

## Changes in bronchial responsiveness, circulating leucocytes and *ex vivo* cytokine production by blood monocytes after PAF inhalation in allergic asthmatics

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**ABSTRACT:** We investigated the effects of inhaled platelet-activating factor (PAF) on methacholine bronchial responsiveness, circulating leucocyte counts, and *ex vivo* tumour necrosis factor alpha (TNF $\alpha$ ) and interleukin-1 (IL-1) production from blood monocytes in eight allergic asthmatics.

Bronchial responsiveness was defined as the provocative concentration of methacholine causing a 20% decrease in forced expiratory volume in one second (PC20). Circulating leucocytes were counted by means of an automatic haemocytometer, and cytokines were measured with specific immunoassays. The different variables were measured before and 4, 24, 48, 72 and 168 h after a PAF (225  $\mu$ g), a lyso-PAF (225  $\mu$ g) and a saline bronchial challenge.

When compared with lyso-PAF and saline, inhalation of PAF resulted in a significant decrease in PC20 over a period of one week. Two falls in bronchial responsiveness were identified, the first by 4 h and the second beginning 48 h and reaching a maximum by 168 h. The increases in spontaneous TNF $\alpha$  and IL-1 production which occurred during the week after both PAF, lyso-PAF and saline, did not differ significantly. Likewise, the changes in circulating neutrophil counts, characterized by a transient rise by 4 h after PAF and lyso-PAF but not saline, followed by a fall by 24 h and a persistent decrease until 168 h, were not significantly different after PAF, lyso-PAF and saline. On the other hand, in comparison with lyso-PAF and saline, inhaled PAF caused a significant protracted augmentation in circulating eosinophil counts, which was maximal by 48 h but did not correlate with the delayed decline in PC20.

Our data indicate that inhaled PAF causes an increase in bronchial responsiveness in asthmatics, which is not directly related to changes in circulating leucocyte counts or production of IL-1 and TNF $\alpha$  from monocytes.

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Platelet-activating factor (PAF) is a phospholipid mediator endowed with potent inflammatory effects. This mediator is principally produced by macrophages, eosinophils and neutrophils. Because asthma is an inflammatory airways disease characterized by eosinophilic mucosal infiltration, it has been thought that PAF might play an important role in asthma [1]. Several studies performed in animals as well as in normal subjects have demonstrated that, besides an acute bronchial obstruction, PAF inhalation can cause a sustained increase in nonspecific bronchial responsiveness, although data obtained in human are controversial [2–9]. By contrast, such a phenomenon has not yet been confirmed in asthmatics [10, 11], but it can be argued that doses of inhaled PAF used in asthmatics were too small to produce a clear prolonged bronchial effect. On the other hand, little is known about the mechanism whereby PAF

induces a sustained increase in bronchial responsiveness.

Due to its rapid metabolism [1], PAF itself has a very short half-life in body fluids, so that prolonged biological effects following PAF exposure probably involve intermediate mediator or cytokines generated by resident or recruited inflammatory cells. In this context, PAF has been shown to stimulate the production of cytokines, including tumour necrosis factor alpha (TNF $\alpha$ ) and interleukin-1 (IL-1), from monocytes and macrophages *in vitro* [12–14]. Some data suggest that TNF $\alpha$  may contribute to bronchial hyperresponsiveness in asthma [15]. Moreover, increased levels of TNF $\alpha$  and IL-1 have been found in bronchoalveolar lavage (BAL) fluid from symptomatic asthmatics [16].

Inhalation of PAF is known to cause a transient fall in circulating neutrophils followed by rebound neutrophilia

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Table 1. – Patient characteristics

Subject No.	Age yrs	Sex	Smoker	FEV <sub>1</sub>		FEF <sub>25–75</sub>		sGaw kPa <sup>-1</sup> ·s <sup>-1</sup>	PC20 mg·ml <sup>-1</sup>	Medication
				l	% pred	l·s <sup>-1</sup>	% pred			
1	23	F	+	3.60	96	3.36	79	1.32	0.43	IB, O
2	25	F	-	3.16	103	3.58	88	1.27	2.36	IB, O
3	28	M	-	5.36	125	5.71	116	5.63	0.28	IB, O
4	30	M	-	5.20	122	5.66	116	1.84	3.13	IB, O
5	27	F	-	2.86	86	2.57	63	1.19	0.09	IB+IC, R
6	30	F	-	3.48	108	3.51	89	3.89	0.17	IB, O
7	28	M	-	6.20	142	5.97	133	2.39	1.20	IB, O
8	26	M	-	3.12	91	3.26	78	1.58	0.43	IB, O
Mean	27			4.12	109	4.20	95	2.39	0.56*	
SEM	1			0.45	6	0.48	8	0.56		

\*PC20 is expressed as a geometric mean. F: female; M: male; FEV<sub>1</sub>: forced expiratory volume in one second; FEF<sub>25–75</sub>: forced mid-expiratory flow; sGaw: specific airway conductance; PC20: provocative concentration of methacholine producing a 20% decrease in FEV<sub>1</sub>; IB: inhaled bronchodilators; IC: inhaled corticosteroids; O: occasionally; R: regularly.

[10, 17]. However, at the present time, no data are available on possible sustained changes in circulating blood cells following PAF inhalation in man. In this study, we investigated the effects of a higher dose of inhaled PAF (225 µg) on methacholine bronchial responsiveness, circulating leucocytes, and *ex vivo* TNF $\alpha$  and IL-1 production from peripheral blood monocytes in mild allergic asthmatics.

## Materials and methods

### Subjects

Eight atopic asthmatics participated in this study. Patients characteristics are shown in table 1. Asthma was diagnosed on the basis of clinical history and methacholine bronchial hyperresponsiveness (provocative concentration causing a 20% decrease in forced expiratory volume in one second (PC20) <4 mg·ml<sup>-1</sup>). Atopic status was determined on the basis of clinical history and skin-prick tests to at least one of the common aeroallergens (*Dermatophagoides pteronyssinus*, cat dander, grass pollen, tree pollen, weed pollen, moulds).

### Study design

In a single-blind randomized manner, each subject successively underwent a PAF and a lyso-PAF bronchial challenge one month apart. One day prior to the PAF or the lyso-PAF exposure, a methacholine challenge was performed in order to define the basal PC20. The PC20 was subsequently measured 4, 24, 48, 72 and 168 h following PAF and lyso-PAF challenge. Blood samples were taken 5 min prior to PAF and lyso-PAF challenge to provide control values. Further blood samples were obtained immediately before each methacholine challenge. The blood samples were used to determine circulating leucocyte counts and cytokine production from adherent monocytes. Five of the eight subjects underwent bronchial challenge with saline; three others

were lost to follow-up. Analysis of the several parameters was performed according to the same protocol as described above. Bronchial challenges were always performed at 11 a.m., with the exception of the methacholine challenge carried out 4 h after the first challenge. Metered-dose inhaler of  $\beta_2$ -agonist was stopped at least 12 h before each bronchial test. No respiratory tract infection had occurred in any subject for at least 4 weeks before each study week. Each subject gave his/her written informed consent after the protocol has been approved by the local Ethics Committee.

### Methacholine challenge

Methacholine chloride solutions (biochemicals) were dissolved in saline solution, stored at 4°C, and used within 14 days after preparation. Bronchial responsiveness was assessed according to the method described by COCKROFT *et al.* [18] and using a compressed air nebulizer, the characteristics of which have been described previously [17]. Provided forced expiratory volume in one second (FEV<sub>1</sub>) did not fall by more than 10% of baseline after saline, doubling concentrations of methacholine (starting at 0.03 mg·ml<sup>-1</sup>) were inhaled every 5 min until a 20% fall in FEV<sub>1</sub> had occurred. FEV<sub>1</sub> was measured 30 s after each concentration and the best of three curves was recorded (Flow screen, Jaeger). The provocative concentration that produced a 20% fall in FEV<sub>1</sub> (PC20) was read from the log dose-response curve by linear interpolation.

### PAF, lyso-PAF and saline challenge

The PAF (1-0-alkyl-2-0-acetyl-sn-glycero-3-phosphorylcholine, Sigma) was purchased as 2 mg·ml<sup>-1</sup> solution in chloroform. PAF solutions were prepared on the morning of each study day as described previously [17]. Lyso-PAF (1-0-alkyl-sn-glycero-3-phosphorylcholine, Sigma) was purchased as 1 mg dry powder and was dissolved in saline to obtain solution. Aerosols were delivered as described above. Subjects successively inhaled 15 µg

(50  $\mu\text{g}\cdot\text{ml}^{-1}$ , 1 min), 30  $\mu\text{g}$  (50  $\mu\text{g}\cdot\text{ml}^{-1}$ , 2 min), 60  $\mu\text{g}$  (200  $\mu\text{g}\cdot\text{ml}^{-1}$ , 1 min) and 120  $\mu\text{g}$  (200  $\mu\text{g}\cdot\text{ml}^{-1}$ , 2 min) of PAF or lyso-PAF during quiet tidal breathing every 15 min, to achieve a cumulative dose of 225  $\mu\text{g}$ . In two subjects, the cumulative dose of PAF was limited to 45  $\mu\text{g}$  because of a fall in  $\text{FEV}_1$  of more than 35% after the second dose. Saline challenge consisted of four LP (NaCl 0.9% inhalations performed every 15 min. Measurements of specific airway conductance (sGaw) (plethysmography, Body test, Jaeger) followed by those of  $\text{FEV}_1$  and forced mid-expiratory flow (FEF<sub>25-75</sub>) (derived from forced flow volume curve, Flowscreen, Jaeger) were carried out 5 and 15 min after each dose of PAF, lyso-PAF or saline. In order to evaluate the acute airway obstruction, the changes in functional parameters were expressed as a percentage of baseline values.

#### *Preparation of human adherent monocytes*

Ten millilitres of fresh blood was collected from each donor. Blood samples were adjusted to a volume of 30 ml by addition of Hank's solution at pH 7.2, layered on Ficoll Hypaque gradient (Nye-gaard, Oslo, Norway) and centrifuged at  $400\times g$  for 30 min at 20°C. The supernatant was removed and the peripheral blood mononuclear leucocytes (PBML) in the interface were collected. The recovered PBML were washed twice with approximately 30 ml of Hank's solution, and suspended in RPMI-1640 (Gibco, UK) containing 5% heat-inactivated foetal calf serum (FCS) and examined for viability using 0.2% trypan blue. Two millilitres of PBML suspended at  $2\times 10^6$  cells $\cdot\text{ml}^{-1}$  in 5% FCS-RPMI-1640 was transferred to a Petri dish coated with normal human AB serum. After incubation in a 5%  $\text{CO}_2$  incubator at 37°C for 2 h, the supernatant was removed. The plate was washed three times with Hank's solution and the supernatant was replaced by 2 ml of 5% FCS-RPMI-1640. The final preparation contained more than 80% monocytes as judged by staining with Diff-Quick solution. Triplicates (200  $\mu\text{l}$ ) of adherent human monocytes (AHM) were incubated for 24 h (37°C, 5%  $\text{CO}_2$ ) in plastic microwells (Nunc, Denmark), in the presence or absence of lipopolysaccharide (LPS) (Sigma, St. Louis, Mo, USA) to a final concentration of 1  $\mu\text{g}\cdot\text{ml}^{-1}$ . After incubation, the supernatants were harvested and stored at -20°C until  $\text{TNF}\alpha$  and IL-1 determination.

#### *TNF $\alpha$ and IL-1 assays*

Cytokines were measured with specific enzyme-linked immunoassays (ELISA) from Medgenix Diagnostic (Medgenix Diagnostic, Fleurus, Belgium). The standard curves were run in human plasma since the samples contained culture medium (5% FCS-RPMI). In our hands, intra- and interassays coefficients of variation for  $\text{TNF}\alpha$  were 6 and 7%, respectively, and the limit of detection is 5  $\text{pg}\cdot\text{ml}^{-1}$ . Intra- and interassay coefficient of variation for IL-1 were 3 and 7%, respectively, and the limit of detection is 4  $\text{pg}\cdot\text{ml}^{-1}$ . There was no cross-reactivity between  $\text{TNF}\alpha$  and IL-1.

#### *Circulating cell counts determination*

Venous blood (5 ml) was taken into a vacutainer containing ethylenediamine tetra-acetic acid (EDTA). Total and differential leucocyte, platelet and red cell counts were determined employing an automatic haemocytometer (Technicon H1).

#### *Statistical analysis*

Results are expressed as mean $\pm$ SEM unless otherwise indicated. Comparison between the curves after PAF, lyso-PAF and saline were performed using Zerbe test for paired data, which allows both the assessment of differences between the two groups point-wise and over any time interval [19]. Assessment of the significance of changes in one variable within one treatment group over the week was made by repeated measure analysis of variance (ANOVA). Any significance detected by ANOVA was further investigated by Dunnett test in order to determine which time-point differs significantly from the baseline.

Changes in methacholine responsiveness, expressed in terms of doubling dilution, were calculated by the following ratio:  $\log_{10}$  (post-treatment  $\text{PC}_{20}$ /pretreatment  $\text{PC}_{20}$ )/ $\log_{10}^2$ . For the five subjects undergoing saline challenge, the individual 95% reference interval of the variation in  $\text{PC}_{20}$  was calculated by taking into account the values measured at baseline and during the week after saline challenge. This reference interval was derived from the mean and standard deviation of six measurements of  $\text{PC}_{20}$  expressed in doubling dilution against the geometric mean. A McNemar paired test was used to compare the percentage of  $\text{PC}_{20}$  value below 95% reference interval after PAF and lyso-PAF challenge.

Changes in haematological variables were transformed as a ratio of baseline before analysis by Zerbe test. Being not normally distributed, the values of spontaneous cytokine production were  $\log_{10}$  transformed before analysis. Correlation between two variables was assessed by calculating the Spearman rank coefficient.

## **Results**

#### *Acute airway obstruction*

Six of the eight subjects underwent the full bronchial provocation test inhaling 225  $\mu\text{g}$  of PAF. In two subjects, the cumulative dose of PAF was limited to 45  $\mu\text{g}$  because of a decrease in  $\text{FEV}_1$  >35% of control after the second dose (30  $\mu\text{g}$ ). Mean maximal decrease in sGaw,  $\text{FEV}_1$  and FEF<sub>25-75</sub> after inhalation of PAF reached  $52\pm 7\%$ ,  $15\pm 4\%$  and  $25\pm 6\%$ , respectively. By contrast, neither lyso-PAF nor saline produced any significant airway obstruction (data not shown). In each subject,  $\text{FEV}_1$  returned to within 5% of baseline value 90 min after the inhalation of the last dose of PAF.

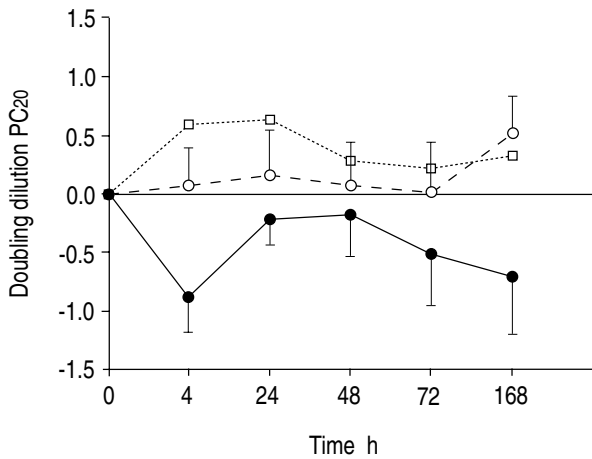


Fig. 1. — Variations in PC<sub>20</sub> over the week following PAF (—●—) (n=8), lyso-PAF (---○---) (n=8), and saline (····□····) inhalation in allergic asthmatics. Results are expressed as means±SEM except for saline, where SEM was omitted for clarity. Baseline geometric mean PC<sub>20</sub> the days before PAF, lyso-PAF and saline inhalation was 0.43, 0.49 and 0.43 mg·ml<sup>-1</sup>, respectively. PC<sub>20</sub>: provocative concentration of methacholine causing a 20% decrease in forced expiratory volume in one second; PAF: platelet-activating factor.

#### Modulation of methacholine airway responsiveness

There was no significant difference between all FEV<sub>1</sub> values measured before each methacholine bronchial challenge ( $F=0.29$ ;  $p>0.05$ ). As compared to lyso-PAF, inhalation of PAF caused a slight and sustained decrease in PC<sub>20</sub> (fig. 1). When considering the curves of the entire week, the differences between PAF and lyso-PAF, as well as between PAF and saline, were significant (Zerbe test,  $F=4.56$ ;  $p<0.05$ ; and  $F=6.48$ ;  $p<0.01$ , respectively). When looking at individual time-points, significant differences between PAF and lyso-PAF were found at 4 h ( $-0.88\pm 0.28$  doubling dilution after PAF vs  $0.08\pm 0.31$  after lyso-PAF) and at 168 h ( $-0.68\pm 0.48$  doubling dilution after PAF vs  $0.54\pm 0.31$  after lyso-PAF;  $p<0.05$ ). A significant difference between PAF and saline was only found at 4 h ( $-0.88\pm 0.28$  doubling dilution after PAF vs  $0.59\pm 0.58$  after saline;  $p<0.05$ ) whereas there was a strong trend at 168 h ( $-0.68\pm 0.48$  doubling dose after PAF vs  $0.35\pm 0.55$  after saline;  $p=0.06$ ).

Individual changes in PC<sub>20</sub> are shown in figure 2. Four out of five subjects had a PC<sub>20</sub> value below their 95% reference interval after PAF challenge, whilst this occurred

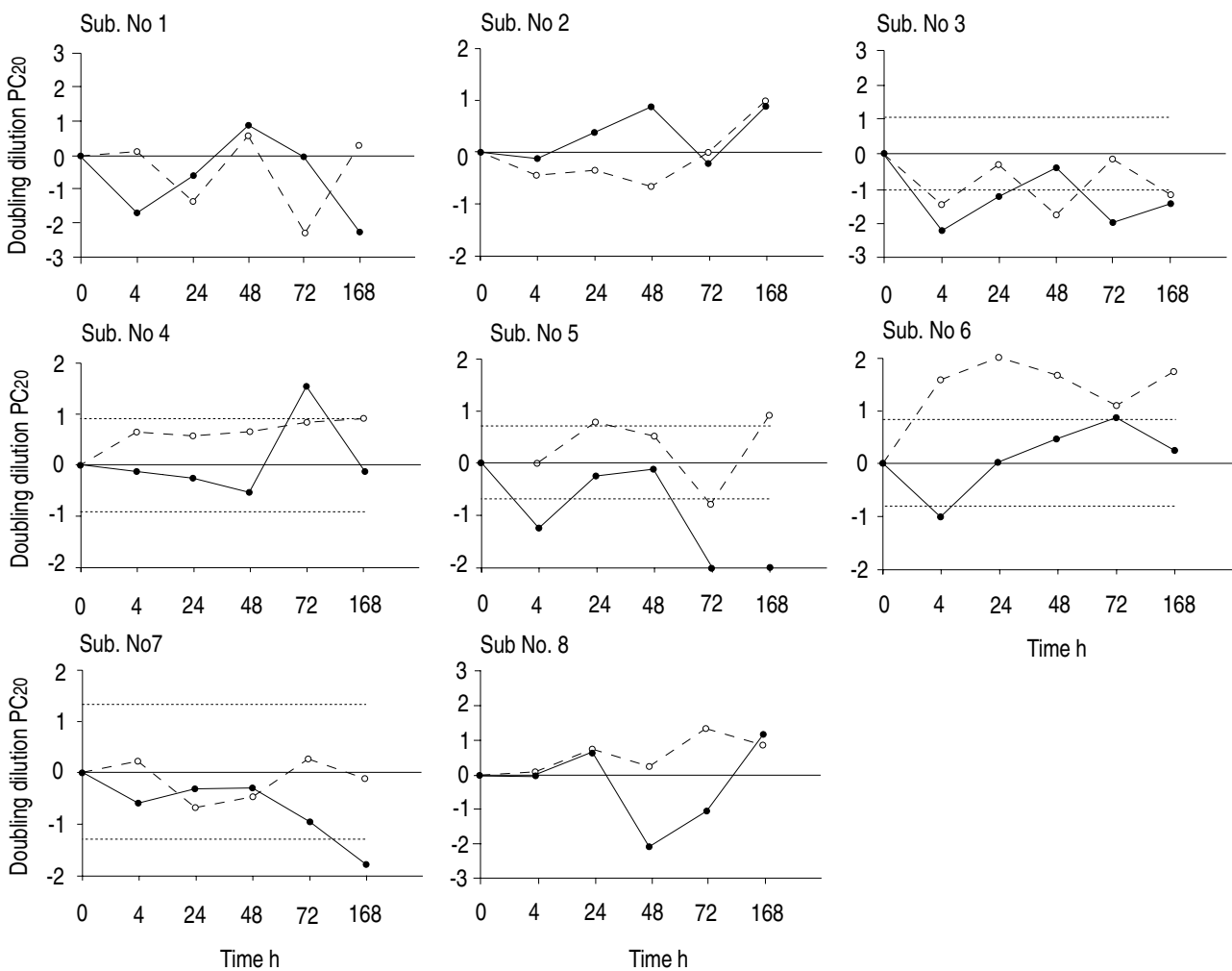


Fig. 2. — Individual variations in PC<sub>20</sub> over the week following PAF (—●—) and lyso-PAF (---○---) inhalation. Dotted lines (····) represent the 95% reference interval for the variations in PC<sub>20</sub> in five subjects calculated from data obtained after saline. Sub: subject. For further abbreviations see legend to figure 1.

Table 2. – Spontaneous and LPS-induced production of TNF- $\alpha$  from peripheral blood monocytes

	T 0 pg·ml <sup>-1</sup>	4 h pg·ml <sup>-1</sup>	24 h pg·ml <sup>-1</sup>	48 h pg·ml <sup>-1</sup>	72 h pg·ml <sup>-1</sup>	168 h pg·ml <sup>-1</sup>	
<b>a) Spontaneous production of TNF-<math>\alpha</math></b>							
PAF	186	501	630*	645*	616*	316	F=5.65
n=8	(10–1606)	(38–5006)	(18–4927)	(57–3900)	(82–2700)	(39–2536)	p<0.001
Lyso-PAF	224	967	876	1000	744	696	F=1.97
n=8	(49–5500)	(108–26678)	(84–24958)	(49–13068)	(40–10266)	(167–12000)	p>0.05
Saline	262	1315*	952	2747*	1905*	50	F=13
n=5	(75–2750)	(370–13360)	(320–12495)	(1071–6028)	(684–5540)	(25–776)	p<0.0001
<b>b) LPS-induced production of TNF-<math>\alpha</math></b>							
PAF	24101	31892	25333	25377	27822	24554	F=0.77
n=8	$\pm$ 3839	$\pm$ 3933	$\pm$ 3008	$\pm$ 4174	$\pm$ 5578	$\pm$ 5891	p>0.05
Lyso-PAF	21605	31964	19872	33357	26071	29385	F=2.07
n=8	$\pm$ 1841	$\pm$ 5388	$\pm$ 4690	$\pm$ 7256	$\pm$ 3218	$\pm$ 4368	p>0.05
Saline	23052	46677	30645	40927	39795	27889	F=1.45
n=5	$\pm$ 6785	$\pm$ 7171	$\pm$ 9018	$\pm$ 13267	$\pm$ 6718	$\pm$ 7951	p>0.05

Results for spontaneous production of TNF- $\alpha$  are expressed as geometric mean and range in parenthesis. Results for LPS-induced production of TNF- $\alpha$  are expressed as arithmetic means $\pm$ SEM. \*: indicates a significant difference (p<0.05) in comparison with T 0 (Dunnet test after repeated one-way ANOVA). T 0: baseline; 4–168 h = 4–168 h following challenge; PAF: platelet-activating factor; LPS: lipopolysaccharide; TNF- $\alpha$ : tumour necrosis factor- $\alpha$ ; ANOVA: analysis of variance.

in only two of five subjects after lyso-PAF. Furthermore, considering the five postbronchial challenge measurements in the five subjects, the number of PC<sub>20</sub> values below the lower limit of 95% reference interval was greater after PAF (9 out of 25) than after lyso-PAF (3 out of 25) (p<0.01).

There was no correlation between both the magnitude of maximal fall in FEV<sub>1</sub> and the decrease in PC<sub>20</sub> 4 h after PAF inhalation ( $r_s = 0.21$ ; p>0.05).

#### Modulation of TNF $\alpha$ and IL-1 production from peripheral blood monocytes

Spontaneous production of TNF $\alpha$  by peripheral blood monocytes was significantly higher by 24, 48 and 72 h after PAF and by 4, 48 and 72 h after saline challenge as compared to baseline. There was a trend to an increased

production of TNF $\alpha$  during the week after lyso-PAF challenge (table 2a). However, no significant difference could be observed between spontaneous secretion of TNF $\alpha$  after PAF, lyso-PAF and saline challenge. In addition, LPS-stimulated TNF $\alpha$  production by the cells did not change significantly during the week following PAF, lyso-PAF or saline bronchial challenge (table 2b).

Spontaneous production of IL-1 from blood monocytes increased significantly after PAF challenge, with a maximal augmentation at 72 h as compared to baseline. In contrast, despite a trend to a raised spontaneous production of IL-1 both after lyso-PAF and saline, this increase did not achieve significance at any time-point. However, no significant difference could be observed between spontaneous IL-1 secretion after PAF, lyso-PAF and saline (table 3a). There was no significant change in LPS-stimulated IL-1 production (table 3b).

Table 3. – Spontaneous and LPS-induced production of IL-1 from peripheral blood monocytes

	T 0 pg·ml <sup>-1</sup>	4 h pg·ml <sup>-1</sup>	24 h pg·ml <sup>-1</sup>	48 h pg·ml <sup>-1</sup>	72 h pg·ml <sup>-1</sup>	168 h pg·ml <sup>-1</sup>	
<b>a) Spontaneous production of IL-1</b>							
PAF	48	65	143	138	161*	92	F=3.19
n=8	(6–334)	(9–378)	(16–1038)	(42–410)	(98–570)	(29–339)	p<0.05
Lyso-PAF	126	240	220	234	74	95	F=1.88
n=8	(26–814)	(30–5814)	(24–8533)	(54–3337)	(25–1866)	(23–1200)	p>0.05
Saline	263	575	446	691	144	100	F=2.64
n=5	(72–705)	(102–5473)	(81–7943)	(171–3825)	(57–2242)	(58–978)	p=0.05
<b>b) LPS-induced production of IL-1</b>							
PAF	15170	18371	13515	13591	18542	11166	F=1.43
n=8	$\pm$ 4385	$\pm$ 3192	$\pm$ 3140	$\pm$ 2559	$\pm$ 5718	$\pm$ 1355	p>0.05
Lyso-PAF	13937	19725	13215	17727	16488	20596	F=0.53
n=8	$\pm$ 2025	$\pm$ 2129	$\pm$ 2944	$\pm$ 2789	$\pm$ 2369	$\pm$ 3169	p>0.05
Saline	14845	24936	20146	18120	18157	19258	F=0.78
n=5	$\pm$ 4728	$\pm$ 1932	$\pm$ 4978	$\pm$ 3028	$\pm$ 1426	$\pm$ 6819	p>0.05

Results for spontaneous production of IL-1 are expressed as geometric mean and range in parenthesis. Results for LPS-induced production of IL-1 are expressed as mean $\pm$ SEM. \*: indicates significant difference as compared with baseline (Dunnet test after repeated measure one-way ANOVA). T 0: baseline; 4–168 h: 4–168 h following challenge. For abbreviation see legend to table 2.

### Variations in circulating blood cells

The variations in circulating leucocytes following PAF and lyso-PAF challenge were characterized by a transient increase at 4 h, followed by a fall at 24 h and a persistent decrease until 168 h (fig. 3a). After saline inhalation, circulating leucocyte counts progressively decreased until 168 h. These variations were essentially due to neutrophils (fig. 3b). The fall in circulating neutrophils by 72 and 168 h after PAF challenge was

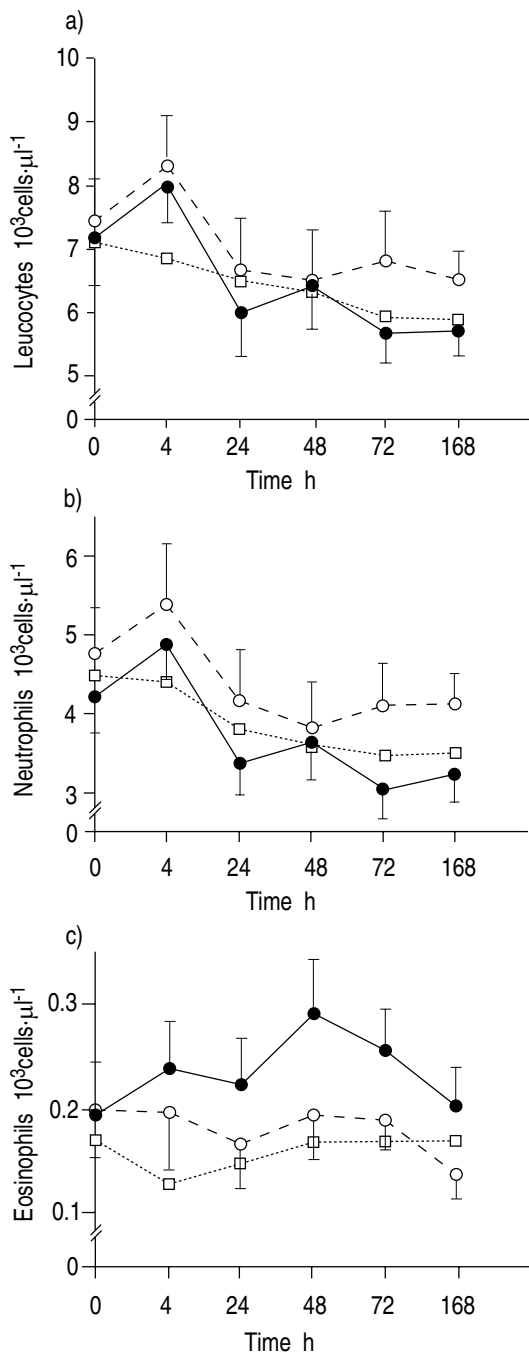


Fig. 3. — Variations in circulating leucocyte counts over the week following PAF (●—●) (n=8), lyso-PAF (○- -○) (n=8), and saline (□····□) (n=5) inhalation in allergic asthmatics. a) total leucocyte counts; b) neutrophils; c) eosinophils. Results are expressed as mean $\pm$ SEM, except for saline where SEM was omitted for clarity. Note that vertical axes are cut-off from zero. PAF: platelet-activating factor.

significant as compared to baseline ( $F=8.12$ ;  $p<0.001$ ). Following lyso-PAF challenge, there was not significant change in circulating neutrophil counts as compared to baseline. However, the count measured by 48 h was significantly lower than that measured by 4 h ( $F=3.87$ ;  $p<0.01$ ). After saline inhalation, there was a progressive decline in neutrophil counts over the week ( $F=2.54$ ;  $p=0.06$ ). Overall, there was no significant difference between the changes in neutrophils observed after PAF, lyso-PAF and saline bronchial challenge.

In contrast, there was a striking and significant difference between PAF and lyso-PAF, as well as between PAF and saline, with respect to the variations in circulating eosinophil counts over the week (Zerbe test,  $F=4.26$ ;  $p<0.05$  and  $F=3.93$ ;  $p<0.05$ ) (fig. 3c). Inhaled PAF, but neither lyso-PAF nor saline, resulted in a sustained increase in eosinophil counts which was maximal at 48 h (from  $196\pm39 \text{ cells} \cdot \mu\text{l}^{-1}$  at baseline to  $293\pm50 \text{ cells} \cdot \mu\text{l}^{-1}$  after PAF vs from  $193\pm139 \text{ cells} \cdot \mu\text{l}^{-1}$  to  $186\pm137 \text{ cells} \cdot \mu\text{l}^{-1}$  after lyso-PAF vs from  $172\pm38 \text{ cells} \cdot \mu\text{l}^{-1}$  to  $172\pm33 \text{ cells} \cdot \mu\text{l}^{-1}$  after saline). No correlation was observed between the maximal percentage increase in circulating eosinophil counts at 48 or 72 h and the maximal decrease in PC20 between 24 and 168 h. Changes in other circulating leucocytes, platelets and erythrocytes were small and not significantly different following the three treatment regimens (data not shown).

### Discussion

Our study shows that inhalation of a dose of 225  $\mu\text{g}$  of PAF caused a slight and sustained increase in methacholine bronchial responsiveness in mild allergic asthmatics. Overall, we could separate two waves of increased methacholine bronchial responsiveness. The first appeared 4 h after the PAF challenge and was followed by an almost complete resolution at 24 h. The second slowly evolving increase in responsiveness was observed at day 7.

Our results partly confirm those obtained by HOPP and co-workers [11], who found a transient increase in methacholine responsiveness 2 h after a mild PAF challenge (30  $\mu\text{g}$ ) in asthmatics, but are apparently in contrast with those reported by CHUNG and BARNES [10]. In their study however, Chung and Barnes did not evaluate the methacholine bronchial responsiveness at 4 h, and they found a slight and progressive, but not significant, decrease in bronchial responsiveness between day 1 and day 7 after inhalation with a moderate dose of PAF (132  $\mu\text{g}$ ). Furthermore, our data, like those of CHUNG and BARNES [10], revealed that individual curves showed a high variability regarding their profile over the week.

Our data suggest that the two waves of increase in methacholine bronchial responsiveness may result from different mechanisms. The rise in responsiveness observed at 4 h is not related to residual airway obstruction after PAF-induced acute bronchial response, since there was no significant difference between FEV<sub>1</sub> values measured before each methacholine challenge. Furthermore, there was no correlation between the degree of acute airway obstruction after PAF challenge and the magnitude of decrease in methacholine responsiveness at 4 h. Hence,

the first increase in bronchial responsiveness might be due to local attraction and activation of the granulocytes in the lung as a direct consequence of the chemotactic and stimulating property of PAF [5, 20, 21]. By contrast, the delayed increase in nonspecific bronchial responsiveness, a phenomenon previously described in normal subjects [4–6], remains difficult to explain.

Recently, SMITH *et al.* [22] reported that the magnitude of increase in methacholine bronchial responsiveness was correlated to the level of BAL neutrophil counts measured 24 h after PAF challenge. However, these authors did not mention the time at which the increase in responsiveness occurred. Conversely, WARDLAW *et al.* [5] reported an inverse relationship between the level of BAL neutrophil counts at 4 h and the magnitude of increase in responsiveness at 72 h following bronchial challenge. When compared with those induced by an allergen, the changes in methacholine bronchial responsiveness after PAF inhalation were smaller in magnitude and exhibited a different time-course [23].

Since alveolar macrophages, the most abundant cells in airway tract, are believed to originate from blood monocytes [24], we thought it would be of some interest to evaluate the activation state of monocytes following PAF bronchial challenge. Both increased TNF $\alpha$  and IL-1 spontaneous secretion occurred after PAF as well as lyso-PAF and saline challenge. In addition, when monocytes were stimulated *in vitro* by LPS, no significant change in TNF $\alpha$  and IL-1 secretion was observed throughout the week. Since there is an increase in spontaneous cytokine release in the patients with repeated bronchial challenges, the variations in cytokine production may be related to the multiple procedure. Whether these changes are the consequence of either the multiple bronchoconstrictions caused by methacholine or the multiple aerosolizations themselves, has not been addressed in this study.

The variations in TNF $\alpha$  and IL-1 production could contribute to the changes in circulating neutrophil counts observed during the week. For instance, TNF $\alpha$  has been reported to inhibit the proliferation of myeloid cells *in vitro* [25], and both IL-1 and TNF $\alpha$  are potent inducers of expression of adhesion molecules on vascular endothelium [26]. Therefore, decreased level in neutrophils could be related to a decline in the bone marrow production and/or a higher proportion of cells adherent to vascular wall and, thereby, not detectable in blood sample. Since increased production of TNF $\alpha$  and IL-1 as well as neutropenia occurred after the three types of bronchial challenge, our results indicate that neither the change in cytokine production nor that in circulating neutrophil counts directly account for the variations in methacholine bronchial responsiveness after PAF challenge. However, we cannot rule out the possibility that a trapping of neutrophils in the lung, favoured by increased production of IL-1 and TNF $\alpha$ , may constitute a necessary step for the development of bronchial hyperresponsiveness. Indeed, the activation state of the recruited neutrophils probably depends on the local pulmonary environment, which might be influenced by the type of mediator previously inhaled. Thus, studies examining markers of neutrophil

activation in the airways may be helpful to clarify this point.

In contrast to lyso-PAF or saline, inhaled PAF results in a prolonged increase in circulating eosinophils. Delayed eosinophilic tissue infiltration following local PAF exposure has been reported previously in several species, including man [27–30]. Tissue eosinophil accumulation depends on the migration of the cells from the circulation; our findings suggest that PAF inhalation may promote blood eosinophilia and a delayed tissue accumulation. PAF has recently been shown to induce eosinophil proliferation from both murine and human haematopoietic cells [30, 31]. In the murine model, this effect is induced by a still unidentified intermediate that is different from interleukin-3 (IL-3), granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-5 (IL-5) [30]. In human haematopoietic precursor cells from umbilical cord blood, IL-3 contributes to this phenomenon [31]. In our study, we did not evaluate the production of IL-5, GM-CSF and IL-3, a set of cytokines involved in eosinophil proliferation and maturation [32, 33]. However, the fact that eosinophils were the only type of leucocytes whose numbers rose in the blood of our study subjects, suggests that the factor responsible may be specific for eosinophils and, therefore, different from GM-CSF or IL-3, which also strongly promote other cell lines. Whether blood eosinophilia following PAF inhalation would also occur in normal subjects remains to be determined, though a previous study showed that skin eosinophilia after local PAF exposure was restricted to atopics [27].

In both atopic and nonatopic asthmatics, bronchial hyperresponsiveness has been correlated with an increased level of circulating eosinophils [34]. In contrast, we did not find any correlation between the percentage increase in blood eosinophils and the magnitude of the decrease in PC20 between 24 and 168 h. Furthermore, the rise in circulating eosinophil counts has been shown to remain low in comparison with that following allergen inhalation [35]. However, the numbers of eosinophils within the lung may be different from that in the blood, and both the cell activation state and the release of mediators may be even more important than the eosinophil count alone. In non-human primates, WEGNER *et al.* [29] reported a marked increase in BAL eosinophil counts 3 days after multiple PAF inhalation, but they failed to find any correlation between the level of eosinophil influx in the lung and the magnitude of decrease in airway responsiveness to methacholine. Clearly, further studies focusing on markers of airways inflammation are warranted to elucidate the mechanism by which PAF produces its delayed bronchial effects in man.

In conclusion, the data presented here show that PAF causes an early and a late increase in methacholine bronchial responsiveness accompanied by a delayed rise in circulating eosinophils. Thus, our data support the hypothesis that PAF plays a role in the development of bronchial hyperresponsiveness in asthmatics.

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