

Allergens, IgE, mediators, inflammatory mechanisms

Nasal neutrophilia and release of myeloperoxidase induced by nasal challenge with platelet activating factor: Different degrees of responsiveness in atopic and nonatopic subjects

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Background: Nasal challenge with platelet activating factor (PAF) is able to induce local neutrophilia, with a different degree of responsiveness in atopic subjects and in nonatopic subjects. We investigated whether nasal accumulation of neutrophils induced by PAF is accompanied by the release of neutrophil-derived mediators.

Methods: Nasal lavages were performed before and after challenge with PAF (500 nmol), lyso-PAF (500 nmol), and saline solution in 10 patients with allergic rhinitis and 10 normal subjects to evaluate changes in neutrophil counts and the release of myeloperoxidase (MPO) and immunoreactive leukotriene B₄.

Results: PAF caused neutrophilia, which appeared after 30 minutes in atopic subjects and after 3 hours in nonatopic subjects. Furthermore, when compared with saline insufflation, PAF caused a significant release of MPO in the nasal lavage fluids collected 30 minutes, 3 hours, and 24 hours after challenge in atopic subjects and 3 hours after challenge in nonatopic subjects, with higher values in the former than in the latter. Neutrophil counts correlated with MPO levels in the nasal lavages collected after PAF challenge. A lower degree of neutrophilia was found 3 hours after stimulation with lyso-PAF in both groups of subjects, with a marginal release of MPO in atopic subjects only. No significant increase of immunoreactive leukotriene B₄ levels in nasal lavages was found after challenge with either PAF or lyso-PAF.

Conclusion: These results indicate that PAF-induced neutrophilia in the nose is accompanied by the release of MPO, which appears earlier and is more marked in atopic subjects than in nonatopic subjects. (*J ALLERGY CLIN IMMUNOL* 1996;97:947-54.)

Key words: Leukotriene B₄, lyso-PAF, myeloperoxidase, neutrophil, nasal lavage, PAF

Platelet-activating factor (PAF) is a lipid mediator produced by most inflammatory cells after immunologic activation and is directly involved in

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Abbreviations used

LT: Leukotriene

MPO: Myeloperoxidase

PAF: Platelet activating factor

inflammatory and allergic reactions.¹ Several clinical and experimental findings suggest that PAF can participate in the pathogenesis of respiratory allergy.² PAF is released into plasma during allergen-induced bronchoconstriction,³ and deficiency of serum PAF acetylhydrolase, the enzyme that

inactivates PAF, is associated with severe respiratory symptoms in children with asthma.⁴ PAF has a wide range of biologic activities that are important in the mechanisms underlying allergic manifestations.¹ It is a potent chemoattractant and activator for neutrophils and eosinophils⁵⁻⁷ and can induce airway microvascular leakage,⁸ bronchoconstriction,^{9,10} bronchial hyperresponsiveness,⁹⁻¹¹ and cellular infiltration.¹² Some of PAF's effects in vivo may be mediated indirectly by the release of secondary mediators.¹³⁻¹⁶ PAF can induce leukotriene (LT) synthesis and release from perfused rabbit lung¹⁷ and from human neutrophils¹⁸ and eosinophils.¹⁹ Moreover, cyclooxygenase inhibitors and LT or thromboxane antagonists can modulate PAF-induced bronchoconstriction, which suggests that these substances could mediate some PAF-induced effects on respiratory airways.¹⁴⁻¹⁶

Previously, we evaluated the role of PAF in the development of nasal allergic response. We found that PAF and its precursor and metabolite lyso-PAF are released in human nasal airways during the early antigen-induced reaction, with a preponderance of lyso-PAF release.²⁰ This is in line with the observations of other groups of researchers.²¹⁻²³ We also showed that PAF insufflation in the nose can provoke an increase in nasal airway resistance²⁴ associated with clinical symptoms, most notably nasal obstruction and hyperreactivity.²⁵ Furthermore, nasal challenge with PAF is followed by local accumulation of neutrophils and eosinophils, with a more rapid and marked response in atopic subjects than in nonatopic subjects.²⁶ PAF-induced nasal eosinophilia is accompanied by the release of eosinophil cationic protein, again with a more marked response in atopic subjects than in nonatopic subjects.²⁷ This indicates that the two populations have different degrees of responsiveness to challenge with PAF.

The aim of this study was to determine whether the nasal neutrophilia caused by challenge with PAF is accompanied by the release of neutrophil-derived mediators. We evaluated cytologic changes and mediator release (LTB₄ and myeloperoxidase [MPO]) in nasal lavage fluids after nasal challenge with PAF in 10 patients with allergy and in 10 normal subjects.

METHODS

Subjects and study design

Ten patients with allergic rhinitis (5 men and 5 women; mean age $\pm$ SEM, 32.8 ± 2.6 years) and 10 healthy subjects (6 men and 4 women; mean age $\pm$ SEM, 29.7 ± 2.4 years) were selected for this study. Allergic

rhinitis was diagnosed by clinical history and skin prick tests. Five patients were allergic to grass pollen, and five to *Parietaria judaica*. Our study was performed during the winter season when the patients were free of symptoms and not taking any medication. Informed consent was obtained from each subject before the study.

Each subject underwent nasal stimulation with PAF, lyso-PAF, and saline solution. Nasal lavages were performed at baseline and at different postchallenge times (30 minutes, 3 hours, and 24 hours) to evaluate changes in nasal cytology and mediator release. The results obtained after nasal insufflation of saline solution were used as controls.

Nasal challenge and nasal lavage procedure

PAF, purchased from Bachem (Bubendorf, Switzerland) and lyso-PAF (Bachem) were dissolved in 100% ethanol (Merck, Darmstadt, Germany; final concentration 1%) and diluted in 0.9% wt/vol NaCl solution containing 0.25% wt/vol human serum albumin (Institut Mérieux, Lyon, France). These solutions were divided into small aliquots and frozen at -20°C . After thawing, PAF, lyso-PAF, or saline solution was sprayed into the nose by means of a disposable metered-dose nebulizer, calibrated to deliver $100 \pm 10\ \mu\text{l}$ per puff. The dose of 500 nmol of PAF was chosen because we had previously observed that this could provoke nasal symptoms and changes in nasal cytology in most subjects.^{24, 26}

To evaluate changes in nasal neutrophilia and mediator levels, nasal lavage fluids were collected with a technique previously described.^{20, 26} Briefly, 5 ml of 0.9% wt/vol NaCl was instilled into each nostril. After 10 seconds, the subject expelled the mixture of saline solution and mucus, which was collected in conical polypropylene tubes (Falcon, Becton Dickinson Labware, Oxnard, Calif.). Recovery of nasal lavage fluid ranged from 50% to 80% of the starting volume (10 ml for each washing). Nasal lavages were performed before and 30 minutes, 3 hours, and 24 hours after nasal stimulation. Nasal lavage fluids were shaken to disperse mucus, and aliquots were collected for total cell counts, which were performed in duplicate in a Fuchs Rosenthal chamber (Walter Schrenck, Hofheim am Taunus, Germany) after staining with Turk liquid (0.01% wt/vol gentian violet, 0.2% wt/vol 95% ethanol, and 5% wt/vol acetic acid in distilled water; prepared by Farmacia Ospedale Policlinico, Milan, Italy). The lavage samples were then centrifuged at 1000 g for 10 minutes at 4°C . The supernatant of each sample was aspirated, and the cell pellet was resuspended in 1 ml of 0.9% wt/vol NaCl with 0.1% wt/vol human serum albumin. Two cytospin slides for each sample were prepared (100 μl with 2 to 4×10^4 cells for each slide, centrifuged at 500 rpm for 10 minutes in a Shandon cytocentrifuge [Shandon Southern Ltd., Runcorn, Cheshire, U.K.]). Cytocentrifuge slides were stained with May-Grünwald-Giemsa stain (staining solutions provided by Farmacia Ospedale Policlinico) and examined by light microscope under oil immersion

to obtain neutrophil counts. The reader was blinded as to whether the subject had received PAF, lyso-PAF, or saline solution.

Mediator assays

MPO. The concentration of MPO was measured by radioimmunoassay with a commercially available kit (Kabi-Pharmacia, Uppsala, Sweden). The sensitivity of the assay is 8 ng/ml, and cross-reactivity of the anti-MPO antiserum is less than 0.1% with lactoferrin, lysozyme, eosinophil cationic protein, and eosinophil peroxidase.

LTB₄. Aliquots of the supernatants for LT assay were aspirated, decanted, and extracted according to the method of Anderson et al.²⁸ The lavage supernatants were mixed with 4 volumes of absolute ethanol and left at 2 to 8° C for 30 minutes. The protein-rich precipitate was removed by centrifugation at 3000 g for 30 minutes at 4° C; the ethanol supernatant was collected, decanted, and then removed by rotary evaporation under vacuum. The samples were stored at -20° C and then redissolved in distilled water for radioimmunoassay.

The determination of immunoreactive LTB₄ was made by radioimmunoassay with commercially available kits (Amersham International Ltd., Little Chalfont, Bucks, U.K.). The LTB₄ rabbit antiserum has no cross-reactivity with peptide LTs, but cross-reacts as much as 3.9% with diastereomers of LTB₄. The sensitivity of the assay is 0.016 ng/ml, and the intraassay and interassay coefficients of variation do not exceed 10%. Each sample was assayed in duplicate, and the results were expressed as nanograms per milliliter of lavage fluid.

High-performance liquid chromatography of LTB₄

Selected samples, containing at least 3 ng of immunoreactive LTB₄, were extracted with a solid-phase C₁₈ cartridge (Supelclean; Supelco, Bellefonte, Pa.), after addition of 20 ng of prostaglandin B₂ as internal standard. The methanolic eluate (2 ml) was dried with an evaporating centrifuge (Speedvac, Farmingdale, N.Y.), reconstituted in 400 µl of mobile phase A, and injected into a high-performance liquid chromatography gradient pump system (model 126; Beckman Analytical, Cassina de' Pecchi, Milan, Italy) connected to a diode array UV detector (Beckman model 168). UV absorbance was monitored at 280 nm, and full UV spectra (240 to 320 nm) was acquired at a rate of 0.5 Hz. A computer-controlled multilinear gradient from solvent A (10% methanol, 10% acetonitrile, 0.02% acetic acid, pH 5.5 with ammonium hydroxide) to solvent B (50% methanol, 50% acetonitrile) was used at a flow rate of 1 ml/min (column: Lichrosphere RP-18 end capped, 5 µm, 250 × 4 mm; Merck, Darmstadt, Germany). This method provides complete separation of LTB₄ from 5S, 12S-dihydroxyeicosatetraenoic acid, as well as from Δ⁶trans-isomers of LTB₄. LTB₄ present in biologic samples, positively identified by two-way chemical analysis, was quantitated by using a standard curve (5 to 500 pmol) of

synthetic LTB₄ (slope, 69 ± 1; offset, 3.2 ± 2.9; values ± 95% confidence limits, $r = 0.998$).

Statistical analysis

Results were expressed as means ± SEM. The two-tailed Student's *t* test for paired data was used to analyze the changes in cell populations and mediator levels in the nasal lavages. (Prechallenge values were compared with postchallenge values obtained at different times; moreover, the values obtained after PAF or lyso-PAF challenge were compared with the values obtained after insufflation of saline solution.) Pearson's correlation coefficient was used to analyze the correlation between cells and mediator levels. Values of *p* lower than 0.05 were considered significant.

RESULTS

Neutrophil counts in nasal lavage fluids after stimulation with PAF, lyso-PAF, or saline solution

Total cell counts in prechallenge nasal lavages were 234.6 ± 78.3 (mean ± SEM × 10³) in atopic subjects and 206.8 ± 65.3 (mean ± SEM × 10³) in nonatopic subjects, so that the difference between the two kinds of subjects was not statistically significant. Nasal stimulation with PAF provoked an increase in total cell counts in the nasal lavages collected 3 hours after stimulation in both atopic and nonatopic subjects (420.6 ± 88.2 × 10³, $p < 0.01$ and 388.2 ± 56.4 × 10³, $p < 0.05$, respectively). In contrast, no significant increase in total cell counts was observed after lyso-PAF or saline insufflation.

Challenge with PAF induced nasal neutrophilia, which was found after 30 minutes in atopic subjects and after 3 hours in nonatopic subjects, and peaked after 3 hours in both atopic and nonatopic subjects. A lower degree of neutrophilia was found 3 hours after lyso-PAF challenge in both atopic and nonatopic subjects (Fig. 1).

Saline insufflation was devoid of any significant effect on cellular composition of nasal lavage fluids.

Mediator levels in nasal lavage fluids

MPO. MPO was found in prechallenge nasal lavages in both atopic and nonatopic subjects (mean ± SEM, 153.1 ± 96.9 ng/ml in the former and 146.3 ± 41.8 ng/ml in the latter, without any significant difference between the two populations). An increase in MPO levels, when compared with baseline values, was found 30 minutes and 3 hours after PAF challenge in atopic subjects ($p < 0.01$) and 3 hours after challenge in nonatopic subjects ($p < 0.01$). Furthermore, when MPO

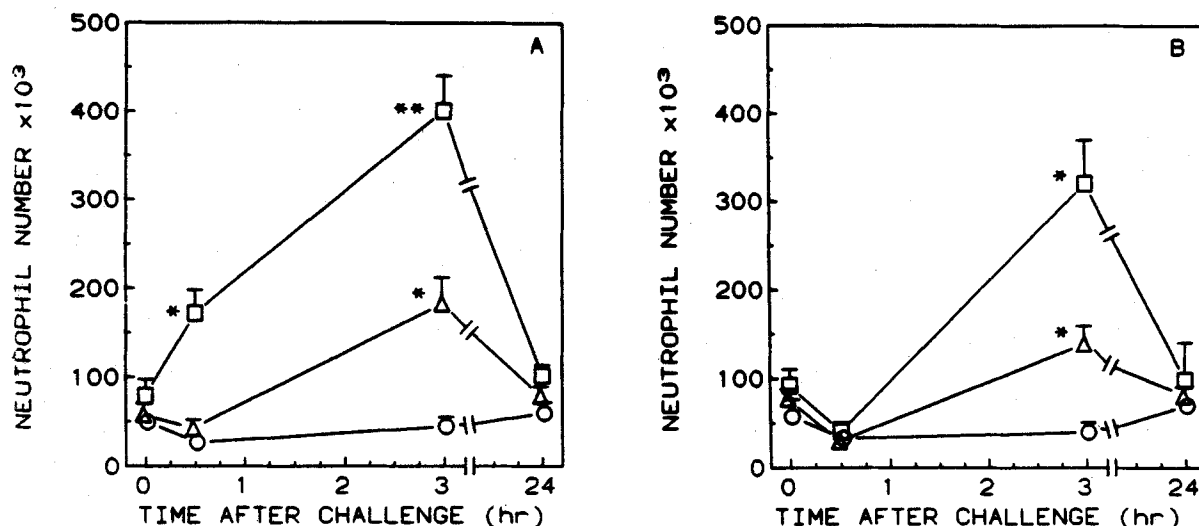


FIG. 1. Time course of neutrophil counts in nasal lavage fluids collected after challenge with PAF (squares, 500 nmol), lyso-PAF (triangles, 500 nmol), or saline solution (circles, 0.9% wt/vol NaCl solution) in 10 atopic subjects (A) and in 10 normal subjects (B). Results are expressed as means $\pm$ SEM. *Significantly different from control value after insufflation of saline solution at $p < 0.05$. **Significantly different from control value after insufflation of saline solution at $p < 0.01$.

levels after PAF challenge and after saline insufflation were compared, significantly higher values were found in the nasal lavages collected 30 minutes ($p < 0.01$), 3 hours ($p < 0.01$), and 24 hours ($p < 0.05$) after PAF challenge in atopic subjects and 3 hours ($p < 0.05$) after PAF challenge in nonatopic subjects (Fig. 2). MPO levels 30 minutes and 3 hours after PAF challenge were higher in atopic subjects than in nonatopic subjects ($p < 0.005$ and $p < 0.05$, respectively). A marginal but significant release of MPO was found 3 hours after challenge with lyso-PAF in atopic subjects only ($p < 0.05$ vs the corresponding value after saline insufflation).

A significant correlation was observed between neutrophil counts and MPO concentrations in the nasal lavages collected 30 minutes and 3 hours after challenge with PAF in atopic subjects ($r = +0.78$, $p < 0.01$ and $r = +0.82$, $p < 0.005$, respectively) and 3 hours after challenge in nonatopic subjects ($r = +0.77$, $p < 0.01$).

LTB₄. Mean immunoreactive LTB₄ concentration in basal lavage samples was 0.66 ± 0.14 ng/ml in atopic subjects and 0.79 ± 0.15 ng/ml in nonatopic subjects, without any significant difference between the two populations. A trend toward a decrease in LTB₄ concentration was observed in the nasal lavage fluids collected up to 3 hours after nasal provocation with PAF. At the 24-hour control, LTB₄ levels were similar to the baseline

values. A similar trend was observed after nasal insufflation of lyso-PAF or saline solution (Fig. 3). Analysis of selected samples by high-performance liquid chromatography coupled to diode array detection allowed positive identification of LTB₄ in nasal washings (Fig. 4).

DISCUSSION

As observed previously,²⁶ nasal challenge with PAF causes nasal neutrophilia, which appears earlier and is more marked in atopic subjects than in nonatopic subjects. The results of this study indicate that nasal accumulation of neutrophils is accompanied by the release of MPO, a protein specific for primary azurophilic granules of neutrophils.^{29,30}

It has been demonstrated that PAF is a potent chemotactic factor for both neutrophils and eosinophils *in vitro*³; *in vivo* experiments have shown that PAF is able to attract neutrophils and eosinophils in human beings, both in the skin³¹ and in the nose.²⁶ Furthermore, a different degree of responsiveness has been evidenced in atopic and nonatopic subjects, with a predominant accumulation of eosinophils at the site of PAF challenge in the former group. We found that nasal challenge with PAF in atopic subjects is followed not only by a more rapid and marked accumulation of neutrophils and eosinophils, but also by an enhanced release of the eosinophil-derived cationic pro-

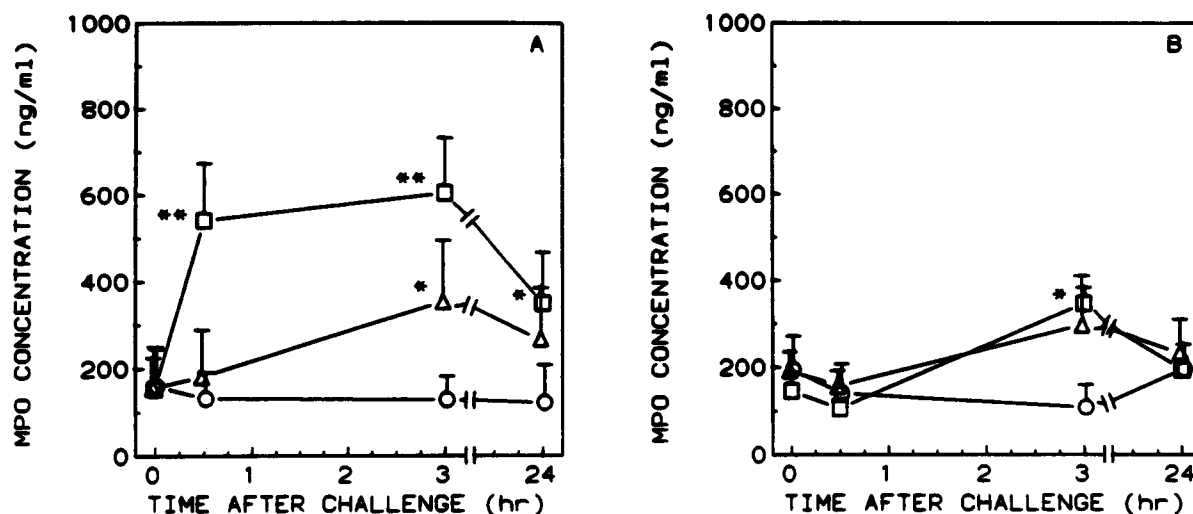


FIG. 2. MPO levels in the nasal lavage fluids collected from 10 atopic subjects (A) and 10 normal subjects (B) after nasal challenge with PAF (squares, 500 nmol), lyso-PAF (triangles, 500 nmol), or saline solution (circles, 0.9% wt/vol NaCl). Results are expressed as means $\pm$ SEM. *Significantly different from control value after insufflation of saline solution at $p < 0.05$. **Significantly different from control value after insufflation of saline solution at $p < 0.01$.

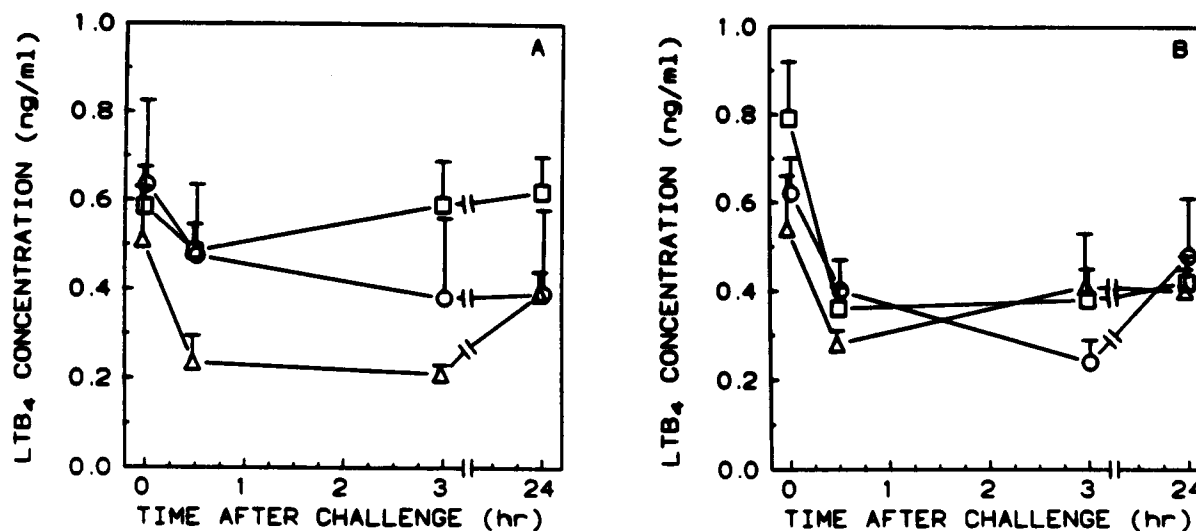


FIG. 3. LTB₄ levels in the nasal lavage fluids collected from 10 atopic subjects (A) and 10 normal subjects (B) after nasal challenge with PAF (squares, 500 nmol), lyso-PAF (triangles, 500 nmol), or saline solution (circles, 0.9% wt/vol NaCl). Results are expressed as means $\pm$ SEM.

tein.²⁷ This study also shows that MPO is released in the nose after PAF challenge, with an enhanced degree of responsiveness in atopic subjects compared with nonatopic subjects. Therefore the neutrophils attracted in the nose by PAF are activated and release granule components, such as MPO. It is not known whether PAF directly causes the secretion of MPO or whether it acts as a priming agent, and the release of MPO is triggered by other stimuli present in the nose (possibly, bacterial

components). High-affinity binding sites for PAF have been detected on neutrophils³²; furthermore, in vitro experiments have indicated that PAF can stimulate the release of components of azurophilic granules (MPO and β -glucuronidase) and specific granules (lactoferrin and lysozyme) of neutrophils.⁶ According to Shaw et al.,⁶ PAF can cause the release of components of neutrophil granules only in the presence of cytochalasin; however, Taylor et al.³³ have shown that PAF itself can

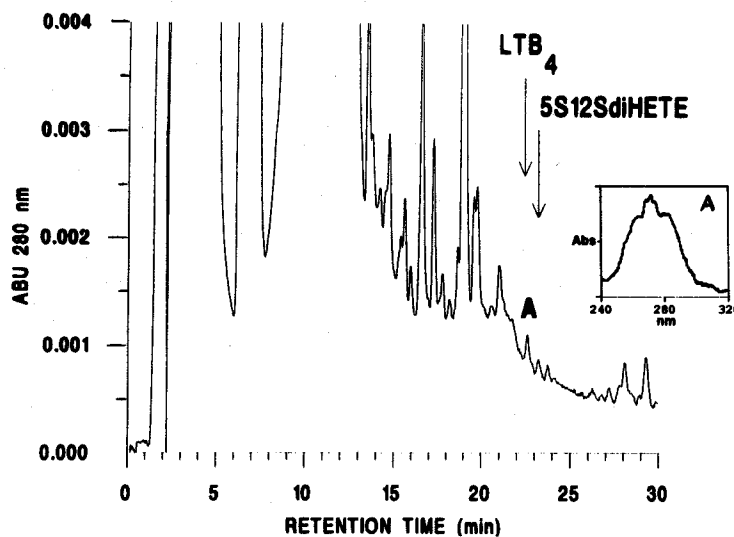


FIG. 4. Reverse-phase high-performance liquid chromatography chromatogram of a nasal washing 24 hours after administration of PAF. Conditions are described in the Methods section. Arrows indicate the retention time of LTB_4 and 5S, 12S-dihydroxyeicosatetraenoic acid (5S12SdiHETE), and inset shows the UV spectrum of peak A.

induce a modest but significant *in vitro* secretion of lactoferrin by blood neutrophils even in the absence of cytochalasin. Interestingly, these authors have found that the neutrophils of atopic subjects are more responsive to PAF and release higher amounts of lactoferrin than the neutrophils of nonatopic subjects. In this regard, our findings raise several interesting questions. It is conceivable that increased response of neutrophils from atopic subjects to PAF may reflect an increased binding of this lipid mediator; furthermore, this could be the consequence of a sort of "preactivation" or "priming" of inflammatory cells in atopic subjects, resulting in an increased reactivity to activating signals. One can also speculate that the more rapid recruitment of inflammatory cells at the site of PAF challenge in atopic subjects is the consequence of an enhanced expression of adhesion molecules and their ligands, also reflecting a primed status of atopic subjects.

In contrast to the presence of MPO, which was correlated with neutrophil accumulation, PAF challenge was not followed by the release of LTB_4 , a newly synthesized mediator that can be generated and released by neutrophils.³⁴ *In vitro* data indicate that PAF can stimulate LTB_4 synthesis in human polymorphonuclear neutrophils.¹⁸ Furthermore, Nieminen et al.³⁵ have found an increase in plasma LTB_4 levels after bronchial challenge with PAF in human subjects. Our failure to find a significant release of LTB_4 after nasal challenge with PAF could be due to the generation and

release of very low amounts or its rapid binding to receptors or trapping by mucous proteins.

As we observed previously, a marginal neutrophilia was also found after challenge with lyso-PAF in both atopic and in nonatopic subjects, with a more marked response in the former than in the latter.²⁶ This neutrophilia was accompanied by the release of MPO, which was found in increased amounts 3 hours after challenge in atopic subjects. Lyso-PAF is a precursor and a metabolite of PAF, which is generally considered inactive *in vitro*. However, lyso-PAF is able to induce bronchoalveolar eosinophilia in nonhuman primates,³⁶ and it can cause an increase in lung resistance and airway microvascular leakage when inhaled by guinea pigs.³⁷ The effects observed in guinea pigs, which are completely abolished by a PAF antagonist (WEB 2086), have been attributed to lyso-PAF conversion into PAF in guinea pig airways by a lyso-PAF, acetyl coenzyme A acetyltransferase. Therefore the activity of exogenous lyso-PAF in the human nose could be the consequence of acetylation and transformation into PAF, occurring in respiratory airways. In fact, it has been demonstrated that PAF can be produced by cultured bacteria supplemented with lyso-PAF,³⁸ and PAF formation by eukaryotic cells is strictly dependent on the concentration of lyso-PAF available.³⁹ It should be noted that an increase in neutrophil counts was found only 3 hours after lyso-PAF challenge, whereas the effect of PAF was already evident 30 minutes after challenge. This delay

could be due to either the requirement for lyso-PAF acetylation or the relative weakness of the direct impact of this compound when compared with PAF.

Nasal challenge with saline solution had no effect on the cellular composition of the nasal lavages or the release of MPO in both atopic and nonatopic subjects. Therefore we conclude that the changes in nasal cytology and the release of MPO provoked by PAF and lyso-PAF are not nonspecific.

The dose of PAF used for nasal challenge is higher than amounts found in nasal washings after local antigen challenge in patients with rhinitis.²⁰ However, the relationship between the amount of PAF generated and that found in nasal lavages is not known. It has been demonstrated that more than 95% of exogenously administered PAF binds quickly to its putative receptors in respiratory airways of experimental animals.⁴⁰ It is reasonable to suppose that the amount of PAF found in nasal washings is much lower than that generated; therefore, the dose of PAF used in our study may have a physiopathologic relevance.

The results of this study indicate that PAF has the capacity to attract and activate neutrophils when insufflated in the human nose. Although the role of neutrophils in allergic inflammation is not yet fully understood and requires further evaluation, neutrophils have been found in nasal washings during the late response to antigen challenge,⁴¹ and neutrophil-derived MPO has been considered, together with eosinophil cationic protein, as a marker of inflammation in allergic rhinitis.⁴² Therefore we suggest that the capacity of PAF to attract and activate neutrophils in the nose may be relevant to the pathogenesis of respiratory allergy.

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