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DESENSITIZATION TO PAF-INDUCED BRONCHOCONSTRICTION AND TO ACTIVATION OF ALVEOLAR MACROPHAGES BY REPEATED INHALATIONS OF PAF IN THE GUINEA PIG

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Guinea-pig alveolar macrophages are activated in the presence of PAF-acether (PAF), as shown by $O_2^{\cdot -}$ production, suggesting that these cells, abundant in the lungs, are involved in PAF-induced bronchoconstriction. Alveolar macrophages collected after *in vivo* desensitization to the bronchoconstrictor effect of PAF became refractory to it *in vitro*, whereas the $O_2^{\cdot -}$ production in response to f-met-leu-phe persisted, although it was diminished suggesting a partial cross-desensitization. A similar desensitization to PAF was also observed in alveolar macrophages *in vitro*, demonstrating a stimulus-specific process. This study suggests that alveolar macrophages may be involved in bronchoconstriction induced by aerosol of PAF.

PAF-acether (Platelet activating factor, PAF), a putative mediator of anaphylaxis (1,2), induces platelet aggregation and secretion of their granule constituents. PAF stimulates the arachidonic acid metabolism in many cells (3) and triggers superoxide production by neutrophils and macrophages (5, 6). Intravenous PAF induces bronchoconstriction (BC), neutropenia and thrombocytopenia in the guinea pig, which are cyclooxygenase-independent (7). In this case BC is platelet-dependent, but when PAF is inhaled, BC is induced by a platelet-independent mechanism (8). If mast cells were involved in this process, release of histamine would be expected. However, as mepyramine, an H_1 receptor antagonist, had little effect on BC induced by PAF aerosol (8) it seems unlikely that these cells participate in the platelet-independent mechanism. Alveolar macrophages, which are the most numerous cells on the air surface interface (9) constitute the first line of defense for lungs. These cells release substances which cause the contraction of isolated bronchi (10, 11). Therefore, one possible mechanism for PAF-induced broncoconstriction is

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in situ activation of alveolar macrophages. Furthermore, alveolar macrophages can be stimulated by IgE (11-13), and may thus be important for bronchial asthma.

We now investigated whether alveolar macrophages are involved in BC by aerosolized PAF, using the generation of superoxide anions ($O_2^{\cdot -}$) as an index of activation. The following questions were addressed : is desensitization by and to repeated PAF inhalations concomittant with macrophage deactivation? In this case, is macrophage deactivation stimulus-specific for PAF? And finally, is ex vivo desensitization paralleled with a similar effect in vitro?

MATERIALS AND METHODS

Sodium pentobarbitone (Nembutal) was from Lathevet, France; Propranolol was from ICI; Pancuronium (Pavulon) was from Organon, France; PAF (1-alkyl-2-acetyl-sn-glycero-3-phophorylcholine) was kindly provided by Prof. J.J. Godfroid (University of Paris VII), 5-hydroxytryptamine (5HT), bovine serum albumin (Fraction V), lidocaine and N-formyl-methionyl-leucyl-phenylalanine (FMLP) were from Sigma Chemical Co, St Louis, MO; ferricytochrome c was from Boehringer, Mannheim, Germany; superoxide dismutase (SOD, from bovine erythrocytes, 3500 UI/mg) was from Serva, Germany; Eagle's Minimal essential medium (MEM) was from Eurobio, Paris, France. All other chemical products were of analytical grade.

Stock solutions of PAF were dissolved in MEM solution containing 0.25 % bovine serum albumin. FMLP was dissolved at 10 mM in dimethylsulfoxide and stored at -20°C. Dilutions of the stock solution were prepared fresh for each experiment.

Guinea pigs (300-400 g) were anesthetized by the intraperitoneal injection of sodium pentobarbitone (30 mg/kg). The carotid artery, the jugular vein and the trachea were cannulated and ventilation was started (Palmer miniature respiratory pump, 60 strokes/min), spontaneous breathing being arrested with pancuronium (4 mg/kg, iv). Bronchial resistance was recorded as previously described (14) and sensitivity was checked with 5HT (1 µg, iv). After constant responses were obtained, the aerosol of PAF (330 µg/ml in the aerosolator) was started and continued for 2 minutes as previously described (8). This was performed three times at 45 min intervals. In control experiments the vehicle was aerosolized to paired control animals.

Alveolar macrophages were obtained 10 min after the third aerosol by eight to ten repeated lung lavages with 5 ml of sterile phosphate buffered saline , pH 7.4 at 37°C, containing 10 mM lidocaine. Cells were then thoroughly washed and resuspended in MEM supplemented by 20 mM Hepes, pH 7.4 at 37°C. In control experiments we observed that macrophages collected in absence or in presence of lidocaine reacted similarly. The cell suspension contained more than 90 % of alveolar macrophages as shown histochemically (15). Viability (>90%) was assessed by Trypan blue exclusion and the cell number was adjusted to 3×10^6 viable cells/ml MEM. The cells were incubated with the suspension medium at 37°C for 2 hours. Macrophages were stimulated in presence of 100 µM cytochrome c by adding either PAF or FMLP in the spectrophotometer's

cuvette thermostated at 37°C. Production of $O_2^{\cdot}$ was measured by the superoxide dismutase-inhibitable reduction of cytochrome c, the absorbance changes being recorded at 550 nm. The amount of reduced cytochrome c was calculated using an extinction coefficient of 21.1 mM^{-1} cm^{-1} (16). In a few experiments, PAF or FMLP were added successively in the cuvette to the macrophage suspension, and formation of $O_2^{\cdot -}$ was monitored. When in vitro desensitization was studied, cells were collected and washed as indicated above and incubated for thirty minutes in MEM medium in presence of PAF (0.01 and 1 µM). Cells were washed again twice, and resuspended in MEM for two hours before stimulation.

RESULTS

Bronchoconstriction (BC) started within 0.5-1 min after the first administration of PAF by aerosol which induced a partial desensitization to a second administration. When PAF was inhaled for a third time, bronchoconstriction was desensitized almost completely (Fig.1). During this experiment, peripheral arterial blood was collected for the determination of the number of circulating platelets and leukocytes, which did not vary. Desensitization was specific to PAF since the responses to 5HT were maintained. An aerosol of the vehicle had no effect on intrapulmonary pressure.

Non-desensitized macrophages generated more $O_2^{\cdot -}$ when stimulated in vitro with 1µM than with 0.01 µM of PAF or of FMLP (Fig.2). This figure also shows that when the macrophages were collected from animals desensitized to the bronchoconstrictor effects of PAF, the formation of

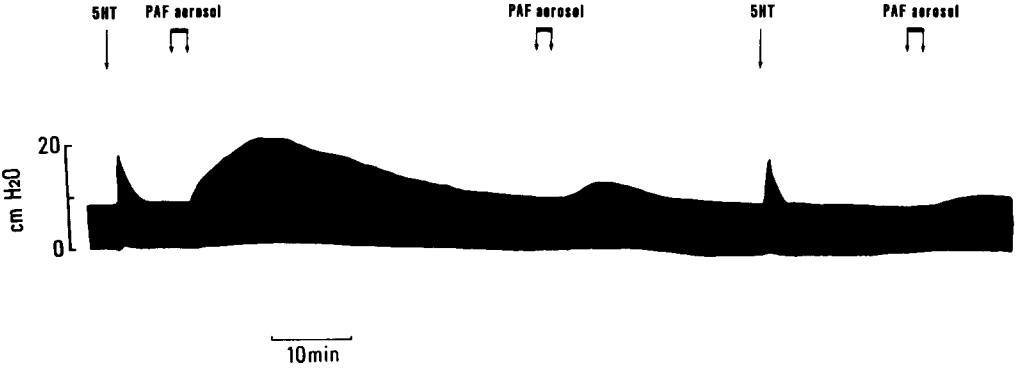

Figure 1
Desensitization to the bronchoconstrictor effect of aerosolized PAF. Bronchial resistance to inflation (scale = 20 cm H_2O) of a guinea pig was recorded. 1 µg of 5 hydroxytryptamine (5HT) was given iv as a control bronchoconstrictor agent. PAF was aerosolised for 2 min at 45 min intervals, as indicated in "Methods".

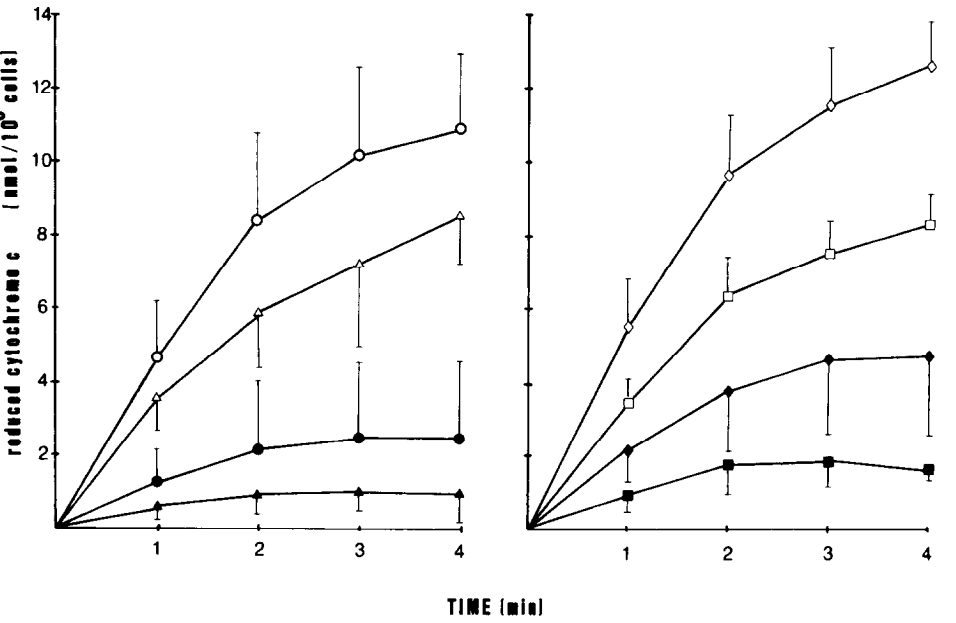

Figure 2
In vitro production of superoxide anions by macrophages collected after three successive inhalations of PAF, as in Figure 1, or of the vehicle.
Alveolar macrophages were collected by lung lavages, incubated and stimulated as described in "Methods". Superoxide dismutase-inhibitable cytochrome c reduction was measured at 550 nm.
Left panel: PAF (μM): ○ ● 1; △ ▲ 0.01.
right panel: FMLP (μM): ◇ ◆ 1; □ ■ 0.01.
open and closed symbols represent control and PAF-desensitized macrophages, respectively.
Results are expressed as mean ± SD of four or five experiments.

$O_2^{\cdot -}$ by PAF and by FMLP was decreased. This suggested that desensitization was stimulus-independent. Nevertheless, as shown in Fig. 3 with the appropriate scale, when the macrophages were stimulated in vitro after an interval of 6 instead of 2 hours, the responses to FMLP had partially recovered, whereas desensitization to PAF persisted. It is important to note that the responses to PAF and to FMLP of non-desensitized macrophages were not modified by the 6 hours incubation.

These results suggested that desensitization by PAF was, at least in part, stimulus-dependent, and this was further investigated. PAF (0.1 nM-10 nM) and FMLP (10 nM) were added to the cell suspension successively, at intervals selected according to the time required to reach the plateau of the previous stimulation, which varied from 2-9 minutes. In order to minimize unspecific cell deactivation, PAF was used at the lowest possible

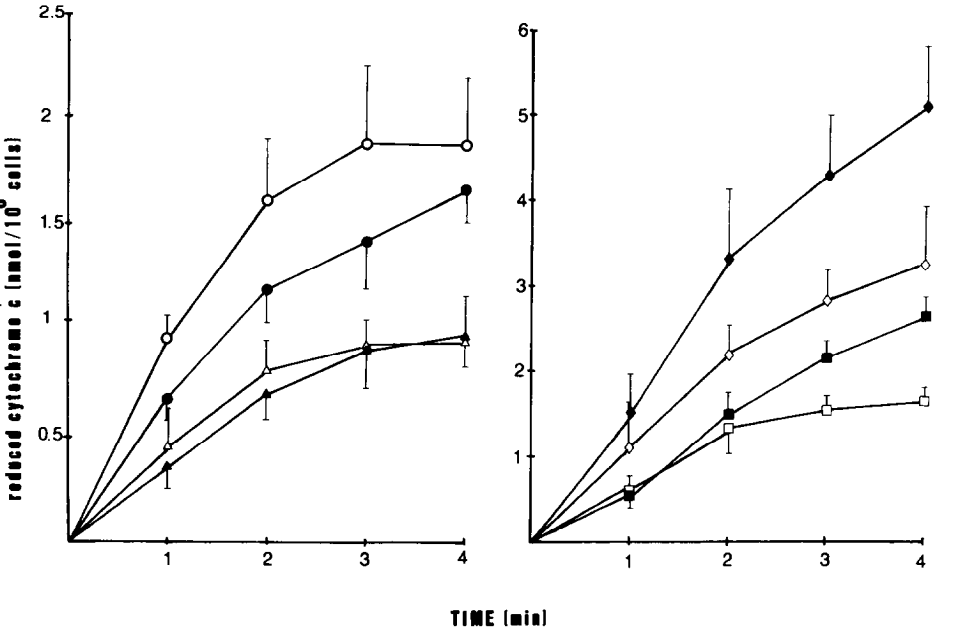

Figure 3
In vitro production of superoxide anions by macrophages collected after successive inhalations of PAF and stimulated with PAF or with FMLP after incubation at 37°C for 2 (open symbols) or 6 hours (closed symbols).
Left panel: PAF (μM): ○ ● 1; △ ▲ 0.01.
right panel: FMLP (μM): ◇ ◆ 1; □ ■ 0.01.
Results are expressed as mean ± SD of three experiments.

concentration that provided a signal. Table 1 demonstrates that macrophages were auto-desensitized to similar concentrations of PAF after a single exposure, and desensitized to 100 fold higher concentrations used as a third stimulus. Under these conditions, the responses to FMLP although reduced, persisted (Table 1).

In order to mimic as much as possible the in vivo situation, control cells were incubated for thirty minutes with 0.01 or 1 μM of PAF, and then washed. As shown in Table 2, preincubation with PAF (1 μM) induced a complete desensitization to PAF itself, whereas the response to FMLP was unaffected. Preincubation with PAF at the lower concentration (0.01 μM) was able to induce a marked desensitization to 0.01 μM and to 1 μM PAF.

DISCUSSION

Successive application of PAF as aerosols led to desensitization to bronchoconstriction and of the alveolar macrophage responses. Macrophages

Table 1

Acute *in vitro* desensitization of alveolar macrophages to PAF

(A)		(B)	
Drug addition (nM)	reduced cytochrome c (nmol/10^6 cells)	Drug addition (nM)	reduced cytochrome c (nmol/10^6 cells)
1) 0.1 PAF	2.21 ± 0.65		
2) 0.1 PAF	0		
3) 10 PAF	0.41 ± 0.28	10 PAF	6.51 ± 3.93
4) 10 FMLP	3.59 ± 0.98	10 FMLP	7.95 ± 1.82

(A): successive additions of drugs. Values represent the amount of reduced cytochrome c obtained at the time necessary for the maximal response to the indicated agonist: 1) 9 min; 2) 2 min; 3) 5 min; 4) 7 min. (B): single additions of drugs. Values represent the amounts of reduced cytochrome c produced 5 min after the agonist addition (time for reaching the plateau). Results are expressed as mean±SD of 3-6 experiments.

collected from PAF-desensitized lungs and stimulated 2 hours later by FMLP exhibited a partial desensitized response. This desensitization was reversed, at least in part, when the stimulation was performed 6 hours after the collection of cells while refractoriness to PAF persisted. The experimental

Table 2

In vitro desensitization of alveolar macrophages to PAF

	Reduced cytochrome c (nmol/10^6 cells)		
Drug addition (μM)	control	preincubation with 0.01 μM PAF	preincubation with 1 μM PAF
0.01 PAF	2.05 ± 0.30	0.56 ± 0.18	0.17 ± 0.13
1.0 PAF	4.16 ± 0.95	0.82 ± 0.62	0.19 ± 0.16
0.01 FMLP	4.85 ± 1.78	5.19 ± 2.68	5.48 ± 1.70
1.0 FMLP	13.02 ± 4.53	12.48 ± 3.04	12.84 ± 3.43

Cells were preincubated with the indicated concentrations of PAF for 30 min and washed twice. After 2 hours at 37°C cells were stimulated by the addition of the indicated concentrations of PAF or of FMLP, and the amounts of reduced cytochrome c produced after 5 min are expressed as mean±SD of 4 experiments.

conditions did not allow the observation of the responses after longer intervals and to look for a better dissociation between stimulus-specific and unspecific desensitization. For this reason the in vitro experiments were performed. When PAF was used and followed by PAF itself, macrophage activation was prevented, but some cross-desensitization to FMLP appeared. This effect was not the result of a chemical reaction between FMLP and PAF in the incubation medium. Indeed, when the response to PAF was blocked by PAF-antagonists, no desensitization to FMLP was observed (unpublished results) suggesting that reduction of the macrophage response observed in Table 1 is of metabolic origin.

However, since this protocol did not allow to distinguish specific from unspecific deactivation, macrophages were exposed to PAF and washed before restimulation. Under these conditions, no cross desensitization was seen, whereas the effect of PAF was suppressed. The in vitro exposure to PAF alone thus only duplicates to a limited extent the in vivo situation. Since in vivo PAF partially desensitized to in vitro FMLP, it is likely that formation of substances which desensitized to FMLP should occur in vivo, when PAF is aerosolized.

Alveolar macrophages can be activated by occupancy of IgE receptors (12, 13, 17) and, when challenged with specific allergens, human alveolar macrophages form PAF (18) which could account, at least in part, for allergic BC. Our results now demonstrate that in vitro exposure of macrophages to PAF activates the cells as shown by the generation of superoxide anions by the stimulus-dependent membrane associated NADPH-oxidase. A similar in vivo macrophage activation is likely to be accompanied by the formation of mediators involved in BC. In fact, in unpublished experiments we observed the release of thromboxane B_2 by PAF-stimulated alveolar macrophages, as reported for elicited guinea-pig peritoneal macrophages (6). Free radicals formed by stimulated macrophages may amplify BC, through the formation of eicosanoids (19, 20), some of which (TxA_2 , PGF_{2a} , PGD_2 , leukotrienes) are

bronchoconstrictor agents or, alternatively through deterioration of beta-adrenergic functions (21).

In summary, our results demonstrate that alveolar macrophages are stimulated by PAF and that in vivo BC induced by PAF aerosol is likely to result from macrophage activation. Work in progress will demonstrate wether similar events occur when antigen replaces PAF as a stimulus.

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