 nm was monitored. As an internal standard, (13S,6Z,9Z,11E)-13-hydroxy-6,9,11-octadecatrienoic acid was used.

Preparation of polymorphonuclear granulocytes

Human polymorphonuclear granulocytes were obtained from atopic and nonatopic volunteers by means of density gradient centrifugation of total blood (Histopaque 1077/1119; Sigma, Deisenhofen, Germany). Polymorphonuclear leukocytes were resuspended at 1×10^6 cells/mL in RPMI-1640 supplemented with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 1% nonessential amino acids (complete RPMI; Life Technologies, Chagrin Falls, Ohio) and kept at room temperature. As analyzed by means of flow cytometry, these cells were 96% pure neutrophils with a vitality of greater than 95% (data not shown). Eosinophil contamination was 2% or less (data not shown).

Migration assay

The chemotactic activity of the substances was evaluated, measuring the neutrophil migration through 5-µm pore polycarbonate filter in 24-well transwell chambers (Corning Costar, Cambridge, Mass), as previously described.[21] Briefly, PMNs were added to the top chamber and suspended in complete RPMI plus 0.5% BSA at 1×10^6 cells/mL. Various concentrations of the different chemokines were added to the bottom chamber of the transwell (0.6 mL). After 1 hour of incubation at 37°C with 5% CO_2, cells that had transmigrated into the lower chamber were recovered and counted with a FACScan (Becton Dickinson, Heidelberg, Germany) for 60 seconds at a flow rate of 60 µL/min. Results are shown as the migration index, which represents the ratio between cells migrated to the lower chamber in the presence of the chemotactic substance and cells migrated in response to the medium alone. When indicated, cells were preincubated with a selective LTB_4 receptor antagonist (LY293111; 40, 400, and 4000 nmol/L), which was kindly provided by Lilly Pharma (Greenfield, Ind) and immediately used for the migration assay.

Checkerboard analysis

Standard checkerboard analysis was performed to analyze chemotactic versus chemokinetic activity of APEs. Briefly, various concentrations of APEs were placed in upper wells, lower wells, or both upper and lower wells of the chemotaxis chamber to determine whether the number of migrated cells was greater with a positive gradient, no gradient, or a negative gradient of APEs.

Measurement of Ca^{2+}

PMNs isolated as described above were adjusted to 5×10^6/mL and loaded with 2 μmol/L fura2/AM (Molecular Probes, Eugene, Ore), according to the instructions of the manufacturer. The calcium flux monitoring was performed on PMNs that were stimulated with APEs (30 mg of pollen/mL), LTB_4 (3.9×10^{-10} mol/L), and lipid mediators from pollen (hexan-isopropanol [HIP] extracts and RP-phase extracts). Furthermore, desensitization experiments were performed by using LTB_4, followed by pollen extracts or vice versa.

Measurement of surface expression of CD11b

PMNs were isolated as indicated above and stimulated with the different stimuli as indicated. Staining with the mAbs for CD11b (Clone ICRF44) was performed, as indicated by the manufacturer and analyzed by means of flow cytometry.

Statistical analysis

The Student paired *t* test was used to compare groups of differently stimulated cells (checkerboard analysis). The unpaired *t* test was used to compare migratory behavior of PMNs of allergic and nonallergic patients. A *P* value of less than .05 was considered significant.

RESULTS

HPLC analysis of pollen extracts

Because we could show that pollens harbor eicosanoid-like substances, we first analyzed total lipid extracts from pollen for their amount of hydro(pero)xy polyenoic fatty acids by means of HPLC analysis (Fig 1). Birch pollen contained substantial amounts of nonesterified linoleic acid (288 nmol/g fresh weight) and linolenic acid (295 nmol/g fresh weight), as quantified with RP-HPLC analysis. Moreover, we observed a strong preponderance of linoleic acid–derived hydroxy fatty acid derivatives (13-hydroxy-octadecadienoic acid [13-HODE], 9.1 nmol/g fresh weight; 9-HODE, 6.3 nmol/g fresh weight; Fig 1, *A*). In contrast, all hydroxy derivatives derived from linolenic acid were less prevalent (1 and 1.6 nmol/g). For grass pollen, we found a different situation (Fig 1, *B*). Again, we detected substantial amounts of nonesterified linoleic acid (821 nmol/g fresh weight) and linolenic acid (3.8 μmol/g fresh weight). In addition, the amount of hydroxy fatty acids was equally distributed between linoleic acid and linolenic acid derivatives, ranging between 20 and 33 nmol/g fresh weight (Fig 1, *B*). Also depicted in Fig 1 (inserts) is the separation of all stereochemical isomers of hydroxy derivatives, either of linoleic acid or of linolenic acid. As described recently, the analysis, either by means of the quantitative distribution of positional isomers (Fig 1, *A* and *B* and inserts) of the hydroxy derivatives of linolenic acid or the stereochemistry of the hydroxy fatty acids formed from all polyenoic fatty acids, are suitable measures to determine whether they are formed by autoxidation or whether they are formed enzymatically (eg, by lipoxygenases).[22] In all cases we found only products derived from autoxidation.

Chemotaxis of PMNs to APEs

Previous results of our group revealed that pollen releases substances that cross-react with antibodies against LTB_4. We were interested whether those LTB_4-like substances would also have LTB_4-like effects, such as induction of chemotaxis. The concentrations of pollen extracts were chosen at a range of 0.034 to 34 mg pollen/mL because 34 mg/mL pollen elicited, in the LTB_4-ELISA, an equivalent concentration of 3.9×10^{-10} mol/L LTB_4, which is known to induce significant migration in PMNs. Interestingly, PMNs revealed an extraordinary capacity to migrate in response to APEs from birch and grass pollen independent from the allergic status of the patient ($P = .87$), pointing to chemotactic mediators released from pollen (Fig 2, *A*). Birch and grass pollen extracts at 34 mg/mL exhibited up to 80% of the chemotactic activity of LTB_4 (3.9×10^{-10} mol/L), suggesting a comparable potency of these LTB_4-like substances in equivalent concentrations. The LTB_4 receptor was involved in chemotaxis because LY293111, an LTB_4 receptor antagonist,[23,24] efficiently blocked migration of PMNs (Fig 2, *B*). At 400 nmol/L LY293111, the migration toward LTB_4 was blocked up to 90%, whereas migration to IL-8 was not affected (Fig 2, *B*). Migration of APEs was blocked up to 70%, indicating that more than one signal pathway is involved in the chemotaxis induced by APEs.

Checkerboard analysis

The results of checkerboard analysis to grass pollen performed in quadruplicate are shown in Table I. Similar results were observed to birch pollen extracts (data not shown). The values along the diagonal, reflecting migratory responses to uniform concentrations of APEs on both sides of the chamber (chemokinesis), are always significantly lower than the values below the diagonal, reflecting responses to a positive gradient (chemotaxis; paired *t* test, $P < .05$). The values above the diagonal represent responses to a negative gradient (chemokinesis) and are also always significantly lower than the values under the diagonal.

Chemotaxis of PMNs in response to lipid mediators from pollen

To further characterize the substances responsible for the migratory behavior of PMNs, pollens were submitted to the HIP procedure for extraction of all lipophilic substances. Taking those HIP extracts that were quantified by means of HPLC for their amount in 13-HODE, we found a significant migration in a concentration range of 1×10^{-10} to 1×10^{-15} mol/L 13-HODE, with a maximum at the concentration of 1×10^{-15} mol/L (Fig 2, *C*), suggesting that lipid mediators from pollen were contribut-

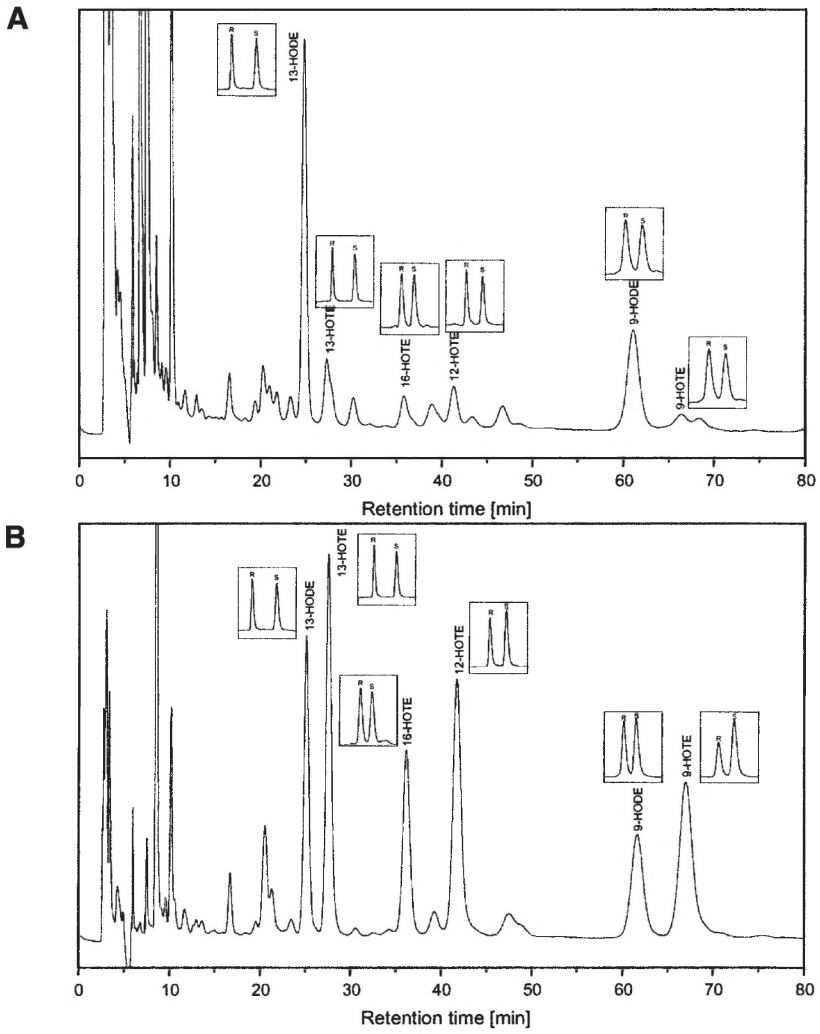

FIG 1. Analysis of polyenoic fatty acid–derived oxylipin formation in pollen lipid extracts. Lipids were extracted, and free oxylipins were isolated by means of RP-HPLC. The regioisomers of hydroxy fatty acids were separated by means of straight-phase HPLC, and the enantiomers were analyzed by means of chiral-phase HPLC. The estimated ratio of the stereoisomers (S/R ratio) of HODEs and HOTEs are shown in the insets: **A,** birch; **B,** grass.

ing to the chemotactic activity of APEs. Taking the fraction from the RP-phase containing primarily 13-HODE and 13-hydroxy-octadecatrienoic acid/hydroxy-linolenic acid (13-HOTE; quantified for the concentration of 13-HODE), we still found chemotaxis of PMNs, even though migratory potency of those extracts was much lower compared with that of APEs (Fig 2, *D*). Furthermore, much higher concentrations (1×10^{-11} mol/L 13-HODE equivalent) were necessary to induce a significant migration. This indicates that the reduction of the chemotactic activity was due to lacking summative effects of multiple activating and chemotactic substances released from pollen.

Pollen extract signaling in PMNs

To address the question of whether pollen-derived mediators participate in receptor-mediated signaling, we measured calcium mobilization in PMNs. Interestingly, they induced a significant calcium release in PMNs, indicating that the induction of chemotaxis was receptor mediated. APEs from grass and birch pollen at 30 mg/mL were approximately equipotent to LTB_4 (3.9×10^{-10}) in mobilizing cytosolic calcium in PMNs (Fig 3, *A-C*). Moreover, HIP extracts, as well as RP-phase (1×10^{-7} mol/L 13-HODE equivalent) substances, were also able to induce calcium flux in PMNs (Fig 3, *F-K*).

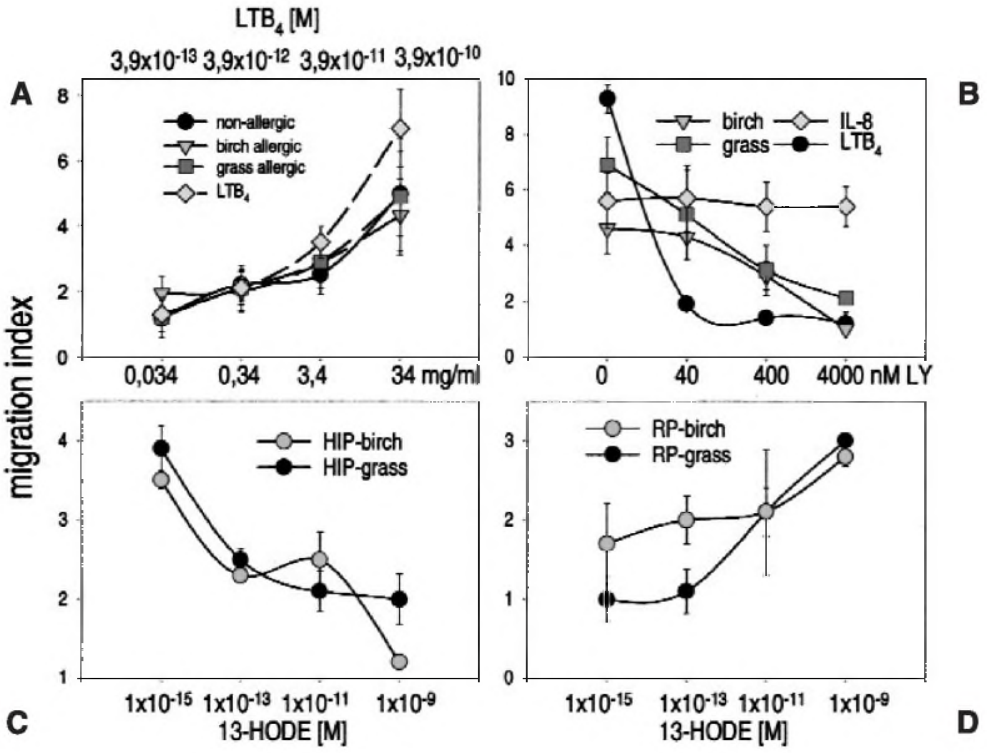

FIG 2. Migratory response of PMN to APEs, lipid mediators from pollen, and LTB_4. **A,** Dose-dependent migration of PMNs to APEs from birch and grass pollen in comparison with migration induced by LTB_4. Data are presented as pooled migration indices from 3 donors with grass allergy, 9 donors with birch allergy, and 11 nonallergic donors (mean ± SEM). **B,** Influence of the LTB_4 receptor antagonist LY293111 on PMN migration. For APEs, one representative experiment of 6 (3 allergic and 3 nonallergic donors) by using PMNs from one allergic donor is shown (mean and range of duplicates). For LTB_4 and IL-8, one representative experiment of 3 (3 allergic donors) is shown. **C** and **D,** PMN migration to lipid extracts of pollen. The mean ± SEM of pooled data from 9 donors (3 nonallergic donors, 3 donors with birch allergy, and 3 donors with grass allergy) is shown.

TABLE I. Checkerboard analysis for APEs from grass pollen

	Upper chamber (mg/mL)				
	0	**0.034**	**0.34**	**3.4**	**34**
Lower chamber (mg/mL)					
0	1 ± 0.01	1.4 ± 0.2	0.8 ± 0.01	0.9 ± 0.1	0.7 ± 0.05
0.034	1.1 ± 0.02	1.5 ± 0.1	1.3 ± 0.2	1.2 ± 0.1	0.8 ± 0.01
0.34	1.2 ± 0.02	1.1 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	0.6 ± 0.1
3.4	2.1 ± 0.1	2.0 ± 0.2	1.8 ± 0.2	1.8 ± 0.01	1.2 ± 0.01
34	5.6 ± 0.3	4.6 ± 0.2	5.6 ± 0.1	5.0 ± 0.1	2.7 ± 0.1

Values represent means ± SD of calculated chemotactic indices from one experiment performed in quadruplicate by using the transwell migration assay, as indicated in the "Methods" section.

Because an LTB_4 receptor antagonist could inhibit the migration to pollen extracts, we performed cross-desensitization studies stimulating first with LTB_4 followed by pollen extracts and vice versa. Interestingly, the responsiveness of PMNs to LTB_4 was lost on prior incubation with APEs from grass and birch pollen (Fig 3, *B* and *C*). Similarly, APEs induced only weak calcium fluxes in PMNs that had previously been exposed to LTB_4 (Fig 3, *D* and *E*). These results strongly suggest that LTB_4 receptor agonists released by pollen contribute to trigger functional responses in PMNs.

APEs upregulate CD11b on PMNs

As a well-established parameter of PMN activation, we analyzed CD11b upregulation after stimulation with aqueous and lipid pollen extracts in comparison with

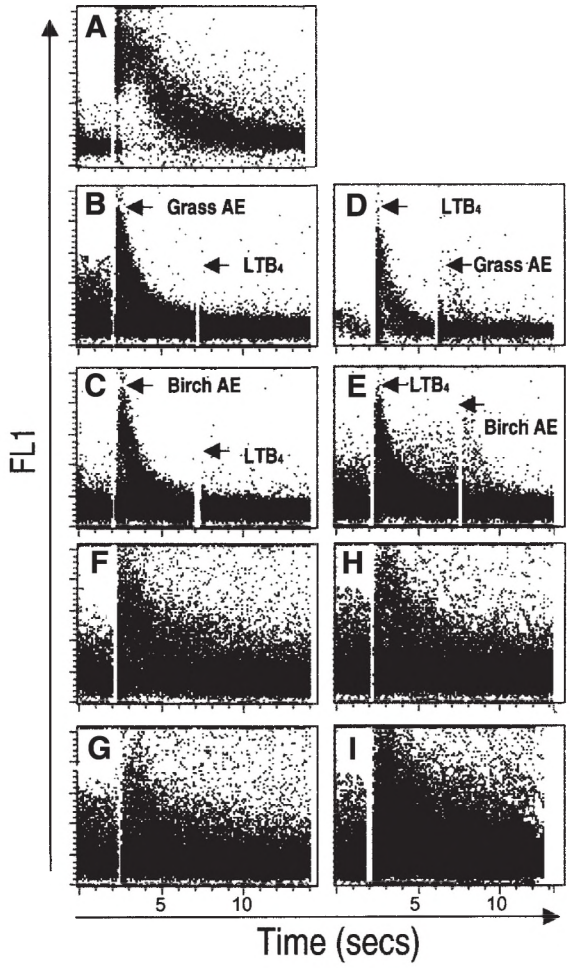

FIG 3. Intracellular calcium flux induced in PMNs by pollen extracts and lipid mediators. **A,** LTB_4 (3.9×10^{-8} mol/L). **B** and **C,** Aqueous extracts *(AE)* from grass pollen and birch pollen, respectively. **F** through **I,** Calcium mobilization in PMNs by lipid extracts from pollen: HIP extracts from birch pollen **(F);** RP-phase from birch pollen **(G);** HIP extracts from grass pollen **(H);** and RP-phase from grass pollen **(I)**. For cross-sensitization experiments, PMNs were first stimulated with APEs of pollen, followed by LTB_4 (**B** and **C**) or vice versa (**D** and **E**). Shown is 1 representative experiment (donor with grass allergy) of 6 performed with PMNs from different donors: 2 from donors with birch allergy, 2 from donors with grass allergy, and 2 from nonallergic donors.

LTB_4. Freshly isolated PMNs were resuspended in RPMI complete plus 10% FBS, and stimulators were added in the concentrations indicated. Flow cytometry was used to assess CD11b expression from 5 to 60 minutes and 2 and 12 hours after stimulation. In Fig 4 the expression after 1 hour of stimulation is shown.

APEs of birch pollen were slightly but not significantly more potent compared with those in grass pollen in inducing CD11b (Fig 4, *B* and *C*). APEs and LTB_4 (3.9×10^{-10} mol/L) were comparably potent in inducing CD11b expression. Even after 12 hours, a high expression of CD11b, usually only transiently expressed, as described by Davey et al,[25] was observed after stimulation with APEs (data not shown). HIP extracts and the RP-phase from both grass (Fig 4, *E* and *F*) and birch pollen (data not shown) showed upregulation of CD11b, pointing to the importance of the lipid ingredients in pollen for the observed effects. The allergen rPhl p 5, itself in concentrations comparable with the concentration in aqueous extracts,[3] did not induce any upregulation of CD11b (Fig 4, *D*).

DISCUSSION

Our recent data point to pollen as a rich source of bioactive mediators, which are rapidly released on contact with an aqueous phase.[3] Here we show that mediators released by pollen (birch and grass) activate and recruit PMNs in vitro. Pollen-derived neutrophil chemotactic activity may be attributable to one or more lipids that are functionally and structurally closely related to LTs. Pollen extracts contained substantial amounts of linolenic acid–derived hydroxy fatty acid derivatives, namely 13-HODE and 13-HOTE, which lead to activation and migration of PMNs.

Neutrophil extravasation into the interstitium at the site of inflammation is a prerequisite for PMN defense function. This is initiated in response to lipid mediators and chemokines by means of selectin-dependent PMN rolling along the endothelial lining, followed by firm PMN adhesion and transendothelial diapedesis.[26] Our finding of LTB_4-like substances in APEs prompted us to further investigate the effect of those substances on PMNs.

LTB_4 activates and recruits granulocytes into inflamed lesions.[17,19] Furthermore, data have accumulated on the contribution of LTB_4 in inflammatory disorders,[27,28] including bronchial asthma and allergic diseases.[29,30] Mice lacking LT production are deficient in their response to some acute and chronic inflammatory stimuli.[31,32] Therefore LTB_4 receptor antagonists are under development as potent anti-inflammatory and antiallergic drugs.[23,33]

Leukotactic activity of pollen-derived (birch and grass) neutrophil chemotaxins was found to be of similar efficacy as that of well-characterized chemotaxins, such as LTB_4 and IL-8 (Fig 2). All migrating cells can be activated by certain inflammatory mediators without the induction of directional migration but increase the random movement of cells as a result of cell activation. Cells exposed to such primers typically show signs of enhanced oxidative metabolism and upregulate the expression of receptors, so that the response to bona fide chemotactic factors and the interaction with adhesion molecules on endothelial cells are enhanced.[34] In addition to chemotactic responses, APEs also provoke chemokinetic PMN responses (Table I). Although the chemokinetic index is significantly lower than that of chemotaxis and occurs only at high concentrations (30 mg/mL), optimal stimulation for both chemotaxis and chemokinesis occurs at the same concentration of APEs known also for other chemokines.[35]

APEs contain considerable amounts of minor and major allergens.[3] Thus it seemed possible that the

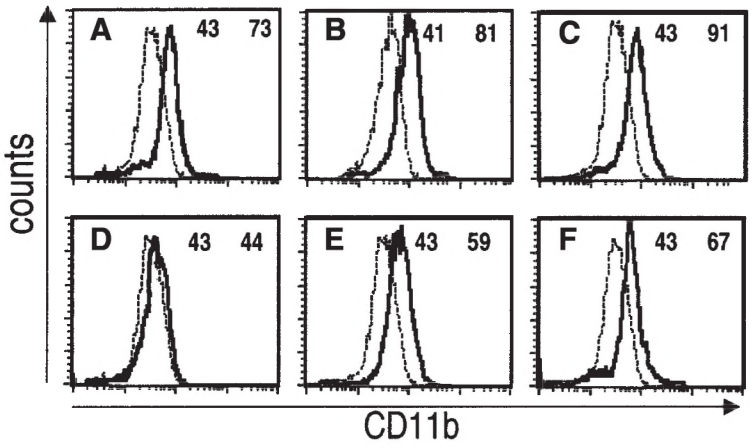

FIG 4. Upregulation of CD11b on PMNs by APEs and lipid mediators from pollen. **A-F,** Histograms of CD11b expression 1 hour after stimulation with 3.9×10^{-9} mol/L LTB_4 **(A)**, APE 34 mg of grass pollen/mL **(B)**, APE 34 mg of birch pollen/mL **(C)**, 50 μg/mL rPhl p 5 **(D)**, HIP birch pollen extracts **(E)**, and RP-phase from birch pollen **(F)**. *Numbers* indicate net mean fluorescence intensity: *left,* unstimulated control; *right,* stimulated probe. *Dotted lines* indicate unstimulated control, and *solid lines* indicate stimulated probes. The experiment stands for 6 similar experiments performed from PMNs of 2 donors with grass allergy, 2 donors with birch allergy, and 2 nonallergic donors.

observed effects were induced by allergen-specific mechanisms. Aside from low- and high-affinity receptors for IgG, it was reported recently that PMNs express different receptors for IgE: the heterotrimeric high-affinity receptor for IgE (FcεRI)[36-38]; the low-affinity receptor for IgE (FcεRII/CD23)[39]; and the Mac-2/IgE–binding protein.[40] However, the occurrence of chemotaxis in both allergic and nonallergic individuals makes a specific IgE-mediated mechanism unlikely. Additionally, we performed migration assays with recombinant Phl p 5, the major grass pollen allergen from *Phleum pratense* L, in concentrations comparable with those found in APEs.[3] Here neither PMN chemotaxis nor chemokinesis could be observed, suggesting that the APE-induced migration was indeed mediated through allergen-independent mechanisms. We could also demonstrate that the migration to APEs could be inhibited by an LTB_4-receptor antagonist (LY293111). This suggests that the LTB_4 receptor is involved in the induction of migration. Recent data on hydroxyeicosanoids binding to the low-affinity LTB_4 receptor (BLT2)[41] demonstrated that this receptor is rather promiscuous, binding to a broader spectrum of ligands similar to LTB_4.

The concept of LTB_4 receptor involvement in the induction of migration was supported by cross-desensitization experiments. The responsiveness of PMNs to LTB_4 by means of calcium mobilization was lost after prior desensitization with APEs. This indicates that the LTB_4 receptor was already engaged after stimulation with pollen extracts.

Full activation of PMNs is dependent on the expression of adhesion receptors of the β2-integrin family, which is known to be critically involved in the interaction with the endothelium during cell recruitment into inflamed tissue. The upregulation of a β2-integrin by APEs and lipid components of pollen indicates that these substances not only act as chemoattractants but also as agonists of PMN activation.

In conclusion, we provide evidence that pollen grains release mediators that recruit and activate PMNs in vitro. It is tempting to speculate that similar mechanisms may be effective in vivo. Our observations suggest that rapid release of bioactive lipid mediators from pollen during contact with the aqueous phase of mucosal or respiratory membranes may act as allergen-independent proinflammatory factors, contributing to the manifestation or aggravation of allergic inflammation.

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