

Enhanced production of platelet activating factor by peripheral granulocytes from children with asthma

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Granulocytes from 23 asthmatic children aged 4–15 years and 32 age-matched healthy children were studied. Cells were purified by Dextran sedimentation and Percoll gradient centrifugation from heparinized blood. After *in vitro* stimulation by ionophore A23187 the amount of newly synthesized PAF and LTC₄ was assessed by radio receptor assay or radioimmunoassay respectively. Eight patients had symptoms of asthma within the last 3 weeks before examination. Granulocytes from the symptomatic patients showed a significantly higher PAF generation (median 125 ng/10⁶ cells, range 7–189 ng/10⁶ cells) when compared to asymptomatic patients ($p < 0.001$, median 14 ng/10⁶ cells, range 6–33 ng/10⁶ cells) or controls ($p < 0.001$, median 11 ng/10⁶ cells, range 3–26 ng/10⁶ cells). In contrast, LTC₄ generation was increased in both patient groups. The results suggest a regulatory role of PAF in the exacerbation of asthma.

Clinical aspects

Granulocytes from the symptomatic patients showed a significantly higher PAF generation (median 125 ng/10⁶ cells, range 7–189 ng/10⁶ cells) when compared to asymptomatic patients ($p < 0.001$, median 14 ng/10⁶ cells, range 6–33 ng/10⁶ cells) or controls ($p < 0.001$, median 11 ng/10⁶ cells, range 3–26 ng/10⁶ cells). In contrast, LTC₄ generation was increased in both patient groups. The results suggest a regulatory role of PAF in the exacerbation of asthma.

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A major feature of asthma is bronchial hyperreactivity which is characterized by increased responsiveness of the airways to both allergic and nonspecific stimuli (4). Inflammatory mediators, such as histamine, prostaglandins (13) and leukotrienes (15) cause immediate bronchoconstriction but not a sustained nonspecific hyperresponsiveness. In this regard, the effect of platelet activating factor (PAF) is unique. Several investigators were able to show that PAF sensitized the airway of normal subjects to metacholine for up to 4 weeks (14, 29).

These findings gave rise to an increasing interest in the role of PAF in the pathogenesis of asthma. PAF is produced by fibroblasts (23), vascular endothelial cells (8), monocytes (8), macrophages (2), neutrophils (32) and eosinophils (20). Relatively little is known concerning the capacity of these cells to produce PAF in various clinical conditions. It was the purpose of this study to analyse PAF production

of granulocytes from asthmatic children in different clinical situations.

Subjects

Patients

The study group consisted of 23 children with atopic asthma 4–15 years of age, median 9.4 years. The asthmatic patients fulfilled the criteria of the American Thoracic Society. They suffered from recurrent attacks of diffuse expiratory wheezes audible on auscultation and accompanied by some respiratory distress. Depending on the severity of the disease, patients were treated with inhaled salbutamol, disodium cromoglycate (DSCG), beclomethasone, oral corticoids or oral theophylline (Table 1). Medication was withheld for at least 12 h before venous puncture and pulmonary function testing. At the time of examination the history of respiratory symptoms in the

Table 1. Clinical features of the patients and mediator generation of granulocytes

Age (years)	Sex	IgE ^a	Pulmonary function		Medication ^d	Symptoms ^e		Mediator generation (ng/10 ⁶ cells)	
			FEV ₁ ^b	MEF ₂₅ ^c		past	acute	PAF	LTC ₄
12.8	m	800	74	65	S	W	—	17	44
7.1	m	25	100	71	S	W	—	33	35
12.8	m	270	78	90	S	D	—	6	99
11.4	m	278	59	33	S	D	—	7	40
8.5	m	486	84	85	S	D	—	28	88
6.4	m	32	76	71	T, S	D	—	8	68
6.6	f	60	82	63	D, S	W	—	18	48
5.1	m	206	90	88	T, S	W	—	13	29
9.5	m	1020	83	72	T, D, S	D	—	16	37
14.5	f	1000	67	44	T, D, S	D	—	18	34
9.4	m	480	75	45	T, D, S	D	—	17	36
9.4	m	1280	64	49	T, D, S	D	—	8	91
10.9	f	210	78	72	B, D, S	W	—	7	28
7.8	m	350	62	40	T, D, B, S	D	—	14	47
14.2	m	1978	77	81	T, D, B, S	D	—	13	60
13.3	m	28	69	61	S	W	D	125	24
9.4	m	678	75	58	T, S	D	W	11	40
9.8	m	470	62	34	D, S	W	W	7	12
6.3	m	780	70	35	T, S	W	W	189	43
14.7	f	634	76	87	T, D, S	W	C	153	49
4.6	m	272	ND	ND	T, D, S	W	W	132	20
8.7	f	396	77	66	T, B, S	W	W	163	37
5.3	m	89	ND	ND	T, B, S	D	W	57	83

^a U/ml.^{b,c} Percent of the expected value.^d T=oral theophylline, B=inhaled beclomethasone, D=inhaled disodium cromoglycate, C=oral corticoids, S=inhaled salbutamol as required.^e Past symptoms were all symptoms the patient ever had. Acute symptoms were reported within 3 weeks before the examination. C=cough, W=wheezing, D=dyspnea associated with wheezing.

past and recent symptoms within the last 3 weeks were recorded using a standard questionnaire completed by the caring physician according to the statements of the patients and their parents. Those patients who asserted not having any changes related to allergic diseases within the last 3 weeks were assigned to the asymptomatic group whereas patients who reported dyspnea associated with wheezing, wheezing alone or cough on exertion were assigned to the symptomatic group (Table 1).

Serum-IgE of each child was detected using a commercial ELISA kit (Behringwerke, Marburg, Germany).

Control group

The control group consisted of 32 children aged 2–14 years (median 6.9 years) who had been admitted for elective minor surgery. The children had negative personal and family histories of atopy and normal levels of total serum IgE. In addition, no specific IgE to inhaled allergens could be detected in the serum using the Phadiatop test (Pharmacia, Freiburg, Germany). Control and patient groups were age matched.

Written consent was obtained from the parents of all patients and controls. At the time of investigation all patients were free of respiratory symptoms, an infection or other diseases.

Material and methods

Pulmonary function test

Respiratory function was assessed in patients older than 5 years. A maximum flow expiratory volume curve was recorded to determine FEV₁ (volume expired at the 1st sec of forced expiration) and MEF₂₅ (maximal expiratory flow at 25% of remaining vital capacity). FEV₁ and MEF₂₅ are given as percent of the expected value on the basis of height and weight of the patient according to the reference values given by Zapletal et al. (35) which were used following the guidelines delineated by Taussig et al. (33). Results are shown in Table 1.

Preparation of cells

For analysis of granulocytes from patients and controls blood was collected in preservative-free hepa-

rin. Five to 10-ml aliquots of the heparinized blood were sedimented with 0.2 vol of 2% dextran 110 in saline for 45 min at 20°C. The supernatant was layered onto discontinuous Percoll gradients prepared by layering two dilutions of Percoll (1.102 g/ml, 1.077 g/ml) in 50 ml conical tubes. The tubes were centrifuged at $1000 \times g$ for 30 min at 4°C, and the cells at each interface were harvested. Granulocytes accumulated predominantly at the 1.102 g/ml / 1.077 g/ml interface, and mononuclear cells at the 1.077 g/ml / plasma interface. Total number of cells and number of eosinophils in the granulocyte fraction were counted. Eosinophils were stained with Randolph stain (28). Contamination by platelets was excluded by a platelet count using procain-HCl.

Granulocytes were washed twice and resuspended in PBS supplement with 1% Nutridoma (Boehringer Mannheim, Mannheim Germany) at a concentration of 3×10^6 cells/ml. Incubations were performed with or without ionophore (2 µg/ml) for 30 min at 37°C in polypropylene test tubes. At the end of incubation the test tubes were centrifuged at $150 \times g$ at 4°C, the supernatant was removed. 100 µl were stored for the assay of LTC₄. From the remaining supernatant the pellet extraction of phospholipids was accomplished by the method of Bligh & Dyer (7).

Competitive PAF binding assay

The binding assay was performed in modification of the methods described by Paulson (27) and Janero (18). Preparation of washed rabbit platelets was accomplished by the method described by Ardlie (1). Briefly, blood was obtained from unanesthetized adult rabbits by puncture of the ear artery. It was collected directly into 50 ml plastic tubes containing acid citrate dextrose as anticoagulant to achieve a final volume of 9:1 blood:citrate and further diluted with phosphate buffered saline (without Ca²⁺, pH 7.4) at a ratio of 1:1 (vol:vol). The erythrocytes were sedimented by centrifugation at room temperature for 18 min at $200 \times g$. The supernatant was saved and washed three times with Tris Tyrodes buffer pH 6.5 containing 0.35% bovine albumin and 2.0 mM Mg²⁺ but no Ca²⁺. Washing was accomplished by centrifugation at room temperature for 18 min at $1000 \times g$. In between washing steps platelets were allowed to stand for 15 min before centrifugation. The washed platelets were finally resuspended to 1.5×10^8 cells/ml in Tyrode's solution pH 7.3 containing 2.0 mM Ca²⁺.

Binding was carried out in polypropylene assay tubes. 60 µl Tris Tyrode's buffer pH 7.3 with Ca²⁺ containing 1 µM lyso-PAF was added to each tube. 340 Bq (157 pM) [³H] PAF (Amersham, Braunschweig, Germany) in 40 µl TTB Ca²⁺ and either un-

labeled PAF (final concentration 10^1 – 10^6 pM) or sample were added to polypropylene assay tubes in 6 µl ethanol total volume. The addition of 500 µl rabbit platelet suspension started the reaction. After incubation for 30 min at room temperature the reaction was stopped by centrifugation for 15 min at $2000 \times g$ at room temperature. The supernatant was carefully removed and platelets with bound PAF were resuspended in 300 µl 1 N NaOH. The suspension was transferred into scintillation vials and mixed with 2 ml Aquasol scintillator. Platelet bound [³H]-PAF was determined by liquid scintillation spectrometry. The inhibition of specific binding was related to the PAF content of the sample by regression analysis from a standard curve of the inhibition of nonlabelled PAF.

In preliminary experiments the assay was shown to be specific by means of thin layer chromatography, sensitivity to phospholipase A2 (6) inhibition of PAF binding by the PAF antagonist Web 2086 (Boehringer Ingelheim, Ingelheim, FRG) and consistent results in serial dilution assays. A representative example of a dilution assay is given in Table 2. Reproducibility of the results obtained from a single sample assayed for several times was $\pm 9\%$.

Quantitation of leukotrienes

The RIA (16) for LTC₄ was performed in 300 µl vol that contained Tris Isogel buffer (0.1 M Tris-HCl, 0.14 M NaCl, 1.5 mM CaCl₂, 5 mM MgCl₂, pH 7.4), culture supernatant or synthetic ³H-LTC₄ (specific activity 34 Ci/mmol, New England Nuclear, Boston, MA) and a rabbit anti LTC₄ antiserum in 1.5 ml polypropylene test tubes. 100 µl of goat anti-rabbit IgG serum were added at slight antibody excess, and incubation was continued for 18 h at 4°C. The immune precipitate was sedimented by centrifugation at $2500 \times g$ for 25 min at 4°C dissolved in 500 µl of

Table 2. Mediator concentration in sequential diluted samples

	PAF ^a (ng)	% of expected ^c value	LTC ₄ ^b (ng)	% of expected ^c value
1:1	3.19×10^{-6}	100	out of range	—
1:5	6.20×10^{-7}	103	out of range	—
1:10	2.83×10^{-7}	88	out of range	—
1:50	6.20×10^{-8}	97	9.3×10^{-9}	100
1:100	2.61×10^{-8}	81	4.9×10^{-9}	105
1:500	6.32×10^{-9}	99	1.0×10^{-9}	107

^a 10×10^6 granulocytes were stimulated with 2 µg/ml ionophore A 23 187. PAF was extracted as described. The extract was diluted sequentially and PAF content measured by radio receptor assay.

^b Cells were treated as in ^a; supernatant was diluted sequentially and the content of LTC₄ was determined by radioimmunoassay.

^c Values were based on the first measurable mediator concentration and the factor of dilution.

0.1 N NaOH, mixed with 4 ml of Readsolve (Beckman) and assessed for radioactivity in a liquid scintillation counter. A calibration curve was established with synthetic LTC₄ in a range between 0.4 and 10 ng. The data are expressed as the mean of duplicate samples. Specificity of the assay was demonstrated by consistent results in serial dilution assays. A representative example of a dilution assay is given in Table 2.

Statistics. Data were analysed using the Mann-Whitney U-test.

Results

Preliminary experiments were carried out to study the kinetics of PAF generation. The experiments revealed that maximal PAF generation was detected after 30 min of culture. Almost all activity was detected in the pellet. Supernatants contained only 1% to 10% of PAF found in the pellet and were often below the level of detection. Therefore we concentrated on the results obtained with pellets.

Granulocytes isolated from the peripheral blood of eight patients with recent symptoms of asthma, 15 asymptomatic children with asthma and 32 healthy controls were compared for their ability to produce PAF after a challenge with ionophore A23187. Fig. 1 shows that granulocytes isolated from the peripheral blood of children with recent symptoms of asthma generated significantly more PAF than cells from asymptomatic patients or controls.

In contrast, LTC₄ generation (Fig. 2) is signifi-

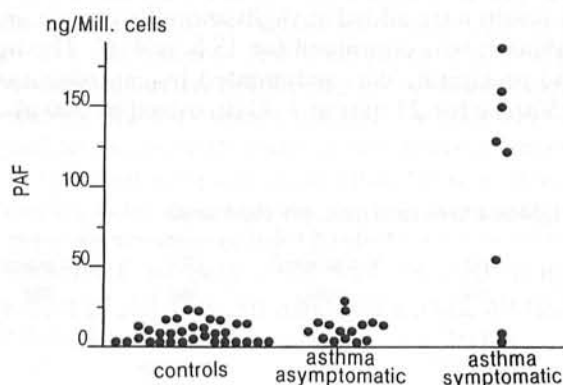


Fig. 1. Relationship between respiratory symptoms and PAF generation by granulocytes from patients with asthma and from healthy controls. Granulocytes (1×10^6 /ml) were isolated from the peripheral blood of 15 patients with no recent respiratory symptoms, 8 patients with wheezing or dyspnea within the last 3 weeks before examination and 32 healthy controls. Granulocytes were stimulated with $2 \mu\text{g}/\text{ml}$ ionophore A23187. Results are expressed as ng PAF generated by 1×10^6 cells. Significant differences between patients with recent symptoms and asymptomatic patients ($p < 0.001$) and patients with recent symptoms and controls ($p < 0.001$).

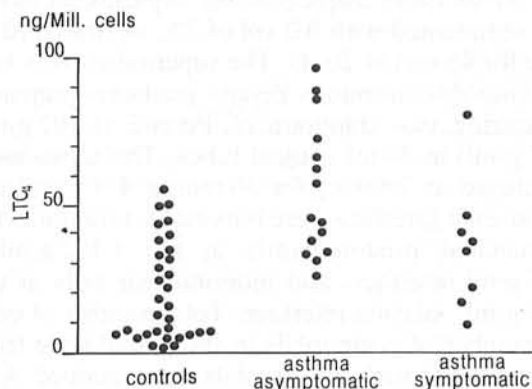


Fig. 2. Relationship between respiratory symptoms and LTC₄ generation by hypodense eosinophils from patients with asthma and healthy controls. Granulocytes (1×10^6 /ml) were isolated from the peripheral blood of 15 patients with no recent respiratory symptoms, 8 patients with wheezing or dyspnea within the last 3 weeks before examination and 32 healthy controls. Granulocytes were stimulated with $2 \mu\text{g}/\text{ml}$ ionophore A23187. Results are expressed as ng LTC₄ generated by 1×10^6 cells. Significant differences between patients with recent symptoms and controls ($p < 0.01$) and asymptomatic patients and controls ($p < 0.01$).

cantly increased in both asthmatic groups compared to controls. Analysis of the proportion of eosinophils in the whole granulocyte preparation revealed that only the asymptomatic asthmatics had a significant rise in the number of eosinophils, whereas there was no difference between controls and symptomatic patients (Fig. 3).

To analyse the correlation between basic lung function and production of PAF and LTC₄ in more

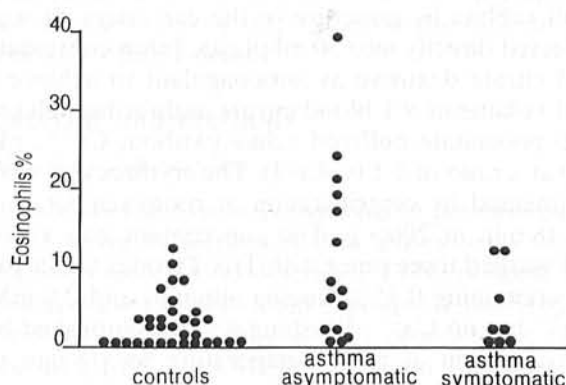


Fig. 3. Relationship between respiratory symptoms and the proportion of eosinophils within the population of purified granulocytes from patients with asthma and healthy controls. Granulocytes were isolated from the peripheral blood of 15 patients with no recent respiratory symptoms, 8 patients with wheezing or dyspnea within the last 3 weeks before examination and 32 healthy controls. Eosinophils were counted by Randolph stain and the proportion of eosinophils within the granulocyte preparation used for the experiments was calculated. Significant increase in the proportion of eosinophils ($p < 0.05$) in asymptomatic asthmatics compared with both the control group and the symptomatic group.

detail, pulmonary function tests were performed in all patients (Table 1). There was no significant correlation between the degree of small airway obstruction (MEF₂₅) or the degree of large airway obstruction (FEV₁) and PAF generation of granulocytes.

Discussion

In this study we were able to demonstrate that granulocytes from asthmatic patients with a history of recent symptoms displayed an enhanced PAF generation upon stimulation with ionophore A23187. In contrast, asthmatic patients without a history of recent symptoms displayed PAF generation within the limits of normal controls. These data are consistent with observations by Chan-Yeung et al. (10) who found a correlation between plasma PAF levels in asthmatic patients and bronchial hyperreactivity. According to a current hypothesis PAF may play an important role in the pathogenesis of asthma. It is thought that PAF is generated locally in the lung and that eosinophils accumulate due to the strong chemotactic activity of PAF (5, 22). Eosinophils from atopic patients have been shown to be more susceptible to the chemotactic activity of PAF than neutrophils (17). Moreover, inhalation of PAF induces preferential eosinophil accumulation into the lower respiratory tract in baboons (3). That the proportion of eosinophils in the peripheral blood is lower in the patient group, with the capacity to produce high amounts of PAF, does support this hypothesis. Activated eosinophils display a strong cytotoxic capacity and could be responsible for the epithelial damage associated with bronchial hyperreactivity (25).

The prominent role of PAF in asthma is further stressed by the difference between PAF and LTC₄ production. In contrast to PAF, LTC₄ generation is significantly elevated in both patient groups regardless of the history of recent symptoms. In previous papers we were able to describe a higher LTC₄ generation by granulocytes of patients with more severe asthma (30) or higher bronchial reactivity (31). However, these differences were not nearly as marked as the difference in PAF generation found in this study. Our data suggest that enhancement of PAF generation within granulocytes is not a feature of asthma as such but is only found during exacerbation of the disease and thus may play a regulatory role.

Although the results presented help to gain insight into the role of PAF in the pathophysiology of asthma some important points remain unclear.

The first question relates to the source of PAF and LTC₄. The cell population studied consisted of neutrophils with varying amounts of eosinophils. Eosinophils were shown to generate considerably higher amounts of PAF and LTC₄ than neutrophils (12, 19). On the basis of this result it seems reasonable to

Table 3. Mediator production in relation to eosinophilia

		PAF ^a (ng/10 ⁶ eos)	LTC ₄ ^a (ng/10 ⁶ eos)	Eosinophils/ μ l
Asthma with symptoms	median	1426.1 ^b	830.4	160
	range	337.0–5089.3	518.0–2766.0	30–390
Asthma without symptoms	median	120.9 ^c	389.1	360 ^e
	range	36.1–561.6	60.6–1650.0	30–1200
Controls	median	259.0 ^d	307.0	130 ^f
	range	13.0–1623.0	10.0–1027.0	30–390

^a Mediator generation/ 1×10^6 eosinophils was calculated taking into account a total of 3×10^6 cells/sample and the proportion of eosinophils as determined by Randolph stain.

Statistically significant differences were observed in: b vs c, $p < 0.001$; b vs d, $p < 0.01$; c vs d, $p < 0.01$ and e vs f, $p < 0.05$.

calculate the mediator production per eosinophil as shown in Table 3. From this point of view, differences between asthmatic patients are even more impressive. These differences may be explained by a high degree of preactivation of eosinophils from symptomatic asthmatics as we have shown in a previous paper (31). However, it has to be taken into account that contaminating neutrophils give rise to an enhanced LTC₄ generation presumably by release of precursor molecules (19) and that although PAF-generation is higher in eosinophils, there is still a considerable production in neutrophils (26). In addition, activated hypodense eosinophils were demonstrated to metabolize PAF more rapidly than normodense eosinophils or neutrophils (12). Thus, we cannot exclude that neutrophils are responsible for at least part of the PAF and LTC₄ generation measured *in vitro*. The source of PAF *in vivo*, however, is even less clear. In addition to neutrophilic and eosinophilic granulocytes, other inflammatory cells such as monocytes (8), and macrophages (9), but also fibroblasts (23) and endothelial cells (8), were shown to be able to generate PAF.

Another matter of debate is the question of whether PAF is released or retained by the producing cell. In accordance with other investigators we found considerable intercellular levels of PAF but negligible amounts in the supernatant (21, 24). This finding had led to the speculation that PAF is primarily a second messenger rather than an intracellular mediator. Since there is evidence from other data suggesting a mediator role of PAF, the question of PAF release has been given considerable attention in recent literature. One explanation for being unable to detect PAF in the supernatant in our experiments may be the stimulus. Although ionophore A23187 is a rather potent stimulus for PAF generation it does not appear to enhance PAF release like other more physiologic stimuli such as zymosan (21). Another

explanation for the low amount of PAF in supernatants is the possible transcellular generation of PAF. Not PAF but lyso-PAF is thought to be released by the cells and taken up and acetylated to PAF by the target cells (34). In addition, some authors suggest that membrane-bound PAF on the cell surface is able to stimulate surrounding cells by cell-cell interaction (24). Finally, it was proposed that PAF is released but is readily bound to membranes of adjacent cells thus appearing to be cell associated. Recently, Cluzel et al. (11) provided experimental evidence for this hypothesis. By superfusing neutrophils with buffer containing ionophore they were able to wash out 50% of the amount of PAF generated within the experiment.

In conclusion, although some important points are yet unsettled it is tempting to speculate on the basis of current knowledge that exacerbation of asthma, and hence increase in bronchial hyperreactivity, is related to an increase of PAF production in inflammatory cells which might trigger a long sustained inflammatory reaction responsible for the destruction of bronchial epithelium. The nature of inflammatory triggers and the precise relationship between PAF production and bronchial hyperreactivity will require further study.

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