Effects of PAF, FMLP and opsonized zymosan on the release of ECP, elastase and superoxide from human granulocytes

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Effects of PAF, FMLP and opsonized zymosan on the release of ECP, elastase and super-oxide from human granulocytes. N.C. Turner, L.J. Wood, M. Foster, T. Gueremy. ©ERS Journals Ltd 1994.

ABSTRACT: Platelet-activating factor (PAF) is a potent chemoattractant for human eosinophils and neutrophils and causes eosinophil and neutrophil recruitment into animal airways. Since eosinophils and eosinophil cationic proteins are thought to play an important role in the pathophysiology of asthma, we have examined the hypothesis that PAF may also stimulate eosinophil cationic protein (ECP) release from human granulocytes.

Granulocytes (93% neutrophils, 3% eosinophils) were isolated from the blood of normal volunteers, using metrizamide density gradients, and stimulated *in vitro* with PAF, L-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) or opsonized zymosan (OPZ). Superoxide generation was measured colorimetrically, granulocyte degranulation by a fluorimetric assay for elastase, and eosinophil activation by specific radioimmunoassay (RIA) for ECP. Granulocyte chemotaxis was also measured.

Whilst both PAF and FMLP were potent chemoattractants for human mixed granulocytes (concentrations producing half the maximal effect (EC_{50}s) *ca* 10 nM), PAF at concentrations below 10 μM was a poor stimulus to superoxide generation, elastase release or ECP release from the same cell population. In contrast, FMLP was a potent stimulus to both superoxide generation (EC_{50} 48 nM) and ECP (EC_{50} *ca* 100 nM) and elastase release (EC_{50} *ca* 1 μM). OPZ was a potent stimulus to superoxide generation, but was a poor stimulus to ECP or elastase release.

Thus, although PAF is a potent chemoattractant for human granulocytes, our results suggest that it alone may not stimulate their subsequent activation and release of cytotoxic products.
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Platelet-activating factor (PAF) is an acetylated ether phospholipid which exhibits a spectrum of potent biological activities that have implicated it as an important mediator of inflammatory reactions. These effects include vasodilatation, increases in microvascular permeability, haemoconcentration, the directed migration of granulocytes, and circulating thrombocytopenia and neutropenia [1–3]. In addition, PAF has been reported to induce a long-lasting nonspecific increase in bronchial reactivity to inhaled spasmogens, in man [4–6] and in experimental animals [7], which is associated with airway eosinophilia [8, 9]. Since bronchial hyperreactivity and airways inflammation, particularly eosinophilia, are the salient pathological features of asthma [10], this has led to the suggestion that PAF may play a central role in this disease.

Among the battery of cytotoxic products generated by inflammatory cells are oxygen free radicals, lysosomal enzymes and cationic proteins. Eosinophil cationic proteins are increased in the serum and bronchoalveolar lavage fluid from asthmatics [11], and have been de-

monstrated to be cytotoxic to the respiratory epithelium and to cause hyperreactivity of respiratory smooth muscle [12, 13]. Furthermore, accumulation of eosinophils in the airway has been observed after antigen-challenge [14], and is one of the characteristic morphological features of asthmatic airways [15]. Thus, there is circumstantial evidence which suggests that eosinophil products and, particularly, cationic proteins contribute to the damage to the epithelium and airways hyperreactivity seen in asthma.

PAF is synthesized by a variety of haematopoietic cells, including monocytes, macrophages, eosinophils, neutrophils, platelets and endothelial cells [1, 2]. These same cells are also targets for the biological actions of PAF *via* interaction with specific high affinity cell surface receptors [16]. Thus, *in vitro* PAF aggregates platelets [1, 2], and is a potent chemoattractant for neutrophils and eosinophils [1, 2, 17, 18]. A number of review articles also suggest that PAF induces granulocyte activation leading to degranulation, superoxide generation and eicosanoid production [1, 2, 19].

In contrast, some reports indicate that PAF fails to stimulate superoxide generation from human granulocytes [20, 21], human eosinophils [22], or guinea-pig peritoneal eosinophils [23] at pharmacologically relevant concentrations. We (unpublished observations) and others [23] have also found that release of eosinophil lysosomal enzymes occurs only at cytotoxic concentrations of PAF, even though PAF in the nanomolar range causes elevations in intracellular calcium in the same cells.

The secretion of eosinophil and neutrophil granule proteins may be an important pathophysiological mech-anism in asthma. Consequently, since PAF is proposed to play a central role in the pathology of asthma through the recruitment and activation of inflammatory cells, we have examined the hypothesis that PAF may stimulate release of granule proteins and, in particular, eosinophil cationic protein (ECP), from human granulocytes; for comparison, superoxide generation and chemotactic responses from the same cells were also studied.

Methods

Purification of granulocytes

Reflecting the paucity of eosinophils in peripheral blood, in preliminary studies we had found that the yield of purified (>90%) eosinophils from the blood of normal donors (found on 22%, 23% and 24% metrizamide gradient interfaces) was low and did not readily lend itself to use in functional studies. In the present experiments, therefore, we have studied a mixed granulocyte population and have utilized ECP as a specific marker of eosinophil activation.

Granulocytes were purified from fresh venous blood from healthy volunteers. One hundred and sixty millilitres of whole blood was collected into Travenol blood collection bags containing 40 ml 4.5% (w/v) dextran T500 in 3.8% (w/v) sodium citrate. The blood was mixed, aliquoted into 50 ml polyethylene centrifuge tubes and incubated at 37°C for 45 min to sediment the red cells. Leucocyte rich plasma was aspirated at 15 min intervals, centrifuged (200–250×g for 10 min) and the cell pellet resuspended in 0.83% (w/v) ammonium chloride for 5 min to lyse the contaminating erythrocytes. Following centrifugation, cells were resuspended in Hank's balanced salt solution (HBSS) without calcium and magnesium, containing 5% foetal calf serum (FCS), 0.1% human serum albumin (HSA) and 50 U·ml^{-1} deoxyribonuclease (DNAse). The mixed cell population (1 ml containing 50×10^6 cells, per gradient) was overlayered onto a discontinuous metrizamide gradient (18, 20, 22%w/v) in HBSS without calcium or magnesium but supplemented with 5% FCS, 0.1% HSA and 50 U·ml^{-1} DNAse. Gradients were centrifuged at 150×g for 30 min at 20°C.

The granulocyte fractions (stages 20 and 22%) were collected and washed twice in HBSS supplemented with 5% FCS, 0.1% HSA and 50 U·ml^{-1} DNAse, and resuspended to a final concentration of 20×10^6 cells·ml^{-1} in HBSS containing calcium and magnesium. Differential cell counts were made on cytospin preparations stained with Wright's stain, counting at least 300 cells under oil immersion.

Granulocyte activation

Cells (93±1.4% neutrophils, 3±0.75% eosinophils; n=12 donors) were aliquoted into sterile, untreated, Nunclon, flat bottomed, 96-well microtitre plates (Gibco, Paisley, Scotland) at a density of 10^6 cells per well, for measurement of superoxide generation or of elastase and ECP release.

Superoxide was measured by reduction of iodonitrotetrazolium violet (INTV). Duplicate aliquots of purified granulocytes were incubated for 5 min at 37°C in HBSS containing 0.5 mg·ml^{-1} INTV±5 µg·ml^{-1} cytochalasin B prior to addition of PAF (10^{-9}–10^{-5} M), L-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (10^{-9}–10^{-5} M) or opsonized zymosan (OPZ) (0.01–1 mg·ml^{-1}) and incubation for a further 30 min. The total incubation volume was 250 µl. At the end of the incubation the plate was spun at 900×g for 10 min, the supernatant discarded, and the cell pellet solubilized in dimethyl sulphoxide (DMSO) containing 5% concentrated HCl. The absorbance of iodonitrotetrazolium formazan in the solubilized cell pellet was measured at 492 nm using a titretek multiscan MCC/340 plate reader.

In parallel experiments, duplicate aliquots of cells were incubated in HBSS containing 5 µg·ml^{-1} cytochalasin B, at 37°C for 5 min, prior to stimulation with PAF (10^{-9}–10^{-5} M), FMLP (10^{-9}–10^{-5} M) or OPZ (0.01–1 mg·ml^{-1}) for a further 30 min. The total incubation volume was 250 µl. The incubation was stopped by centrifugation at 900×g for 10 min. Two hundred microlitres of the supernatant was removed and stored at -20°C prior to assay for elastase and ECP. In a separate series of experiments, following incubation with 10 µM FMLP in the presence of cytochalasin B, the supernatant was removed and the cells lysed in distilled water containing 1% Triton-X. The cell incubate and lysate were stored at -20°C prior to assay for elastase, ECP and lactate dehydrogenase (LDH) activity.

Elastase was measured fluorimetrically, using the method described by Castillo et al. [24], but modified for use in a Monarch centrifugal analyser. Ten microlitres of supernatant was incubated for 10 min at 25°C with 280 µl N-methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methyl-coumarin substrate (0.2 mM in 0.05 M Tris(hydroxymethyl)aminomethane, 0.5 M NaCl, 0.01 M CaCl$_2$ buffer pH 7.5, 10% DMSO). Fluorescence of the liberated 4-methyl-7-coumarin was measured at 370 nm Ex, 460 nm Em. Elastase activity was derived by rate constant calculation from a human granulo cyte elastase standard curve. Interassay coefficient of variations (CoV) <6%; intra-assay variation <8%. The threshold limit of detection was 0.45 µmol·10^6 cells^{-1}.

ECP was measured by double antibody radioimmunoassay (RIA) using purified human eosinophil cationic protein as standard (Kabi Pharmacia Diagnostics, Milton Keynes, UK). The concentrations of ECP in 50 μl sample volumes of granulocyte supernatant were read from the human ECP standard curve (2–200 μg·l^{-1}) and expressed as μg ECP released·10^6 eosinophils^{-1}, as determined from the differential cell counts. The detection limit of the assay is <2 μg·l^{-1} (equivalent to 0.5 ng·well^{-1}); basal unstimulated ECP release was equivalent to 3.8 ng·well^{-1}.

LDH activity was measured using a Roche Diagnostics enzymatic assay on a COBAS bioanalyser.

Chemotaxis was measured in blind well chemotaxis chambers (Neuro Probe, Costar, High Wycombe, UK) using the methodology described by METCALF et al. [25]. PAF, FMLP or 0.1% bovine serum albumin vehicle, in HBSS (200 μl), was placed in the lower well and granulocytes (300,000 in 200 μl HBSS for each well) loaded into the upper compartment of control and test wells. The two chambers were separated by two 13 mm^2, 5 μm pore size, polycarbonate filters, with a nominal thickness of 10 μm (Neuro Probe, Costar, High Wycombe, UK), that had been presoaked in HBSS. The chemotactic activity of PAF and FMLP was tested over the concentration range 10^{-9}–10^{-5} M and compared to HBSS control. Chambers were incubated for 45 min at 37°C. Granulocytes migrate through the upper and into the lower filter, with an apparent barrier to migration offered by the interface between the filters [25]. At the end of the incubation, the filters were removed and the upper filter discarded. The lower filter was fixed in 100% anhydrous methanol, mounted onto a microscope slide and stained with Wright's stain. The number of cells that had migrated onto the lower filter in the area of a graticlue on the ×40 objective was counted.

Results were expressed as the number of cells in five high power fields.

PAF and FMLP were dissolved and diluted in HBSS containing 0.1% BSA. Zymosan was caused to swell by boiling in distilled water for 30 min and opsonized by incubation in pooled human serum for 30 min at 37°C; the opsonized particle was washed three times in HBSS before use.

Results are expressed as mean±SEM. Data were compared using a two-tailed Mann-Whitney U test.

Materials

HBSS with or without calcium and magnesium, FCS and the 96-well mictrotitre plates were from Gibco (Paisley, Scotland). FMLP, zymosan, BSA, metrizamide, DNAse, BSA, HSA, INTV, DMSO, and N-methoxysuccinyl-Ala-Ala-Pro-Val-7amido-4-methyl-coumarin were from Sigma (Poole, Dorset, UK). C-16 PAF was from Novabiochem (Nottingham UK). The chemotaxis chambers and Nuclepore polycarbonate filters were from Costar (High Wycombe, UK).

Results

Superoxide generation

FMLP caused a concentration-dependent stimulation of granulocyte superoxide generation that was markedly enhanced in the presence of cytochalasin B (concentration producing half the maximal effect (EC_{50}) 4.85±3.3× 10^{-8} M,) (fig. 1a). Superoxide generation stimulated by opsonized zymosan was enhanced in the presence
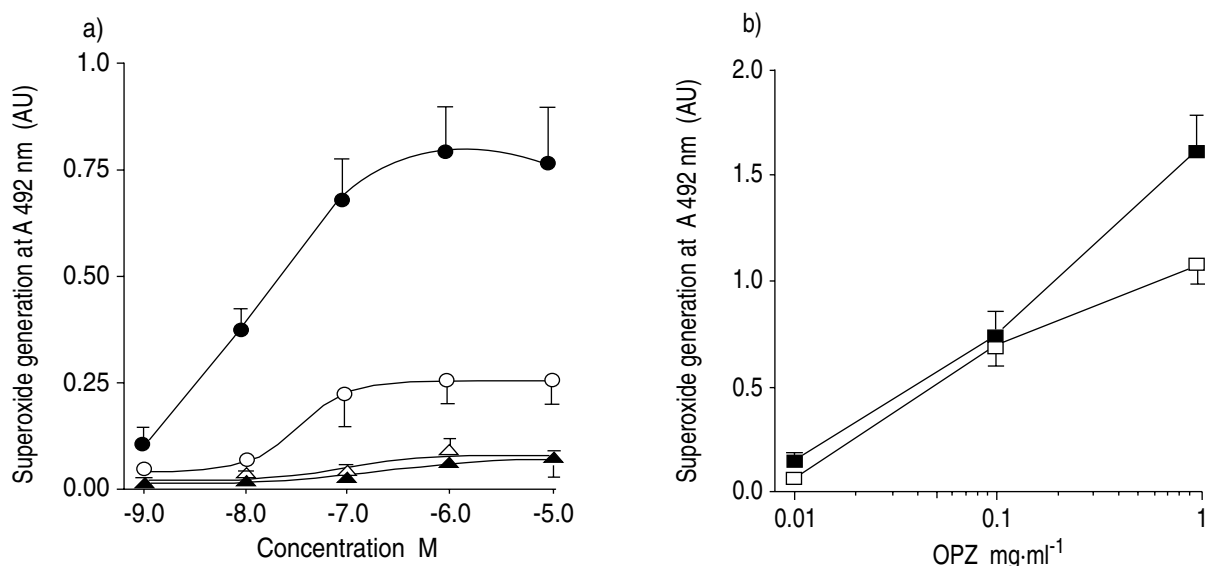

Fig. 1. – Superoxide generation from human granulocytes (10^6) stimulated with: a) platelet-activating factor (PAF) (Δ) or FMLP (○); or with b) opsonized zymosan (OPZ) (☐). Experiments were carried out in the absence (open symbols) or the presence (closed symbols) of 5 μg·ml^{-1} cytochalasin B. Superoxide generation was measured by reduction of iodonitrotetrazolium violet and the data expressed in absorbance units (AU) of the resulting formazan, measured at 492 nm. Data points are the mean±SEM of duplicate wells from 5–11 separate experiments.

of cytochalasin B only at 1 mg·ml^{-1}. At this concentration of OPZ, the oxygen free radical-mediated reduction of INTV was approximately two fold greater than the maximum response to FMLP (fig. 1b). In contrast, PAF was a poor stimulus to superoxide generation, causing only *ca* 10 and 5% of the reduction of INTV elicited by maximum effective (Emax) concentrations of FMLP (1 μM) and OPZ (1 mg·ml^{-1}), respectively, (fig, 1a). There was no effect of cytochalasin B on PAF responses.

Granulocyte degranulation

Elastase release was used as a marker of total granulocyte degranulation; eosinophil degranulation was measured using ECP release as a specific marker of eosinophil function.

In unstimulated cells, basal release of elastase was 0.66±0.125 μmol·10^6 granulocytes^{-1} (n=9), and of ECP was 0.128±0.047 μg·10^6 eosinophils^{-1} (n=12), respectively.

In the presence of cytochalasin B, FMLP (10^{-9}–10^{-5} M) stimulated granulocyte degranulation, causing concentration-related increases in both elastase and ECP release. At 10 μM FMLP, elastase release was increased 100 fold to 68.8±9.75 μmol·10^6 granulocytes^{-1} (n=9) and ECP release 14 fold to 1.79±0.28 μg·10^6 eosinophils^{-1} (n =12) (figs. 2 and 3). The percentage of total enzyme release for elastase by 10 μM FMLP was 20.4±4.4%. At this concentration of FMLP, LDH release was 0.86±0.22% of total and was not greater than that seen in unstimulated cells. In these experiments, although 10 μM FMLP increased ECP release into the incubation medium from 0.048±0.015 μg·10^6 eosinophils^{-1} to 2.24±0.1 μg·10^6 eosinophils^{-1}, intra-

cellular ECP (*i.e.* that present in the cell lysates from unstimulated cells (0.195±0.011 mg·10^6 eosinophils^{-1})) amounted to <10% of that released on stimulation with 10 μM FMLP.

Fig. 3. – Increase in eosinophil cationic protein (ECP) release from human granulocytes (10^6) stimulated by PAF (▲) or FMLP (●). Experiments were carried out in the presence of 5 μg·ml^{-1} cytochalasin B. Data points are the mean±SEM of duplicate wells from at least five separate experiments. For abbreviations see legend to figure 1.
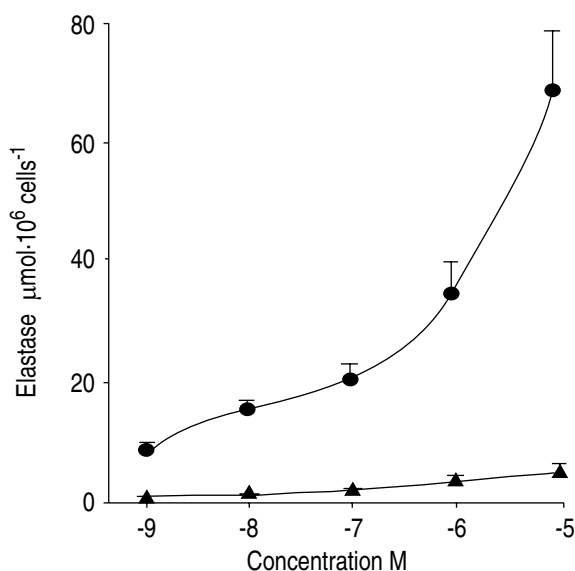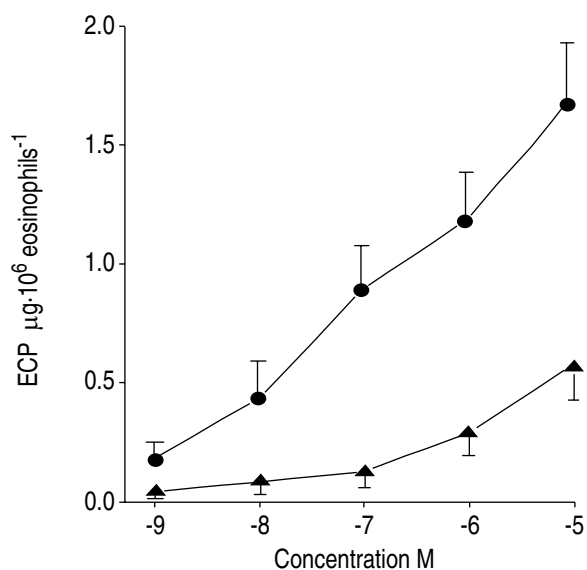
Fig. 2. – Increase in elastase release from human granulocytes (10^6) stimulated by PAF (▲) or FMLP (●). Experiments were carried out in the presence of 5 μg·ml^{-1} cytochalasin B. Data points are the mean±SEM of duplicate wells from at least seven separate experiments. For abbreviations see legend to figure 1.
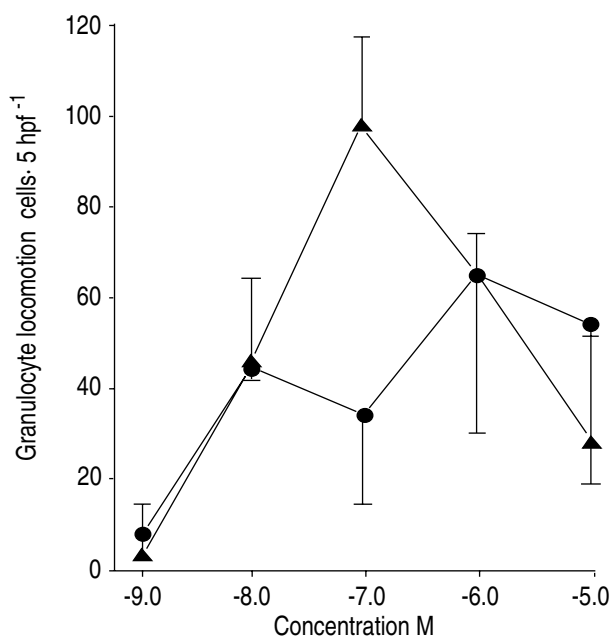
Fig. 4. – Effects of PAF (▲) and FMLP (●) on chemotaxis of human granulocytes. Cells (300,000) were incubated for 45 min at 37°C in blind well chemotaxis chambers separated by two 3 μm polycarbonate membranes. PAF or FMLP were placed in the lower well and cells in the upper. Agonist-induced chemotaxis was assessed by counting cells trapped on the upper surface of the lower filter. Data points are the mean±SEM of duplicate wells from at least 5 and 3 separate experiments, respectively. hpf: high power field. For further abbreviations see legend to figure 1.

Under the same conditions, OPZ and PAF were relatively poor stimuli to granulocyte degranulation. OPZ increased elastase release from 0.83 ± 0.17 $\mu mol\cdot10^6$ granulocytes^{-1}, in unstimulated cells to 8.13 ± 2.7 $\mu mol\cdot10^6$ granulocytes^{-1} at 0.1 mg·ml^{-1} OPZ (n=5) but had no significant effect on ECP release (p<0.05; n=5). PAF (10^{-9}–10^{-5} M) elicited a concentration-related increase in elastase release from 0.4 ± 0.08 $\mu mol\cdot10^6$ granulocytes^{-1} in unstimulated cells to 4.72 ± 1.3 $\mu mol\cdot10^6$ granulocytes^{-1} at 10 μM PAF (n=7), (fig. 2). Over the same concentration range, ECP release was increased from 0.05 ± 0.01 $\mu g\cdot10^6$ eosinophils^{-1} to 0.61 ± 0.15 $\mu g\cdot10^6$ eosinophils^{-1} at 10 μM PAF (n=7), (fig. 3).

Granulocyte chemotaxis

Both PAF and FMLP caused a marked, concentration-dependent increase in granulocyte migration. Both agents caused similar degrees of granulocyte chemotaxis and were equipotent EC_{50} *ca* 10–20 nM, Emax 98 ± 20 and 65 ± 35 cells (n=5 and n=3) in five high power fields, respectively. The PAF concentration-response curve was bell-shaped, with loss of chemotactic activity at PAF concentrations above 10^{-7} M (fig. 4).

Discussion

The recruitment of inflammatory cells, both neutrophils and eosinophils, into the airway of asthmatics is considered to be the major precipitating factor in the pathogenesis of asthma [10], and is associated with late-phase bronchoconstriction, changes in bronchial responsiveness and epithelial desquamation [14, 15, 26]. The increased number of inflammatory cells, notably eosinophils, in asthmatic airways has been widely-reported, and appears to be associated with increasing disease severity [27]. There is also convincing evidence that these cells are activated. Eosinophil cationic proteins are increased in bronchoalveolar lavage fluid from asthmatics compared to control groups [11], and major basic protein (MBP) is increased in asthmatic sputum and mucous plugs [28]. In addition, bronchial biopsy of asthmatic airways has demonstrated that inflammatory cells show morphological signs of activation, including increased numbers of eosinophils staining positive for the cleaved and secretory form of ECP in the epithelium and submucosa [29, 30].

Despite the identification of a host of inflammatory mediators released following antigen challenge of animal and human lung, and during acute asthmatic episodes [2], it remains unclear which mediators are responsible for granulocyte recruitment and subsequent activation. As reported by others (WARDLAW *et al.* [17] and BRUIJNZEEL *et al.* [18]), in our present experiments PAF and FMLP were potent chemoattractants for human granulocytes with EC_{50}s in the region of 10–20 nM. Furthermore, as with the two other studies, the PAF concentration-response curve was bell-shaped with loss of chemotactic activity at 10 μM. This inhibition of cell migration by

supramaximal concentrations of PAF is unexplained at present, but may reflect receptor desensitization, or may be a consequence of granulocyte aggregation in the upper well at concentrations of PAF of 1 μM and above. In contrast, at concentrations up to 10 μM there was no evidence of auto-inhibition of FMLP stimulated chemotaxis.

Although *in vitro* activation of granulocytes by PAF has been reported to be associated with formation of superoxide anions and enzyme secretion [1], it is now becoming clear that the ability of PAF to stimulate free radical generation and degranulation from animal and human granulocytes requires clarification. WORTHEN *et al.* [20] and FLOCH *et al.* [21] have suggested that PAF is a relatively weak stimulus to superoxide generation from rabbit and human granulocytes. Furthermore, we and others [23] have found guinea-pig eosinophils to be unresponsive to PAF, even though PAF is a potent stimulus to the superoxide burst of macrophages from the same animals [31]. In the present experiments, compared to FMLP or OPZ, PAF was a poor stimulus to superoxide generation, and unlike responses to FMLP or OPZ, its activity was unaffected by prior exposure of the cells to cytochalasin B; the maximum response being less than 10% of that to FMLP and less than 5% of that to OPZ.

It is well-established that chemotactic stimuli only consistently stimulate neutrophil secretion when treated with cytochalasin B, or when the stimulus is presented on a surface [32]. In granulocytes cytochalasin B increases the secretory response by virtue of its inhibition of phagocytosis; the component affected being phagosome closure, due to inhibition of microfilament assembly or contraction [33]. The plasma membrane and, in particular, the phagosome membrane has been shown, cytochemically, to be the site of superoxide production, and is activated following binding of particles or soluble stimuli to the plasma membrane and incorporation into the phagolysosome [34]. The enhancement of OPZ stimulated superoxide generation by cytochalasin B, therefore, is most likely to be due to inhibition of phagosome closure, oxygen radicals being free to diffuse into the extracellular matrix. The enhancement of FMLP-stimulated superoxide generation, however, is more surprising, and suggests that superoxide production following the interaction between FMLP and its cell membrane receptors may also involve phagosome or endosome formation. Furthermore, the data suggest that FMLP-stimulated superoxide generation is largely restricted to the phagosome membrane, rather than being a consequence of a disseminated perturbation of the cell membrane.

In addition, we have found FMLP to be a potent stimulus to both elastase and ECP release. The mean maximal release of elastase by 10 μM FMLP being $20.6\pm4.4\%$ of total. However, whilst ECP is a specific marker for eosinophil activation, since the cells used in the present experiments represent a mixed population, we cannot rule out the possibility that FMLP-induced ECP release is secondary to neutrophil activation. Unexpectedly, the ECP content of lysates from control cells

was <10% of that stimulated by 10 μM FMLP; the reason for this apparent lack of release of ECP on cell lysis is unclear. Nevertheless, although we were unable to detect significant total cellular levels of ECP, the LDH content of the lysate (452.3±6.5 U·10^6 cells^{-1}), which was 30 fold greater than that of the incubation media of control cells (15.6±1.2 U·10^6 cells^{-1}), together with the six fold greater levels of elastase in the lysate, indicates that the low total ECP levels cannot be explained by a failure of cell lysis to release cytosolic and granule contents. It is also unlikely that FMLP stimulated ECP release reflects *de novo* synthesis. Immunocytochemical studies, however, have shown that ECP exists both in a storage and secreted form [35]. It is possible, therefore, that under the present experimental conditions, either the antibody used in the RIA does not recognize the form released on cell lysis or, alternatively, that the ECP released on lysis remains tightly associated with intracellular membranes.

As with superoxide generation, PAF was a poor stimulus to granulocyte degranulation, eliciting less that 10% of the response to FMLP. Interestingly, OPZ was also a relatively poor stimulus to elastase release and failed to stimulate ECP release into the extracellular medium. In this regard, it is possible that, even in the presence of cytochalasin B, granule enzymes and proteins remain associated with the phagolysosome, cell membrane and/or opsonized particle following phagocytosis, rather than being free to diffuse extracellularly as with FMLP.

Although the cells used in these studies were a mixed granuloctye population, ECP can be considered a specific marker for eosinophil degranulation [36]. Thus, the lack of effect of PAF on elastase (a product of both neutrophils and eosinophils) and ECP release, suggests that PAF is not a potent stimulus of granular enzyme release from either cell type. The poor activity of PAF as a stimulus to the granulocyte superoxide burst or to granulocyte degranulation, however, does not reflect a generalized insensitivity to this mediator, as it is in marked contrast to its potency as a chemoattractant in the same mixed cell population. Thus, the insensitivity of the secretory response is a specific phenomenon and not due to some nonspecific desensitization to PAF in these experiments.

In conclusion, our results have shown that PAF is a relatively poor stimulus to superoxide generation, elastase and ECP release from a mixed granulocyte population from normal volunteers, despite being a potent chemotactic factor for the same cells. Specific high affinity PAF receptors have been located on the plasma membrane of human granulocytes [16], and our data suggest that these are primarily associated with chemotactic responses. The data suggest that whilst PAF may be involved in cell recruitment into the airways of asthmatics it may not stimulate their subsequent activation and release of granule proteins. We cannot discount the possibility, however, that cells in the asthmatic airway or circulation are more responsive to PAF than those from the peripheral circulation of normals. In this regard, inflammatory cells in the peripheral circulation and airway of asthmatics demonstrate differing degrees of activation [15, 27], and eosinophils from asthmatics have been shown to release more ECP than cells from controls without asthma [37].

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