# Chemotaxis and activation of human peripheral blood eosinophils induced by pollen-associated lipid mediators

**Sabine G. Plötz, MD,[a]* Claudia Traidl-Hoffmann, MD,[a]* Ivo Feussner, MD,[b] Anna Kasche, MD,[a] Alexandra Feser,[a] Johannes Ring, MD, PhD,[c] Thilo Jakob, MD,[a,c] and Heidrun Behrendt, MD[a]** *Munich and Göttingen, Germany*

**Background: Eosinophil accumulation at sites of allergic inflammation is largely regulated by chemokines and lipid mediators released by a variety of cells of the local microenvironment. Recent studies have shown that pollen grains, apart from their function as allergen carriers, are a rich exogenous source of eicosanoid-like lipid mediators that are rapidly released on contact with the aqueous phase and thus may contribute to the generation of local inflammatory responses.**

**Objective: Here we analyze the biological activity of pollen-associated lipid mediators (PALMs) on peripheral human blood eosinophils.**

**Methods: Human eosinophils were coincubated with pollen grains and analyzed by electron microscopy. The lipid mediator composition of aqueous pollen extracts (APEs) was analyzed by HPLC. Human eosinophils were exposed to APEs or lipid fractions from pollen. Effects on eosinophils were tested by transwell migration and surface expression of CD11b.**

**Results: *In vitro* experiments showed adhesion of eosinophils to *Phleum pratense* pollen. In chemotaxis assays eosinophils displayed significant directed migration to APEs. HPLC analysis of APEs from *Phleum pratense* and *Betula alba* pollen demonstrated the occurrence of linoleic and α-linolenic acid as well as their monohydroxylated derivatives. Moreover, total lipid extracts from pollen and RP-HPLC fractions containing monohydroxylated derivatives of linoleic and α-linolenic acid induced similar migratory responses, although to a lesser degree than APEs. In addition, APEs and lipid extracts induced up-regulation of CD11b surface expression and secretion of eosinophil cationic protein. APE-induced chemotaxis was blocked by the leukotriene $B_4$ receptor antagonist LY293111, suggesting that PALMs may serve as ligands for $LTB_4$ receptors.**

**Conclusion: Pollen grains release lipid mediators that recruit and activate eosinophils in vitro. Similar mechanisms may be effective under natural exposure conditions, in which PALMs may play a role in the recruitment of eosinophils to the site of allergic inflammation.**

***Key words:*** *Eosinophil granulocytes, chemotaxis, pollen, lipid mediators*

Eosinophils are important effector cells of many immunologic events including IgE-mediated allergic inflammation.[1-3] Accumulation of eosinophils at the site of tissue inflammation is the result of a multistep cascade that involves tethering and rolling along the endothelium, cell activation and firm adhesion to the endothelium, and finally transendothelial migration. The majority of these steps are guided by a number of chemoattractants released from epithelial, endothelial, and other cells in the local microenvironment.[4] Two main types of chemoattractants are involved, chemokines (eg, eotaxin, binding to the CCR3 receptor) and lipid mediators (eg, arachidonic acid derivatives like leukotriene $B_4$ [$LTB_4$] acting through various membrane receptors). Linoleic as well as α-linolenic acid structurally resemble arachidonic acid and can, as arachidonic acid, easily be converted to hydroxylated derivates either enzymatically or via auto-oxidation.[5,6] Recently, oxidized derivates of linoleic acid have been shown to exhibit a number of biological functions such as modulation of cell proliferation, apoptosis, and inflammation.[7-9] They have also been shown to be potent chemoattractants and activators of human polymorphonuclear granulocytes.[10]

Although it is generally accepted that chemoattractants are generated by activated cells of the local microenvironment, little attention has been paid so far to exogenous sources of these mediators. Recent reports about the effects of parasite-derived prostaglandin $E_2$ in schistosomiasis infection[11] and the existence of dermatophyte-derived lipid mediators inducing granulocyte migration[12] suggest that exogenous lipid mediators may also modulate local immune responses in the host.

Anemophilous pollen grains are the male gametophyte in gymnosperms that are wind dispersed and transported over wide areas. Under natural exposure conditions pollen grains also function as allergen carriers that liberate

From [a]the Division of Environmental Dermatology and Allergy GSF/TUM, Center for Allergy and Environment, Technical University, Munich; [b]Department of Plant Biochemistry, Albrecht-von-Haller Institute of Plant Sciences, Georg-August-University, Göttingen; and [c]Department of Dermatology and Allergy, Technical University Munich.

*Both authors contributed equally to this work.

Supported in part by a grant from BStMLU. S.G.P. is supported by the Hochschulwissenschaftsprogramm III of the Technical University of Munich (HWPIII, TUM). C.T.-H. is supported by the Bayerische Habilitationsförderpreis.

Reprint requests: Heidrun Behrendt, MD, Division of Environmental Dermatology and Allergy GSF/TUM, Center for Allergy and Environment, Technical University Munich, Biedersteiner Str. 29, 80802 Munich, Germany. E-mail: heidrun.behrendt@lrz.tum.de.

*Abbreviations used*
APE: Aqueous pollen extract
ECP: Eosinophil cationic protein
HIP: Hexane isopropanol
HODE: Hydroxy octadecadienoic acid
HOTE: Hydroxy octadecatrienoic acid
$LTB_4$: Leukotriene $B_4$
PALM: Pollen-associated lipid mediator

allergens from internal binding sites under humid conditions[13] and is responsible for induction, maintenance, and elicitation of IgE-mediated allergic diseases.[14] We recently demonstrated that airborne pollen also releases substantial amounts of eicosanoid-like substances as determined by cross-reactivity in $LTB_4$ and prostaglandin $E_2$ ELISA.[15] Because arachidonic acid has been shown to be absent in pollen,[6] large amounts of other unsaturated fatty acids and their derivates that are abundant in plants (eg, linoleic and linolenic acid[16]) may account for the observed cross-reactivity. Indeed, pollen grains from birch and grass contain large amounts of linoleic or α-linolenic acid and of their monohydroxylated derivates.[10,16]

Here we demonstrate for the first time that human eosinophils are targets for pollen-associated lipid mediators (PALMs). We conclude that PALMs may contribute to the induction and exacerbation of allergic inflammation via affecting cells of the innate immune system.

## METHODS

### Donors

Patients included in the study had a positive history of allergic rhinitis, a positive skin prick test result for grass (n = 9) and birch pollen (n = 7), and specific serum IgE (RAST classes >3) for the respective allergen. Healthy control subjects (n = 4) had no history of allergic diseases. 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 giving written informed consent. The investigation was conducted according to the Declaration of Helsinki.

### Reagents and antibodies

Unless otherwise indicated, all fine chemicals were purchased from Sigma (Deisenhofen, Germany), Serva (Heidelberg, Germany), or Pharmingen (San Diego, Calif). $LTB_4$, 13-hydroxy linoleic acid (13-HODE), and 13-hydroxy linolenic acid (13-HOTE) were purchased from Cayman Chemical (Ann Arbor, Mich), and the $LTB_4$ receptor antagonist LY293111 was provided by Lilly Pharma (Greenfield, Ind). Fluorescein isothiocyanate—labeled mouse anti-human CD11b/Mac-1 (clone ICRF44), the corresponding isotype control, and eotaxin were purchased from Becton Dickinson (San Jose, Calif). Anti-CD16 mAb microbeads were from Miltenyi Biotec (Bergisch-Gladbach, Germany).

### Generation of aqueous pollen extracts

Pollen grains (*Phleum pratense* L, *Betula alba* L) were obtained from commercial producers (Allergon, Ängelholm, Sweden) and kept under dry conditions at 4°C until use. Pollen grains in increasing concentrations were incubated in RPMI medium (30 minutes, 37°C at pH 7.4), centrifuged (500*g*), and filtered sterile (0.2-mm filters). These aqueous pollen extracts (APEs) were used for stimulation and migration studies.

### Partial purification of chemotactic factors from pollen by hexan-isopropanol extraction and HPLC

Oxidized fatty acids (oxylipins) were extracted according to the method of Weichert et al[17] as already published.[10] Analysis of APE was carried out by HPLC on an Agilent (Waldbronn, Germany) 1100 HPLC system coupled to a diode array detector (Agilent) for analysis of oxylipins. Thus oxylipins were purified on RP-HPLC. This was carried out on an ET250/2 Nucleosil 120-5 C18 column (Macherey-Nagel, Dueren, Germany; 2.1 × 250 mm, 5-μm particle size) with a solvent system of methanol/water/acetic acid (85:15:0.1) and a flow rate of 0.18 mL/min. Straight phase—HPLC of the hydro(pero)xyfatty 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/2-propanol/acetic acid (100:1:0.1) and a flow rate of 0.1 mL/min. The conjugated double bond system at 234 nm was recorded, and oxylipins were identified by authentic standards. Nonoxygenated fatty acids within APE were analyzed as methyl esters by gas chromatography. Gas chromatography analysis was performed with an Agilent GC 6890 system coupled with an FID detector equipped with a capillary HP INNOWAX column (30 m × 0.32 mm, 0.5-μm coating thickness; Agilent). Helium was used as carrier gas (30 cm × sec). The samples were measured with a split of 20:1 with an injector temperature of 220°C. The temperature gradient was 150°C for 1 minute, 150°C to 200°C at 15°C/min, 200°C to 250°C at 2°C/min, and 250°C for 10 minutes. Fatty acids were identified by authentic standards.

### Eosinophil purification

Eosinophils were isolated as described elsewhere.[18] Total blood was separated by density gradient centrifugation with Histopaque 1077/1119 (Sigma). $CD16^+$ neutrophils were depleted by passing the granulocytes through a magnetic cell separation system (Miltenyi Biotec). Eosinophils were resuspended at $10^6$ cells/mL in RPMI 1640 medium supplemented with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids (all from Life Technologies, Chagrin Falls, Ohio; complete RPMI). As analyzed by flow cytometry, these cells were >96% pure eosinophils with a viability of >95 % (data not shown).

### Morphologic studies

Adhesion of eosinophils to pollen grains was assessed after coincubation of $1 \times 10^6$ eosinophils and $1 \times 10^5$ grass pollen grains (*Phleum pratense* L; Allergon, Uppsala, Sweden) per mL RPMI 1640 for 30 minutes up to 2 hours at 37°C. Cytospin preparations were stained with Pappenheim's stain, and the number of eosinophils attached to pollen was counted (300 pollen per sample; n = 5). For scanning electron microscopy, samples were fixed with 2% cacodylate buffered glutaraldehyde, pH 7.2, post-fixed with osmium tetroxide, dehydrated in graded series of ethanol, and air-dried with hexamethyldisilizane[19] (Sigma). After sputtering with gold in a Balzers SCD050 (Liechtenstein) sputter machine, the samples were mounted and investigated in a JEOL 6300 SEM (Jeol, Tokyo, Japan) scanning electron microscope with 20 kV and tilt angle of 15 to 45 degrees.

### Chemotaxis assay

The chemotactic property of the substances was evaluated by measuring eosinophil migration through a 5-μm pore polycarbonate filter in 24-well transwell chambers (Corning Costar, Cambridge, Mass) as previously described.[10] Briefly, $10^5$ eosinophils suspended in 100 μL RPMI plus 0.5% BSA were added to the top chamber. Various

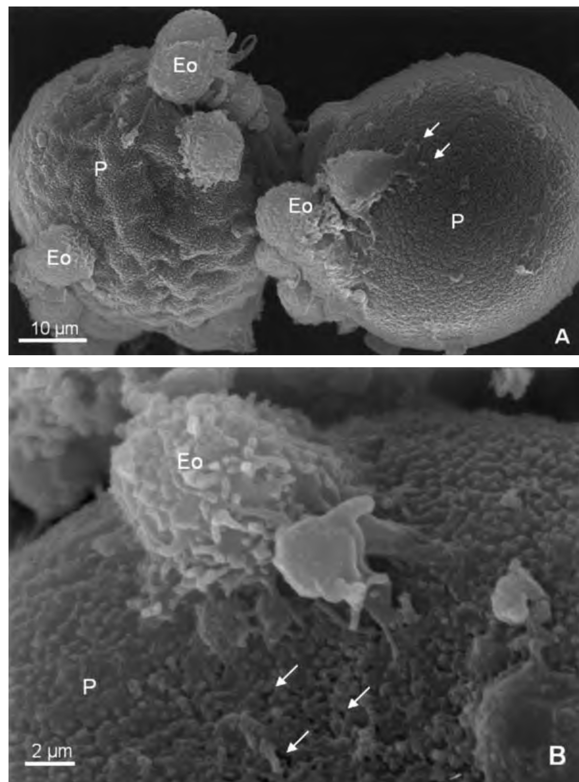


**FIG 1.** Adherence of eosinophil granulocytes to pollen grains. Scanning electron micrographs of *in vitro* interaction between *Phleum pratense* pollen *(P)* and isolated human peripheral blood eosinophils *(Eo)* as seen in coculture experiments ($1 \times 10^6$/mL eosinophils plus $1 \times 10^5$/mL pollen per mL RPMI). **A,** After 30 minutes of coculture the cells show adherence to pollen. Some cells seem to spread over the surface and display cytoplasmic extensions and leading lamellae *(arrows)*. **B,** After 90 minutes of coculture alterations of pollen surface become visible *(arrows)* in the vicinity of an eosinophil adhering to the exine.

concentrations of the different chemokines or APE were added to the bottom chamber of the transwell (600 μL). After 90 minutes of incubation at 37°C with 5% $CO_2$, cells transmigrated into the lower chamber were recovered and counted by flow cytometry (FACS Calibur; Becton Dickinson, Heidelberg, Germany) for 60 seconds at a flow rate of 60 μL/min. In blocking experiments, eosinophils were pretreated with a selective $LTB_4$ receptor antagonist (LY293111) at different concentrations (0.4-40 μmol/L) for 15 minutes on ice and additional 15 minutes at 37°C. After incubation, cells were immediately tested in the migration assay in the presence of the antagonist.

The chemotactic activity of APE was expressed as migration index, which is defined as the ratio of stimulated migration of eosinophils in the presence of the chemoattractant and the migration of nonstimulated eosinophils to the medium control. To analyze chemotactic versus chemokinetic activity of APE, standard checkerboard analysis was performed. Briefly, various concentrations of APE 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 APE.

## Eosinophil CD11b surface expression

Eosinophils ($1 \times 10^5$ cells in 100 μL) were isolated as indicated above and then incubated with eotaxin or APE in increasing concentrations for 30 minutes. Cells were washed twice and stained at 4°C with mAb against CD11b (clone ICRF44) and the appropriate isotype control as indicated by the manufacturer. All data were corrected for the value obtained for the corresponding isotype control and expressed as net mean fluorescent intensity.

## Eosinophil cationic protein release

Eosinophils were maintained in RPMI 1640 medium (Life Technologies, Chagrin Falls, Ohio) supplemented with 0.5% BSA. For stimulation experiments, eosinophils ($1 \times 10^6$/mL) were preincubated with GM-CSF (50 ng/mL) for 20 minutes and then incubated with aqueous and lipid grass and birch pollen extracts or the allergen rPhl p5b for 60 minutes at 37°C (under 5% $CO_2$) in U-bottom microtiter plates (Greiner, Nürtingen, Germany). After incubation, supernatants were harvested and analyzed for eosinophil cationic protein (ECP) content by using the Pharmacia UniCAP System for ECP (Pharmacia, Freiburg, Germany) as described by the manufacturer.

## Statistical analysis

Student paired *t* test was used to compare groups of differently stimulated cells (checkerboard analysis and different stimuli). A *P* value less than .05 was considered significant.

# RESULTS

## Adherence of human eosinophil granulocytes to pollen grains

Scanning electron micrographs of coculture experiments of $1 \times 10^6$ eosinophils coincubated with $1 \times 10^5$ grass pollen grains per mL medium revealed that eosinophils strongly attach to pollen grain surfaces (Fig 1). These eosinophils form extended lamellopodia and exhibit degranulation. Morphometric analysis of 300 pollen per sample indicated that an average attachment of 2.2 ± 0.78 eosinophils per pollen grants from atopic donors (n = 5) attached to pollen grains. As shown by scanning electron microscopy, the exines of the pollen were markedly altered in the vicinity of eosinophil attachment, indicating toxic activity of eosinophils or their products on pollen surfaces (Fig 1, *B*). Indeed, in supernatants of eosinophils coincubated with pollen grains, ECP was detected in higher concentrations compared with medium-incubated eosinophils (21.3 ± 13.2 vs 12.9 ± 8.4 μg/L).

## APEs stimulate human eosinophil chemotaxis

To analyze the crosstalk of pollen grains and eosinophils, we investigated the impact of soluble pollen mediators on eosinophil effector functions. Migratory response of eosinophils to APE was tested in transwell chambers as indicated above. Eosinophils exhibit a strong capacity to migrate toward APE in a dose-dependent manner (Fig 2, *A*). Migratory response was similar for birch or grass pollen−derived APE. In addition, no difference in APE-induced eosinophil migration between cells from atopic or nonatopic donors was detected, pointing to a more general biological mechanism independent from the sensitization status of the donor (data not shown). Furthermore, recombinant *Phleum p 5b* in concentrations comparable with those found in APE did not induce eosinophil migration (migration index below 1.1 for 10, 30, and 60 μg *rPhl p 5b*, respectively).

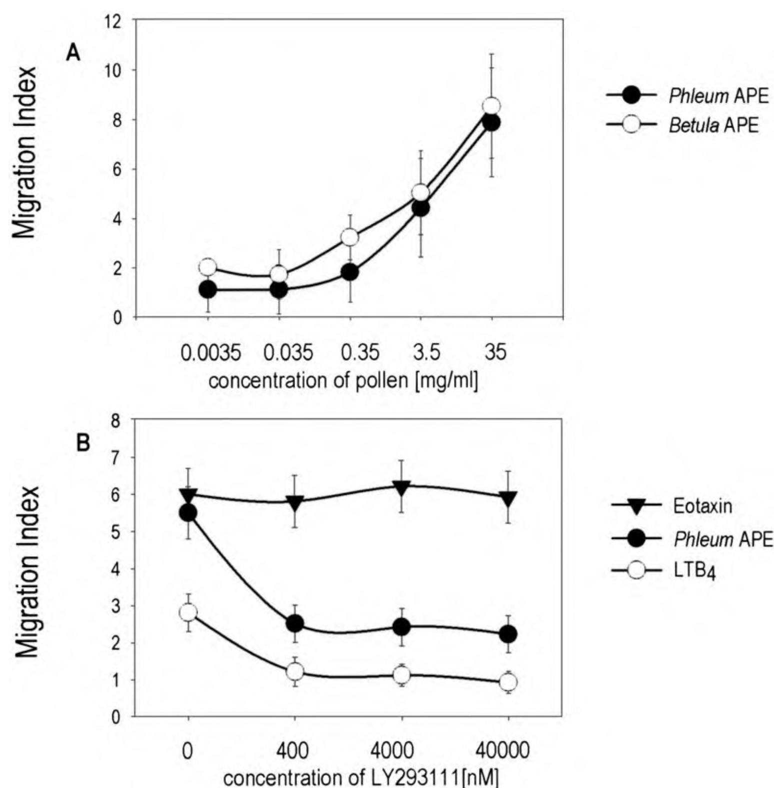


**FIG 2.** Migratory response of eosinophils to APEs. **A,** Eosinophils exhibit dose-dependent migration to aqueous extracts from birch and grass pollen. Data are expressed as migration index (number of cells migrated with APE/number of cells migrated with medium alone; means ± SDs) (n = 5 atopic donors). **B,** The selective $LTB_4$ receptor antagonist LY293111 inhibits migration of eosinophils to APE (3.5 mg/mL) and $LTB_4$ in a dose-dependent manner, whereas the migration to eotaxin (200 ng/mL) is not impaired (means ± SEMs, n = 3 atopic donors).

## Checkerboard analysis

To discriminate whether APE-derived locomotory responses are chemotactic or chemokinetic as a result of unspecific cell activation, standard checkerboard analysis was performed.

As shown in Table I, the values along the diagonal, reflecting migratory responses to uniform concentrations of APE on both sides of the chamber (chemokinesis), are significantly lower compared with the values below the diagonal reflecting responses to a positive gradient (chemotaxis) (paired *t* test; $P < .05$). With increasing concentrations of APE on both sides of the chamber, an increased migratory response can be observed that reflects increased chemokinetic activity at higher concentrations. This is also confirmed by values above the diagonal showing migratory responses to a negative gradient (chemokinesis). However, under all conditions the migratory response to a positive gradient was greater than to no or to a negative gradient, confirming the chemotactic nature of the signal.

## Effect of the $LTB_4$ receptor antagonist LY293111 on APE-induced chemotaxis of human eosinophil granulocytes

Because APE contains substances cross-reacting in the $LTB_4$ ELISA,[10] experiments were performed to investigate whether APE-induced migration was $LTB_4$-receptor dependent. Eosinophils ($1 \times 10^6$/mL) were preincubated with increasing concentrations of the $LTB_4$-receptor antagonist LY293111[20] for 15 minutes on ice and additional 15 minutes at 37°C followed by transwell migration studies in the presence of the antagonist. As shown in Fig 2, *B*, LY293111 induced a dose-dependent decrease of migration toward both APE and $LTB_4$ ($3.9 \times 10^9$ nmol/L) as control stimulus. At a concentration of 400 nmol/L LY293111, APE-induced significant migration (migration index greater than 2) was blocked up to 90% (Fig 2, *B*). Preincubation with LY293111 had no effect on eotaxin-stimulated eosinophils, ruling out toxic (nonspecific) effects of the antagonist.

## APEs increase the surface expression of CD11b on human eosinophils

Surface expression of the adhesion molecule CD11b on eosinophils was measured after incubation of the cells with aqueous extracts of grass and birch pollen and eotaxin as control stimulus. As shown in Fig 3, grass as well as birch pollen–derived APE (15 mg/mL) induced significant upregulation of CD11b ($P < .05$), similar to that of eotaxin (40%, 25%, and 35%, respectively, over basal expression). The CD11b upregulation was significantly ($P < .05$)

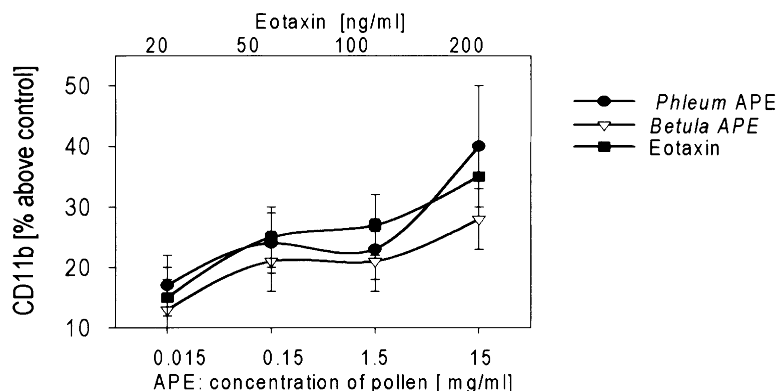


**FIG 3.** Effects of APE and eotaxin on the surface expression of CD11b by eosinophils. Eosinophils ($2 \times 10^5$/100 μL) were incubated for 1 h with birch or grass pollen–derived APE and eotaxin. Results are expressed as percent increases in the mean fluorescent intensities above the control value obtained in the absence of agonist (means ± SEMs, n = 3 atopic donors).

**TABLE I.** Checkerboard analysis for APE from birch pollen

| | Upper chamber (mg/mL) | | | | |
|---|---|---|---|---|---|
| **Lower chamber (mg/mL)** | **0** | **0.034** | **0.34** | **3.4** | **34** |
| 0 | 1 ± 0.01 | 1.4 ± 0.1 | 1.3 ± 0.06 | 3.1 ± 0.2 | 3.0 ± 0.03 |
| 0.034 | 1.2 ± 0.02 | 1.9 ± 0.4 | 1.8 ± 0.05 | 2.8 ± 0.2 | 3.9 ± 0.1 |
| 0.34 | 1.9 ± 0.04 | 2.0 ± 0.2 | 2.1 ± 0.1 | 3.3 ± 0.02 | 3.2 ± 0.9 |
| 3.4 | 3.2 ± 0.2 | 2.6 ± 0.1 | 2.9 ± 0.04 | 4.4 ± 0.5 | 3.6 ± 0.5 |
| 34 | 6.0 ± 0.1 | 7.4 ± 0.2 | 5.9 ± 0.1 | 7.2 ± 0.2 | 6.8 ± 0.1 |

Values represent means ± SEMs of calculated chemotactic indexes from 1 experiment performed in duplicate by using the transwell migration assay. The values along the diagonal, reflecting migratory responses to uniform concentrations of APE on both sides of the chamber (chemokinesis), are always significantly lower compared with the values below the diagonal, reflecting responses to a positive gradient (chemotaxis) (paired *t* test; $P < .05$).

increased as compared with control conditions, starting at a dose of 0.15 mg/mL APE of both birch and grass pollen. The results with 1.5 mg/mL differed significantly from those with 0.015 mg/mL and from control ($P < .05$).

## Identification and partial characterization of eosinophil chemotactic lipids in grass pollen extracts

To identify the compounds that are responsible for eosinophil chemotactic activity, HPLC analysis of APE was performed as recently described.[17] In aqueous extracts from grass pollen, substantial amounts of nonesterified linoleic acid and α-linolenic acid (290 nmol and 1800 nmol per 100 mg pollen, respectively) were found (Fig 4). Among the monohydroxylated products of the unsaturated fatty acids, a preponderance of α-linolenic acid–derived hydroxy fatty acid derivates such as 12-, 13-, 9-, and 16-HOTE was found. Between them, the preponderance of the 12-HOTE indicates that the hydroxy fatty acid isomers are derived from auto-oxidative processes and not from the action of oxidizing enzymes such as lipoxygenases. This observation was further confirmed by determination of the enantiomeric composition of the hydroxy fatty acids; all derivatives turned out to be racemic, indicating an auto-oxidative origin (data not shown).

## Chemotaxis of human eosinophils toward lipid fractions of pollen extracts

In an attempt to further identify the substances responsible for the eosinophil migration, pollen was extracted in hexane isopropanol (HIP) extract and further purified by RP-HPLC as described previously.[10,16] The HIP extract contains total lipophilic substances from pollen. The RP-HPLC (RP extract) merely contains isomers of the hydroxy derivates HODE and HOTE, as recently published.[10] HIP and RP extracts were quantified for the concentration of 13-HODE and expressed in mol/L concentration of 13-HODE equivalent. As shown in Fig 5, HIP extract and to a lesser extent RP extract induced significant migration in eosinophils, indicating that lipid substances from pollen grains are indeed responsible for the observed eosinophil chemotactic activity.

## ECP release in human eosinophils is stimulated by both APE and lipid extracts

As shown in Fig 6, PALMs from grass, but not recombinant phleum p 5b, induced substantial amounts of ECP release in GM-CSF–primed eosinophils. The highest ECP release was observed on stimulation with HIP extract (about 80% of the $LTB_4$-induced release),

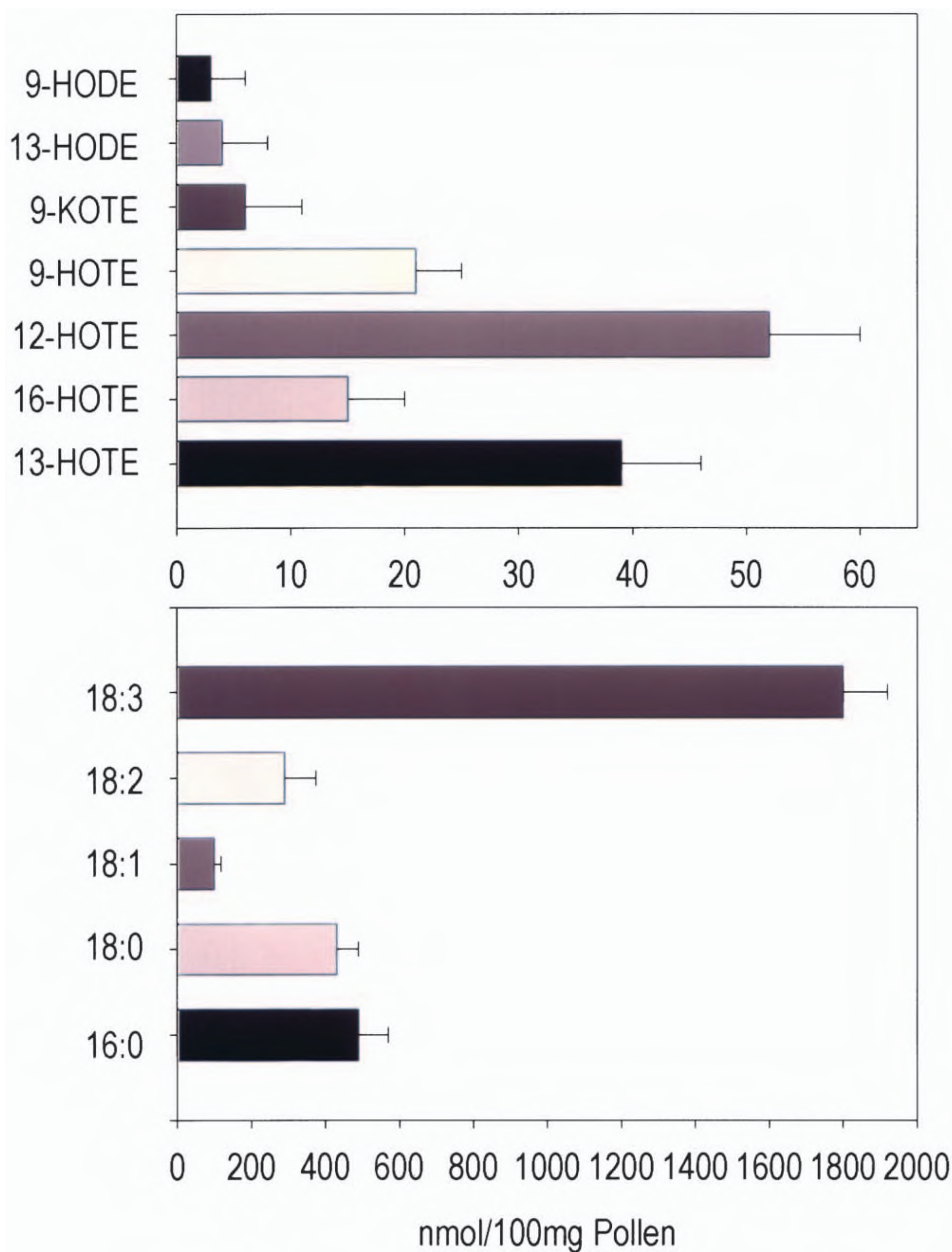


**FIG 4.** HPLC analysis of grass APEs. Grass APEs contained substantial amounts of nonesterified linoleic and linolenic acid. In addition, we observed a preponderance of linoleic acid–derived hydroxy fatty acid derivates (12-HOTE, 13-HOTE, 9-HOTE, and 16-HOTE). Results are presented as means ± SDs of 3 independent experiments.

followed by APE (about 55% of the $LTB_4$-induced release) and RP extract (about 30% of the $LTB_4$ release). These results emphasize the involvement of PALMs in triggering eosinophil effector functions such as ECP release.

## DISCUSSION

Eosinophils acquire the propensity to invade inflamed tissue via the action of numerous well-characterized endogenous mediators present in the tissue microenvironment. Here we demonstrate for the first time the biological activity of exogenous PALMs, particularly hydroxy fatty acid derivatives of linoleic- and α-linolenic-acid, on human eosinophils *in vitro* in terms of recruitment, activation, and mediator release. The results support the hypothesis that migration and activation of eosinophils are not only regulated by the local microenvironment and factors within the host but also by exogenous lipid mediators released by pollen themselves. Therefore, we suggest that

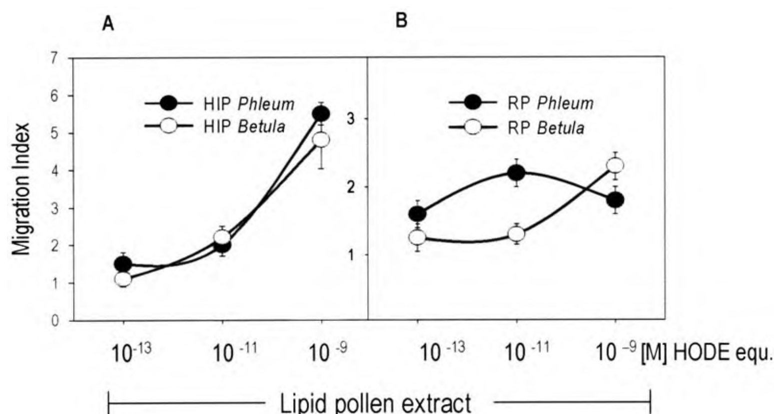


**FIG 5.** Migratory response of eosinophils to lipid extracts of pollen. Both HIP extracts **(A)** and HODEs/HOTEs extracted by RP-HPLC **(B)** from pollen extracts induced significant migration in eosinophils, indicating a chemotactic activity of lipophilic substances isolated from pollen. Means ± SEMs of pooled data from 3 different donors are shown.

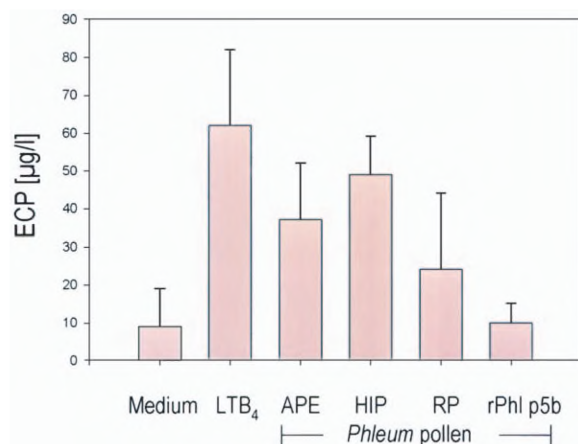


**FIG 6.** ECP release in human peripheral blood eosinophils incubated with APEs and lipid pollen extracts. APE (3.5 mg/mL) as well as lipid extracts (HIP extract and RP-Phase) of grass pollen induced ECP release in isolated human peripheral blood eosinophils. Recombinant Phl p5b (20 µg/mL) did not induce ECP release.

the exposure to PALMs may contribute to the elicitation[21] and aggravation of eosinophil-associated inflammatory reactions. The results are in line with prior observations on human neutrophils from our group.[10] Furthermore, the observed effects seem to reflect a general feature of all airborne pollen so far investigated.

The respiratory tract contains a large number of cell types including epithelial cells and granulocytes such as neutrophils and eosinophils, all of which are virtually capable of interacting with respirable particles including allergen-bound carriers such as pollen. After *in vitro* coincubation of granulocytes and pollen in a serum free medium, adherence between pollen grains and human eosinophils was demonstrated. Adherence was accompanied by degranulation and mediator release from eosinophils as well as alteration of the pollen's surface. These observations extend early studies from Siegel and Sherman,[22] who reported an enhanced adherence of guinea pig white cells to ragweed pollen grains *in vitro* in the presence of serum. Although transferrin was discussed as a serum factor responsible for the induction of adherence of granulocytes to pollen grains,[23] the mechanisms of adherence of eosinophils to pollen grains under serum free conditions remain to be investigated.

Concerning the question via which receptors soluble pollen-associated mediators induce migration in eosinophils, we focused on the $LTB_4$ receptor because of the structural[13,15] and functional[10,14] similarity of PALMs. Interestingly, the preincubation of eosinophils with the $LTB_4$ receptor-antagonist LY293111[20] blocked the effects of APEs, pointing to the involvement of the $LTB_4$ receptor in eosinophil migration induced by pollen-associated mediators. Recent data on hydroxy eicosanoids binding to the low affinity $LTB_4$ receptor (BLT2)[24] suggest that this receptor is rather promiscuous, binding to a broader spectrum of ligands similar to $LTB_4$.

To investigate random movement of eosinophils because of cell activation, standard checkerboard analysis with birch pollen–derived APE was performed. APEs induced both chemokinetic responses as well as directional migration. However, chemokinetic responses preferentially occurred at high concentrations, whereas low concentration induced chemotaxis, which is a common characteristic of chemokines.

Because APEs contain considerable amounts of minor and major allergens[13] and because eosinophils have been reported to express low levels of FcεRI receptors on the surface and high amounts of intracellular hFcεRI,[25] one might consider that the observed effects could be induced by allergen-specific mechanisms. However, preincubation with anti-FcεRI mAbs did not reduce APE-induced eosinophil migration, and the major grass pollen allergen itself (rPhl p 5b), in concentrations comparable with those

found in APE, did not induce chemotaxis (data not shown). Furthermore, pollen-derived lipid extracts induced significant migration, thus ruling out a protein effect. Notably, APE also induced migration in eosinophils from nonatopic donors. These findings further promote the view that the activation and migration of eosinophils on APE are not induced by allergen-specific mechanisms.

Cell extravasation into the interstitium at the site of inflammation is a prerequisite for eosinophil effector functions. $\beta_2$ Integrins, including CD11b, have been shown to be important for the interaction of eosinophils with vascular endothelial cells and transendothelial migration.[26] In eosinophils, $\beta_2$ integrins mediate firm adhesion to vascular endothelium and extracellular matrix proteins[27] and participate in eosinophil superoxide anion production and in degranulation.[1] The effects of APE on CD11b expression (Fig 3) suggest that mediators released from pollen enhance interaction of eosinophils with endothelial cells and their transmigration into tissues.

Concerning further eosinophil effector functions, our data show that APEs and the pollen-derived hydroxy derivates of linoleic and linolenic acid induce the release of ECP in GM-CSF−primed human eosinophils (Fig 6). Release of ECP after stimulation of GM-CSF−primed eosinophils with the lipid mediator platelet-activating factor has been described recently.[28]

Notably, all above described effects of pollen-associated mediators on eosinophil effector function are invariably observed in sensitized and nonsensitized individuals. Most interstingly, Brunekreef et al[29] found in a time-series analysis in the Netherlands a strong association between the day-to-day variation in pollen concentrations and that of deaths due to cardiovascular disease, chronic obstructive pulmonary disease, and pneumonia. The known association between air pollution and the number of daily deaths is hypothesized to be related to the inflammatory potential of very small particles.[30] One possibility might be that particles of biological origin may have similar effects, and pollen was repeatedly shown to release paucimicronic particles. Another hypothesis might be that bioactive lipid mediators released by pollen contribute to this effect.

In summary, our findings suggest that airborne pollen grains not only function as allergen carriers, but it may have, by virtue of releasing bioactive lipid mediators, far more effects on human health than previously anticipated.[14, 21]

We would like to express our gratitude to Martha Grünefeld-Ruchholz, Gabi Pleyl-Wisgickl, Ellis Fraust, and Johanna Grosch for excellent technical support.

## REFERENCES


1. Gleich GJ. Mechanisms of eosinophil-associated inflammation. J Allergy Clin Immunol 2000;105:651-63.
2. Gleich GJ. The eosinophil and bronchial asthma: current understanding. J Allergy Clin Immunol 1990;85:422-36.
3. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, Venge P, et al. Eosinophilic inflammation in asthma. N Engl J Med 1990; 323:1033-9.
4. Bianchi E, Bender JR, Blasi Pardi R. Through and beyond the wall: late steps in leukocyte transendothelial migration. Immunol Today 1997;18: 586-91d.
5. Engels F, Kessels GCR, Henricks PAJ, Nijkamp FP. Preferential formation of 13-hydroxylinoleic acid by human peripheral blood eosinophils. Prostaglandins 1996;52:117-24.
6. Müller MJ. Radically novel prostaglandins in animals and plants: the isoprostanes. Chem Biol 1998;12:323-33.
7. Henricks PAJ, Engels F, Van der Linde HJ, Nijkamp FP. 13-hydrolinoic acid induces neutrophil activation. Eur J Pharmcol 1991;197:233-4.
8. Liu XH, Conolly JM, Rose DP. The 12-lipoxygenase gene-transfected MCF-7 human breast cancer cell line exhibits estrogen-independent, but estrogen and omega-6 fatty acid-stimulated proliferation in vitro, and enhanced growth in athymic nude mice. Cancer Lett 1996;109:223-30.
9. Toboreck M, Blanc EM, Kaiserb S, Mattson MP, Henning B. Linoleic acid potentiates TNF-mediated oxidative stress, disruption of calcium homeostasis, and apoptosis of cultured endothelial cells. J Lipid Res 1997;38:2155-67.
10. Traidl-Hoffmann C, Kasche A, Jacob T, Huger M, Plötz SG, Feussner I, et al. Lipid mediators from pollen act as chemoattractants and activators of polymorph nuclear granulocytes. J Allergy Clin Immunol 2002;109:831-8.
11. Angeli V, Faveeuw C, Delerive P, Fontaine J, Barriera Y, Franchimont N, et al. Schistosoma mansoni induces the synthesis of IL-6 in pulmonary microvascular endothelial cells: role of IL-6 in the control of lung eosinophilia during infection. Eur J Immunol 2001;31:2751-61.
12. Schroder JM, Hasler R, Grabowsky J, Kahlke B, Mallet AI. Identification of diacylated ureas as a novel family of fungus-specific leukocyte-activating pathogen-associated molecules. J Biol Chem 2002; 277:27887-95.
13. Behrendt H, Becker WM. Localization, release and bioavaibility of pollen allergens: influence of environmental factors. Curr Opin Immunol 2001;13:709-15.
14. Traidl-Hoffmann C, Kasche A, Menzel A, Jakob T, Thiel M, Ring J, et al. Impact of pollen on human health: more than allergen carriers? Int Arch Allergy Immunol 2003;131:1-13.
15. Behrendt H, Kasche A, Ebner von Eschenbach C, Risse U, Huss-Marp J, Ring J. Secretion of proinflammatory eicosanoid-like substances precedes allergen release from pollen grains in the initiation of allergic sensitization. Int Arch Allergy Immunol 2001;124:112-21.
16. Feussner I, Wasternack C. The lipoxygenase pathway. Annu Rev Plant Physiol Plant Mol Biol 2002;53:275-97.
17. Weichert H, Stenzel I, Berndt E, Wasternack C, Feussner I. Metabolic profiling of oxylipins upon salicylate treatment in barley leaves-preferential induction of the reductase pathway by salicylate. FEBS Lett 1999; 464:133-7.
18. Plötz SG, Lentschat A, Behrendt H, Plötz W, Ring J, Rietschel ET, et al. The interaction of human peripheral blood eosinophils with bacterial lipopolysaccharides is CD14-dependent. Blood 2001;97:235-41.
19. Bock C. A quick and simple method for preparing soft insect tissues for scanning electron microscopy using carnoy and hexamethyldisilazan. Beitr Elektronenmikroskop Direktabb Oberfl 1987;20:209-14.
20. Jackson WT, Froelich LL, Boyd RJ, Schrementi JP, Saussy DL, Schultz RM, et al. Pharmacologic actions of the second-generation leukotriene $B_4$ receptor antagonist sdLY29311: in vitro studies. J Pharmacol Exp Ther 1999;288:286-94.
21. Fogg M, Ruchelli E, Spergel J. Pollen and eosinophilic esophagitis. J Allergy Clin Immunol 2003;112:796-7.
22. Siegel I, Sherman WB. Pollen-white cell interactions. J Allergy 1970;45: 133-45.
23. Mackay JA, Sass-Kuhn S, Moqbel R, Walsh GM, Kay AB. The requirement for transferrin-dependent adherence of human granulocytes to pollen grains. Allergy 1986;41:169-78.
24. Yokomizo T, Kato K, Hagiya H, Izumi T, Shimizu T. Hydroxyeicosanoids bind to and activate the low affinity leukotriene B4 receptor BLT2. J Biol Chem 2001;276:12454-9.
25. Kayaba H, Dombrowicz D, Woerly G, Papin JP, Loiseau S, Capron M. Human eosinophils and human high affinity IgE receptor transgenic mouse eosinophils express low levels of high affinity IgE receptor, but release IL-10 upon receptor activation. J Immunol 2001;167:995-1003.

26. Bochner BS, Schleimer RP. The role of adhesion molecules in human eosinophil and basophil recruitment. J Allergy Clin Immunol 1994;94:427-38.
27. Simon HU. Integrin expression by eosinophils. Allergy 2000;55:791-2.
28. Simon HU, Weber M, Becker E, Zilberman Y, Blaser K, Levi-Schaffner F. Eosinophils maintain their capacity to signal and release eosinophil cationic protein (ECP) upon repetitive stimulation with the same agonist. J Immunol 2000;165:4065-75.
29. Brunekreef B, Hoek G, Fischer P, Spieksma F. Relation between airborne pollen concentrations and daily cardiovascular and respiratory-disease mortality. Lancet 2000;355:1517-8.
30. Seaton A, MacNee W, Donaldson K, Godden D. Particulate air pollution and acute health effects. Lancet 1995;345:176-8.
31. Wang DY, Clement P. Pathogenetic mechanisms underlying the clinical symptoms of allergic rhinitis. Am J Rhinol 2004;14:325-31.