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# *In vitro* and *in vivo* effects of treatment by platelet-activating factor on N-formyl-met-leu-phe-mediated responses of polymorphonuclear leucocytes

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**Summary.** Two chemoattractants, the peptide N-formyl-met-leu-phe (FMLP), and the ether phospholipid, platelet activating factor (PAF), each stimulate a variety of *in vitro* responses in polymorphonuclear leucocytes (PMN). Because often more than one inflammatory mediator is active during inflammation, we determined the effect on PMN of sequential stimulation with these two agents. Before FMLP stimulation, human PMN were exposed to PAF, at concentrations which gave little or no response when administered alone. PAF enhanced FMLP-elicited superoxide release in a dose-dependent fashion. Likewise, release of granular lysozyme from the cells was increased in PAF treated cells. Similar treatment with other phospholipids, including the lyso derivation of PAF, failed to produce these effects. Incubation with nordihydroguaiaretic acid, an inhibitor of arachidonic acid metabolism, had little effect on the enhancement of lysozyme release by PAF. To determine if enhancing effects by PAF might occur also *in vivo*, we studied rabbits receiving PAF and/or FMLP intravenously. When rabbits received 0·01 μg PAF (a dose which does not elicit the sustained neutropenia observed with higher doses of PAF) followed by 0·05 μg FMLP the absolute granulocyte count (AGC) dropped at 1 min ($46 \pm 11\%$ of original value), and continued to fall ($24 \pm 12\%$ at 10 min). Controls, treated with the suspending fluid for PAF, and then 0·05 μg FMLP, had a similar 1 min AGC value, but at 10 min AGC returned to $65 \pm 6{\cdot}1\%$ ($P < 0{\cdot}001$ for comparison of 10 min values). Thus PAF pretreatment enhanced FMLP-elicited granulocytopenia *in vivo*. Study of *in vitro* human PMN aggregation revealed that, at certain relative concentrations of PAF and FMLP, aggregation was enhanced. These studies show that both *in vitro* and *in vivo* responses of FMLP-stimulated PMN may be exaggerated by pre-exposure to PAF.

During inflammation, the polymorphonuclear leucocyte (PMN) is exposed to a multitude of bioactive substances. Among these substances are likely the chemoattractive formylated peptides, similar to N-formyl-met-leu-phe (FMLP), which are released by microorganisms (Schiffmann *et al*, 1975a, b). Also important are a variety of lipid mediators including the platelet activating factor (PAF), 1-O-alkyl-2-acetyl-3-phosphorylcholine (Godfroid *et al*, 1980; Pinckard *et al*, 1980) which is released from various cells including the PMN themselves (Betz & Henson, 1980; Clark *et al*, 1980; Sanchez-Crespo *et al*, 1980). Each of these agents individually has stimulatory effects on PMN *in vitro*, being particularly effective in chemokinetic/chemotactic activation (Snydermen & Goetzl, 1981; Goetzl *et al*, 1980). The *in vivo* responses of PMN, however, are likely modulated by these agents acting in concert or in sequential fashion. We had observed that although PAF alone fails to elicit an oxidative burst in PMN, this lipid mediator was capable of amplifying the oxygen use by cells which were subsequently stimulated with FMLP (Ingraham *et al*, 1982), and others have made similar observations (Dewald & Baggiolini, 1985). In this paper we report results of an investigation on the *in vitro* and *in vivo* effects of sequential stimulation of PMN by first PAF and then FMLP.

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## MATERIALS AND METHODS

*Cell preparation.* PMN from heparinized venous blood were sedimented through cushions of Ficoll (Bøyum, 1968) and freed of contaminating erythrocytes by hypotonic lysis. Final suspension of cells (98–99% PMN) was in phosphate-buffered saline with 5·5 mM glucose (PBSG) or Kreb's Ringer phosphate with glucose (KRPG).

*Chemicals.* Synthetic PAF, designated L-α-lecithin-β-acetyl-γ-O-alkyl, and its lyso derivative were obtained from Calbiochem-Behring Corp. (La Jolla, Calif.). The PAF preparation which was 98% pure was that of Do & Ramachandran (1980) from chicken egg yolk. The alkyl groups are predominantly hexadecyl and octadecyl species. Preparation and storage of PAF stock solutions was as previously described (Ingraham *et al*, 1982). Other phospholipids used were obtained from Applied Science (State College, Penn.). Cytochrome c (Type VI), bovine serum albumin (BSA), superoxide dismutase, FMLP, nordihydroguaiaretic acid (NDGA), and enzyme substrates were obtained from Sigma Chemical Co. (St Louis, Mo.).

*Measurements of $O_2^-$ ion and released enzymes.* Superoxide ion was measured as previously described (Ingraham *et al*, 1982). Preliminary time course and dose response experiments revealed that PAF ($10^{-9}$–$10^{-8}$ M) treatment could be from 0 to 5 min before FMLP stimulation and produce enhanced effects. For most experiments the conditions described in the legend to Fig 1 were used. Values are the means and standard deviations of nmoles $O_2^-$/5 min/$10^7$ PMN. The results of replicate samples were averaged and converted to nmoles reduced cytochrome c by use of the extinction coefficient of $19{\cdot}1 \times 10^3$ $M^{-1}$ $cm^{-1}$.

For enzyme release, 1–$1{\cdot}5 \times 10^7$ PMN were incubated in 0·9 ml KRPG for 5 min at 37°C. PAF or its suspending medium was added and incubation continued an additional 5 min. FMLP was then added and incubation continued 2·5, 5 or 10 min longer. At the end of the incubation the contents of the tube were centrifuged (400 *g*, 5 min, 4°C) and enzyme activities were determined on the cell-free supernatant. Beta glucuronidase, lysozyme and lactic acid dehydrogenase were determined by previously described methods (Ingraham *et al*, 1982).

*In vivo studies.* For *in vivo* studies, stocks of FMLP were prepared by dissolving the peptide in 0·02 M sodium bicarbonate to a concentration of 1 mg/ml and diluted to final concentration in 1 ml of PBS.

White male New Zealand rabbits (2 ± 0·1 kg) were initially anaesthetized as previously described (Lash *et al*, 1983). The anaesthesia was supplemented with inhalation ether during exposure of the femoral vessel. Intramedic polyethylene tubing was used to catheterize the femoral artery and vein. Normal saline was administered via the venous line at a rate of 10 ml/h. A blood sample was drawn for the zero time value to leucocyte counts. Stimulant PAF (0·01 μg, i.e. 0·005 μg/kg body weight) in a volume of 1 ml PBS with 0·25% BSA was administered via the venous line and blood samples were drawn from either the arterial or venous lines at 1 and 2·5 min. Then a challenge dose of FMLP (0·05 μg) in 1 ml PBS with bicarbonate was administered via venous lines and blood taken at 1, 2·5, 5 and 10 min for determination of the absolute granulocyte counts (AGC). Previously conducted dose–response studies with each of these substances alone showed that 0·01 μg PAF produced a minimal drop in AGC and 0·05 μg FMLP produced an immediate drop of approximately 60% initial value followed by complete recovery between 15 and 30 min post-stimulation. (See Fig 2 legend and Lash *et al*, 1983, for further details.)

*Aggregation.* Procedure for aggregometry through use of a Payton, Model 300B aggregometer was as previously described (Ingraham *et al*, 1982). Details are given in the legend of Fig 3.

*Statistics.* Statistical significance was determined by *t* test comparing PAF-treated samples with controls incubated with 0·25% BSA in 0·9% NaCl, the suspending medium for PAF. For analysis of changes in leucocyte count during *in vivo* stimulation of rabbits, statistical significance of differences in slopes of curves during the recovery of AGC was determined by analysis of variance.

## RESULTS

*$O_2^-$ ion production and enzyme release*

Because we had shown earlier that pretreatment with PAF enhanced $O_2$ utilization by PMN subsequently stimulated with FMLP (Ingraham *et al*, 1982), we began our present work by examining the effect of PAF on FMLP-mediated production of $O_2^-$. PMN pretreatment with $10^{-8}$ M PAF for 5 min and then stimulation with $10^{-7}$ M FMLP markedly increased $O_2^-$ production compared to control cells which were incubated with 0·9% NaCl containing 0·25% BSA before receiving FMLP addition. These differences (243 ± 6 nmole $O_2^-$/5 min/$10^7$ PMN with PAF and 144 ± 5 without PAF) were significant, $P<0{\cdot}001$. Similarly, PAF enhanced $O_2^-$ production at other times tested up to and including 10 min. The enhancement was not due to additive effects of PAF and FMLP because by itself at this concentration and in the

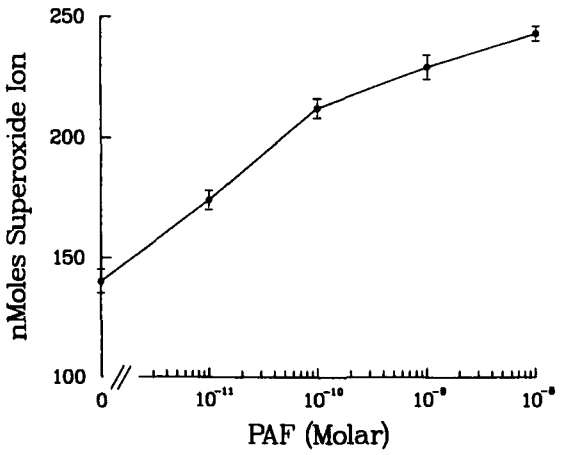


**Fig 1.** Effect of concentration on PAF enhancement of FMLP-mediated $O_2^-$ production. After 5 min equilibration at 37°C PAF was added and incubation proceeded an additional 5 min. Controls received equivalent volumes of 0·9% NaCl with 0·25% BSA, the suspending medium for PAF. The reaction was then initiated by addition of FMLP, $10^{-7}$ M.

absence of cytochalasin B, PAF has virtually no effect on $O_2^-$ production (Ingraham *et al*, 1982). Dose–response studies (Fig 1) showed that exposure to PAF concentrations as low as $10^{-11}$ M gave amplified responses with FMLP ($10^{-8}$–$10^{-6}$ M). To determine if these effects were unique to PAF, we performed similar experiments with other phospholipids including phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and the lyso derivative of PAF (LPAF). None of these compounds at $10^{-8}$ M showed significant effects on enhancement of $O_2^-$ production by PMN after exposure to $10^{-7}$ M FMLP (Table I).

Similar results were obtained when we examined the effects of treatment with PAF on FMLP-mediated release of lysozyme. Pretreatment with $10^{-8}$ M PAF markedly increased the release when PMN were subsequently stimulated with $10^{-7}$ M FMLP (Table II). We cannot attribute the increase to PAF-promoted degranulation because, in the absence of cytochalasin B, PAF does not promote significant enzyme release (Ingraham *et al*, 1982). β-Glucuronidase release from primary granules was negligible whether cells received pretreatment with PAF or not, suggesting that only the specific granules responded to this stimulation. As with $O_2^-$ production, the other phospholipids (PE, PC, LPC and LPAF) at $10^{-8}$ M had little effect on degranulation (data not shown).

Other workers (O'Flaherty, 1985; O'Flaherty *et al*, 1981a, 1983), in examining effects of sequential stimulation by PAF and other stimuli on PMN functions, have results suggestive of a role for endproducts of lipoxygenase action on arachidonic acid. Accordingly, we examined the effect of NDGA, a lipoxygenase inhibitor, on PAF enhancement of FMLP responses. Neither $10^{-6}$ M nor $10^{-5}$ M NDGA diminished the enhancement effect of PAF on lysozyme release, however (Table II).

**Table I.** Effect of phospholipids on FMLP-mediated $O_2^-$ production*

| Phospholipid | Per cent $O_2^-$ | *P* value |
|---|---|---|
| None | 100† | — |
| PAF | 205 | 0·017 |
| LPAF | 126 | 0·381 |
| PE | 90 | 0·466 |
| PC | 88 | 0·465 |
| LPC | 81 | 0·147 |

* PMN were incubated for 5 min at 37°C with $1 \times 10^{-8}$ M phospholipid. FMLP ($10^{-7}$ M) was added and the reaction proceeded an additional 10 min. Details of procedure are given in Materials and Methods section. Abbreviations for phospholipids are LPAF-=lyso PAF, PE=phosphatidyl ethanolamine, PC=phosphatidyl choline and LPC=lyso PC.

†Data presented is result of duplicate determinations in one of two experiments.

**Table II.** Effect of PAF treatment on lysozyme release by human PMN stimulated with FMLP*

| | Fold enhancement by PAF† | |
|---|---|---|
| Experiment | −NDGA | +NDGA‡ |
| 1 | 1·47§ | 2·47§ |
| 2 | 1·59 | 1·48 |
| 3 | 1·22 | 1·20 |
| 4 | 1·27 | 1·18 |
| Average±SD | 1·39±0·17¶ | 1·58±0·61¶ |

* FMLP concentration was $10^{-7}$ M.

† PAF treatment was $10^{-8}$ M for 5 min.

‡ NDGA concentration was $10^{-5}$ M.

§ Results are the ratio: (% release+PAF)/(% release−PAF). In individual experiments average release+PAF ranged from 14% to 35% total lysozyme and −PAF ranged from 10% to 24%. For each experiment comparison of the means for PAF treatment to controls gave *P* values ⩽0·05. Lysozyme release with PAF alone at $10^{-8}$ M is less than 2%. Total lysozyme values were 25·2±11·2 μg egg white lysozyme equivalent per $10^7$ cells. No significant change in FMLP-mediated release of β-glucuronidase or of lactic acid dehydrogenase was seen when cells were pretreated with PAF.

¶ Comparison of means + NDGA and −NDGA gave *P*=0·57.

*In vivo studies*

PAF infusion results in several effects *in vivo* including hypotension, thrombocytopenia and neutropenia (McManus *et al*, 1980), and i.v. administration of FMLP to rabbits causes hypotension and neutropenia (Lash *et al*, 1983). Typically with FMLP (0·1–0·01 μg per 2 kg animal), a marked drop in granulocytes occurs at 1 min with nadir at 2·5 min. Recovery begins at 5 min and is complete by 30 min (Lash *et al*, 1983). To determine if PAF might produce amplified FMLP-mediated responses *in vivo* we employed the rabbit model. Anaesthetized rabbits received an initial dose of 0·01 μg PAF, an amount which caused a brief granulocytopenia with recovery at about 5 min post-stimulation. 3 min after this initial PAF dose the animals received 0·05 μg FMLP, an amount which by itself causes immediate and marked granulocytopenia which begins to reverse at 5 min post-FMLP administration. Initial drop in AGC upon FMLP infusion was similar whether or not animals received pretreatment with PAF (Fig 2). Analysis of variance treatment of the slopes during the recovery phase, an approach which minimized day to day and animal to animal variability, revealed, however, that this phase was dramatically affected by PAF pretreatment. Granulocytopenia was sustained in the animals receiving both agents (Fig 2). Analysis of variance of the regression lines for time points between 2·5 and 10 min post FMLP treatment showed that the difference was statistically significant

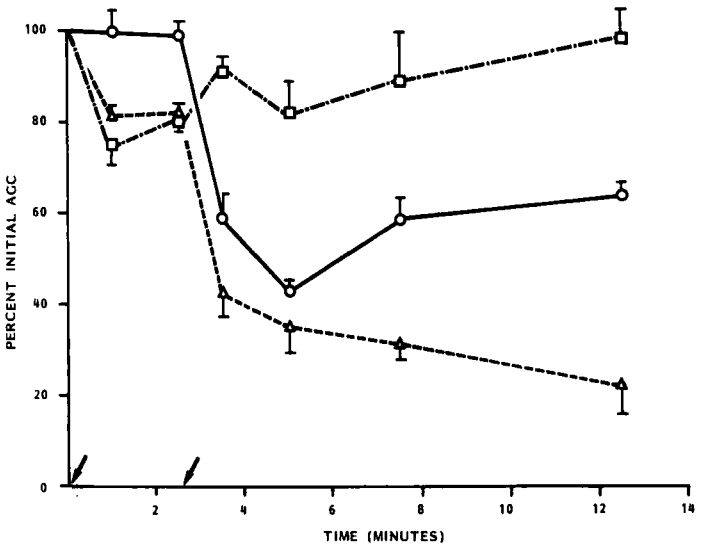


Fig 2. Per cent absolute granulocyte count of rabbits receiving sequential stimulation. Rabbits were anaesthetized and sequential stimuli, each in a volume of 1·0 ml, were administered i.v., the first given at 0 min (first arrow) and the second at 3·0 min (second arrow). □--□=animals receiving 0·01 μg PAF in PBS with 0·25% BSA at 0 min and PBS with bicarbonate at 3 min; ○—○=animals receiving PBS with BSA at 0 min and 0·05 μg FMLP in PBS with bicarbonate at 3 min; △--△=animals receiving 0·01 μg PAF at 0 min and 0·05 μg FMLP at 3 min. Each curve represents results obtained with three or more animals. Values are means ±standard deviations. Details of experimental procedure are given in Materials and Methods.

($P=0·017$) when the slopes were compared for PAF treated and non-PAF treated animals subsequently challenged with FMLP.

*Aggregation*

One means by which granulocyte numbers in the peripheral circulation can be diminished is through aggregation of the cells to one another and their subsequent sequestration in the smaller capillaries. In view of the amplified AGC response in rabbits receiving sequential doses of PAF and FMLP (Fig 2), we wished to determine the effect of sequential stimulation on *in vitro* aggregation. Treatment of human PMN by doses of PAF as low as $10^{-12}$ M gave heightened response to FMLP (Fig 3). The effect was most dramatic at $10^{-8}$ and $10^{-7}$ M FMLP and at the lower doses of PAF, likely because higher doses of FMLP give near maximal aggregation and higher doses of PAF themselves bring about aggregation of the cells. Thus, to study the phenomenon it was important to adjust the relative doses of the two agents. Preliminary study of rabbit granulocytes has shown enhancement similar to that in human cells.

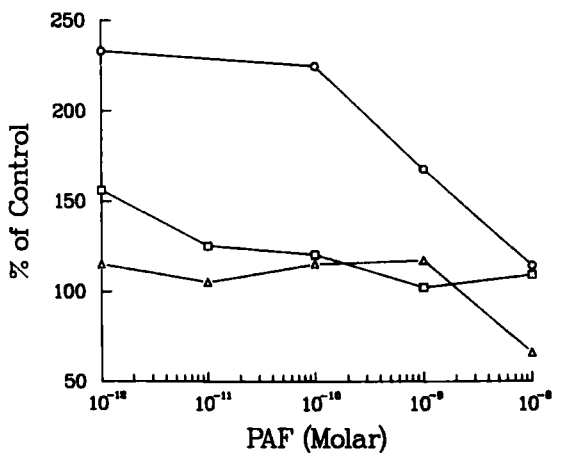


Fig 3. Effect of PAF on FMLP-mediated aggregation of human PMN. PMN ($10^7$/ml KRP) were incubated at 37°C in a siliconized cuvette equipped with teflon stirbar. PAF was added and 3 min later FMLP was added. Quantitation of the area under the aggregation curve was determined through planimetry and was expressed in arbitrary units. The area for cells receiving 0·9% NaCl with 0·25% BSA in place of the PAF suspension were used to give values of 100%. The actual values were 21, 64 and 131 respectively for $10^{-8}$, $10^{-7}$ and $10^{-6}$ M FMLP. Results shown are for one of six experiments. ○, $10^{-8}$ M; □, $10^{-7}$ M; △, $10^{-6}$ M FMLP.

## DISCUSSION

PMN may be exposed to a multitude of bioactive substances during the inflammatory process. Complement is activated giving rise to the active fragments $C_{5a}$ and $C_{3b}$ and chemoattractive formylated peptides, similar to FMLP, are likely released by invading microorganisms (Schiffman *et al*, 1975a, b). In addition to peptides, lipid mediators are released from a variety of tissues including the PMN, themselves, which produce a variety of species of PAF (Pinckard *et al*, 1984) as well as the lipoxygenase endproducts of arachidonic acid metabolism (Borgeat & Samuelsson, 1979; Goetzl & Sun, 1979) hydroxyeicosatetraenoic acids (HETE) and leucotrienes (LT). Each of these agents alone has stimulatory effects on PMN *in vitro* being particularly effective in chemokinetic/chemotactic activation (Snyderman & Goetzl, 1981; Goetzl & Pickett, 1980; Goetzl *et al*, 1980). It is likely that the *in vivo* responses of PMN, however, are modulated by these agents acting in concert or in sequential fashion.

Multiple stimulation of PMN has been studied *in vitro*. The approach most frequently employed has been to 'desensitize' PMN by an agent so that subsequent stimulation results in a diminished response. Upon repeated use of the same agent (i.e. homologous desensitization) PMN become refractory, often through 'down-regulation'. Decreased responsiveness has been demonstrated for repeated exposure of PMN to FMLP (Sullivan & Zigmond, 1980; Smith & Hollers, 1980), as well as to C5a, to PAF, and to 5,12-diHETE (O'Flaherty *et al*, 1981b). Heterologous desensitization is also possible and may produce data suggestive of the sequence occurring in

activation events. For example, pretreatment of PMN with certain agents produces diminished aggregation response to others. Exposure to PAF decreased the effect of subsequent stimulation by $LTB_4$ (Lin *et al*, 1982), while exposure to $LTB_4$ diminished the responses to C5a, FMLP and PAF (O'Flaherty *et al*, 1981a). Pretreatment with 5-hydroperoxyeicosatetraenoic acid induced chemotactic deactivation independent of the nature of the subsequent stimulus (Goetzl *et al*, 1981). Such results are consistent with a role for the arachidonic acid metabolites in stimulation by a variety of agents.

Stimulation with two different agents sequentially may also amplify response. We had earlier demonstrated that PAF and FMLP did not produce heterologous desensitization. Sequential exposure of chlorotetracycline-loaded PMN with combinations of PAF and FMLP showed that PAF treatment did not prohibit later FMLP responsiveness and vice versa (Ingraham *et al*, 1982). Furthermore, we had shown that PAF, which alone fails to elicit oxidative burst, was capable of amplifying the $O_2$ use when PMN were subsequently stimulated with FMLP. Dewald & Baggiolini (1985) have noted similar amplifying effects of PAF treatment on FMLP-induced superoxide ion generation. Similarly, platelets pretreated with PAF, while becoming refractory to subsequent PAF stimulation, had amplified response to other agents (Henson & Pinckard, 1977, see p. 17).

Our present work, showing amplified superoxide production when cells are treated with PAF and then stimulated with FMLP, supports our prior observation showing enhanced oxygen utilization with a similar sequence of stimulation (Ingraham *et al*, 1982). Because $O_2^-$ production and $O_2$ utilization are both expressions of the oxidative burst, it is expected that they would be affected in a similar fashion. We found also that degranulation is similarly affected with PAF-treated cells showing from 1·2 to 2·5 times the release of lysozyme (Table II). O'Flaherty (1985, p. 236) shows similar enhancement of degranulation when PMN are exposed to $2 \times 10^{-8}$ M PAF and then receive stimulation with $0{\cdot}5 \times 10^{-8}$ M FMLP.

The enhancement effect appears to be particular to PAF as other phospholipids tested (PE, PC, LPC and LPAF) at $10^{-8}$ M do not cause significant enhancement (Table I). The mechanism of PAF's effect on FMLP stimulation, however, is unknown. Fletcher & Gallin (1980) were able to show that stimuli promoting degranulation caused an increase in the availability of FMLP receptor sites and a decrease in their binding affinity. This effect was thought to be the result of granules fusing with the plasma membrane and thus expressing additional receptors associated with the granules. PAF might act by a different mechanism, however, because in the absence of cytocholasin B, at PAF concentrations as high as $5 \times 10^{-7}$ M there is no significant release of the granule enzymes (Ingraham *et al*, 1982). PAF binding to PMN appears to occur through high affinity saturable specific sites as well as through non-saturable low affinity sites (Valone & Goetzl, 1983). Either type of PAF binding might have localized effects which lead to alteration in affinity or distribution of FMLP receptor sites on the plasma membrane. Alternatively, the specific binding of PAF might induce specific metabolic changes in the PMN which in turn heighten their ability to respond to FMLP. The possible arachidonic acid metabolites include 5-HETE and $LTB_4$ (Borgeat & Samuelson, 1979; Goetzl & Sun, 1979). The role of the former in PAF enhancement, however, is unclear since pretreatment with 5-HETE itself may (Beckman *et al*, 1985a) or may not (Beckman *et al*, 1985b; O'Flaherty *et al*, 1983) have enhancing effects on FMLP responses. On the other hand, $LTB_4$ might have enhancing effects since pretreatment by this agent does promote $O_2^-$ production by FMLP-stimulated PMN (Gay *et al*, 1984; Dewald & Baggiolini, 1985) while at the same time it diminishes (i.e. desensitizes) PAF-mediated aggregation (O'Flaherty *et al*, 1981a). PAF treatment of PMN itself does result in enhanced production of $LTB_4$ (Lin *et al*, 1982; Chilton *et al*, 1982). Our results with NDGA, however, do not support the idea that PAF-initiated lipoxygenase conversion of arachidonic acid is important in enhancement of FMLP responses (Table II), and the role of arachidonic acid metabolites in PAF stimulation remains unclear. Another consideration that is important in regard to PAF enhancement of PMN responses is the possibility of other cells responding to PAF and producing substances that modulate PMN responses. For example, stimulated platelets may produce arachidonic acid metabolites which are, in turn, modified by PMN enzymes (Marcus *et al*, 1984). Platelets are profoundly affected by PAF and *in vivo* exposure to PAF may stimulate platelets in such a way that their released substances promote various PMN responses. Likewise, *in vitro* preparations of PMN may contain platelets that could function similarly and our results may be reflective of that influence as well as PAF influences directly on PMN.

Our studies on anaesthetized rabbits reveal that enhancing effects of PAF treatment on FMLP-mediated responses may be found not only *in vitro* but at least to some extent in the intact animal as well. Although we did not find significant differences in values of AGC at early times with and without PAF administration at 0·01 μg, prior PAF treatment did promote sustained granulocytopenia at times when AGC of animals receiving only FMLP showed return toward normal levels. Sequestration of granulocytes could be influenced by enhanced aggregation of the cells one to another and their subsequent trapping in the microvasculature. Our studies of FMLP-mediated aggregation in human cells indicates PAF treatment can enhance this *in vitro* response (Fig 3). The effect had strict dependency on relative concentrations of PAF versus FMLP and could be observed only in the dose–response regions in which the cells were neither significantly aggregating to the PAF treatment itself nor aggregating maximally to the FMLP stimulation.

Enhancement through sequential stimulation of PMN is not confined to treatment by PAF. Studies with PMN show that FMLP pretreatment heightens cell response to later stimulation by a variety of soluble (English *et al*, 1981) and particulate substances (Van Epps & Garcia, 1980). Likewise, PMN treated with phorbol myristic acetate (PMA) followed by ionophore A23187 produce enhanced arachidonic acid release (Volpi *et al*, 1985). Further, sequential exposure of PMN to various combinations of the heterologous stimuli FMLP, A23187 and PMA increases the amount of activated

oxidase in membrane fractions (McPhail *et al*, 1984). Thus, the interplay of various agents acting on PMN sequentially can profoundly influence responses. Enhancement of the oxidative responses through sequential stimulation might promote more effective management of microbial infection through greater production of microbicidal reduced oxygen by-products. Likewise, other increased PMN responses may widen the activities of these cells as they perform their role in host defence.

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