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Involvement of leukotriene B4 and platelet-activating factor in cytokine priming of human polymorphonuclear leucocytes

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SUMMARY

Recombinant human (rh) tumour necrosis factor (TNF) alpha and rh granulocyte–macrophage colony-stimulating factor (GM-CSF) prime human polymorphonuclear leucocytes (PMN) for increased superoxide anion (O_2^-) generation and for increased platelet-activating factor (PAF) biosynthesis and leukotriene B_4 (LTB_4) release. Both PAF and LTB_4 are candidate mediators for the enhanced O_2^- generation in cytokine-primed PMN, since exogenous PAF or LTB_4 primes PMN. We measured the generation and release of these mediators and examined their potential roles in cytokine priming using the PAF receptor antagonist, WEB 2086, and the inhibitor of 5-lipo-oxygenase, CGS 8515. rhTNF-α or rhGM-CSF, alone, increased PAF levels in PMN, but did not cause PAF release or LTB_4 synthesis. N-formylmethionyl-leucyl-phenylalanine (FMLP) stimulated the release of detectable and biologically active amounts of both LTB_4 and PAF in primed, but not in non-primed PMN. However, neither blockade of PAF receptors, nor inhibition of LTB_4 synthesis influenced the priming of O_2^- generation by rhTNF-α or rhGM-CSF. Simultaneous pretreatment of PMN with WEB 2086 and CGS 8515 also failed to inhibit priming. Our results do not exclude a role for cell-associated PAF in the priming response, but indicate that the release of PAF and LTB_4 do not mediate this phenomenon. The ability of cytokines to amplify the production and release of lipids may represent a mechanism to attract and localize the pro-inflammatory actions of stimulated PMN to regions where cytokine levels are also elevated.

INTRODUCTION

Circulating polymorphonuclear leucocytes (PMN) undergo a final maturation process during extravasation and migration to local sites of inflammation. This process is known as priming and may be induced *in vitro* by a number of cytokines, including recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF),[1-6] recombinant human tumour necrosis factor-alpha (rhTNF-α)[7,8] and recombinant human interleukin-1 alpha (rhIL-1α).[9] Low levels of endotoxin also induce priming which is associated with an increase in the magnitude of the respiratory burst,[10,11] enhanced generation of leukotriene B_4 (LTB_4)[12] and platelet-activating factor (PAF).[11,13]

The mechanism of priming has not been elucidated, despite intensive investigation.[6,14-21] Priming of PMN by rhGM-CSF is not accompanied by activation of phosphatidylinositol phosphate breakdown or activation of protein kinase C.[15] However, both rhGM-CSF and rhTNF-α elicit the mobilization of arachidonic acid and generation of PAF,[4,19,22-24] suggesting activation of phospholipase A_2. Arachidonic acid may be converted to leukotriene B_4 (LTB_4), which is reported to prime PMN for an enhanced respiratory burst to the chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP).[25] Similarly, several studies indicate that PAF primes the respiratory burst in PMN.[16,25-27] Thus, both PAF and LTB_4 are candidates for autocrine mediators of priming.

In the present study, we have examined the roles of PAF and LTB_4 in the enhanced respiratory burst in cytokine-primed PMN. The role of PAF was evaluated using the specific and competitive PAF receptor antagonist, WEB 2086.[28,29] The role of LTB_4 was evaluated using the selective 5-lipo-oxygenase inhibitor, CGS 8515.[30] In addition, we examined the generation and release of these phospholipid-derived mediators of inflammation from primed and stimulated PMN. The biosynthesis of PAF and LTB_4 was markedly increased in primed PMN, and subsequent stimulation by FMLP led to the release of biologically relevant levels of PAF. However, our data do not support a role for released PAF or for LTB_4 in the enhanced respiratory burst in cytokine-primed PMN.

Abbreviations: FMLP, N-formylmethionyl-leucyl-phenylalanine; GM-CSF, granulocyte–macrophage colony-stimulating factor; h, human; HEPES, N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid; LTB_4, leukotriene B_4; O_2^-, superoxide anion; PAF, platelet-activating factor; PMN, polymorphonuclear leucocytes; r, recombinant; TNF-α, tumour necrosis factor-alpha.

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MATERIALS AND METHODS

Materials

All reagents and solvents were of analytical or higher grade. Chemicals were obtained from the following sources: bovine serum albumin (BSA; grade 5, essentially fatty acid free), cytochrome C (horse heart type III), dextran, clinical grade C formyl-methionylleucyl-phenylalanine (FMLP), HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid), indomethacin, superoxide dismutase (Sigma Chemical Co., St Louis, MO); rh granulocyte–macrophage colony-stimulating factor (rhGM-CSF; batch number 84; Scherring Plough Corporation); rh tumour necrosis factor-alpha (rhTNF-α; lot no. N9017AX; specific activity 4×10^7 U/mg protein; generous gift from Genentech, South San Francisco, CA); rh interleukin-1 alpha (rhIL-1α; specific activity exceeds 10^8 thymocyte mitogenesis U/mg protein; generous gift from Dr S. Gillis, Immunex Corporation, Seattle, WA); hexadecyl platelet-activating factor (PAF; Novachem, Bukendorf, Switzerland); (hexadecyl-1,2-^{3}H(N))-2-acetyl-sn-glyceryl-3-phosphoryl-choline, 59·5 Ci/mmol, [14, 15-^{3}H(N)]-leukotriene B_4, 32 Ci/mmol (New England Nuclear, Boston, MA); leukotriene B_4 antiserum (Dr J. Salmon, Wellcome Research Laboratories, Beckenham, Kent, U.K.); leukotriene B_4 (Dr J. Rokach, Merck Frosst Laboratories, Montreal, Quebec, Canada); WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno (3,2,-f), (1,2,4)-triazolo, (4,3-a), (1,4)-diazepine-2-yl)-1-(4-morpholinyl)-propanone) (Boehringer Ingelheim, Ingelheim, Germany).

Preparation of human polymorphonuclear leucocytes

PMN were obtained from buffy coats supplied by the Red Cross Society, South Melbourne, Australia. The interval between the initial venepuncture and the experiment was approximately 5 hr. Similar results were obtained with PMN obtained from volunteers for which the time between venepuncture and the commencement of the experiment was 2·5–3 hr. PMN were isolated by dextran (T500; Pharmacia, Uppsala, Sweden) sedimentation of red blood cells and density gradient centrifugation through Lymphoprep (endotoxin-tested) using previously described methods.[29,31] Contaminating red blood cells were lysed with NH_4Cl (0·15 M containing 10 mM HEPES, pH 7·0). The PMN were then washed twice in Tyrode buffer containing 0·25% BSA [composition (mM): 5, HEPES; 137, NaCl; 11, D(+)-glucose; 11·9, $NaHCO_3$; 2·7, KCl; 0·26 $MgCl_2$; 0·4, NaH_2PO_4; 1·8 $CaCl_2$] and resuspended in Tyrode at 6×10^6/ml. Cell purity, assessed by morphology, exceeded 98% and viability, assessed by exclusion of trypan blue, exceeded 99%. None of the drugs/vehicles used in this study had any significant effect on cell viability. Buffer reagents were tested for endotoxin contamination by the supplier (Sigma Chemical Co.) and reported to contain less than 0·01 ng/ml. This level is considerably less than that known to prime PMN (10 ng/ml).[11,31] Buffers were prepared using water for injection (BP).

Isolation of platelets

Rabbits (2–4 kg), C.S.L., Parkville, Australia were anaesthetized by intravenous infusion of propanidid and exsanguinated via a sterile cannula inserted in the carotid artery. The blood was immediately mixed with trisodium citrate (0·38% w/v, final concentration) and platelet-rich plasma (PRP) was prepared by centrifugation at room temperature for 20 min at 100 **g**. Washed platelets were prepared as previously described in detail.[32] The platelet concentration was adjusted to 2×10^8/ml and platelets were stored in Tyrode solution overnight at 4° before use on the following day.

Extraction and bioassay of PAF

PAF was bioassayed on washed rabbit platelets as described previously.[33] PMN supernatants were assayed without further extraction using a maximum volume of 50 μl and the amount of PAF-like bioactivity was quantified by reference to the aggregation induced by the hexadecyl PAF standard. The PMN cell pellets were extracted into ice-cold 80% ethanol, the protein precipitate was removed by centrifugation (1000 **g**, 4°, 5 min) and the extract was evaporated to dryness under reduced pressure before reconstitution in the assay buffer (containing 0·25% BSA to facilitate the resuspension of PAF). The criteria for the identification of the bioactivity as PAF included susceptibilty to antagonism by a selective concentration of the PAF receptor antagonist, WEB 2086 (1 μM), and resistance to inhibition by indomethacin (2·8 μM). In addition, samples containing PAF-like bioactivity were pooled, subjected to a Bligh-Dyer extraction and chromatographed on silica gel TLC (chloroform:methanol:water:acetic acid, 65:35:6:0·1) together with authentic [^{3}H]Paf (approximately 4000 d.p.m.). The co-migration of the bioactivity of pooled and re-extracted samples with authentic PAF further indicated that the activity in the non-extracted samples was due to PAF.

Superoxide anion assay

The generation of O_2^- was measured by the superoxide dismutase (SOD)-inhibitable reduction of cytochrome C.[34,35] Following 30 min pretreatment with cytokines or the vehicle, 0·25% BSA in Tyrode buffer, PMN were resuspended at $1–2 \times 10^6$/ml in Tyrode buffer containing 80 μM cytochrome C. The response to FMLP was complete within 5 min, at which time the samples were centrifuged (1000 **g**, 4°, 5 min). The absorbance (550 nm) of the supernatants was determined in a Hitachi U2000 spectrophotometer. The generation of O_2^- was calculated by the superoxide dismutase (30 U/ml)-inhibitable increase in absorbance at 550 nm using the differential molar extinction coefficient, 21×10^3/M/cm.

Radioimmunoassay of LTB_4

LTB_4 was measured in the supernatants of human PMN primed with cytokines then stimulated with FMLP for 5 min. Non-extracted supernatants were subject to radioimmunoassay using the LTB_4-specific antisera and the methods of Salmon *et al.*[36] The limit of detection of the assay was 0·5 pmol/10^6 PMN.

RESULTS

Generation of PAF and release of LTB_4 in cytokine-primed PMN

In freshly isolated PMN incubated for 30 min in the cytokine vehicle (Tyrode, 0·25% BSA) a low level of cell-associated PAF was detected (Fig. 1), but there was no release of PAF into the supernatants (data not shown). Upon stimulation with FMLP (100 nM) cell-associated PAF levels increased significantly ($P < 0·05$; Fig. 1) but no PAF was detectable in the supernatants (Fig. 2). Similarly, no LTB_4 release was detected in non-primed cells stimulated with FMLP (Table 1).

PMN were exposed to concentrations of cytokines known to be near maximally active in other assay systems. Quantitative

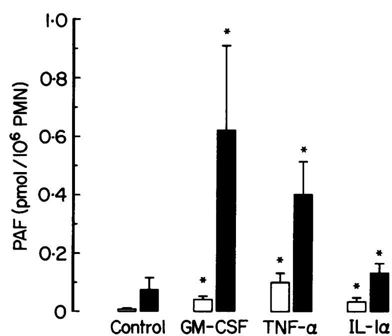

Figure 1. Cell-associated PAF levels in control and cytokine-primed PMN (open histograms) and in those subsequently stimulated with FMLP (filled histograms). Data are presented as the means and standard error of the mean of 3–13 observations. *$P<0·05$ Wilcoxon Rank sum test, compared with corresponding control value.

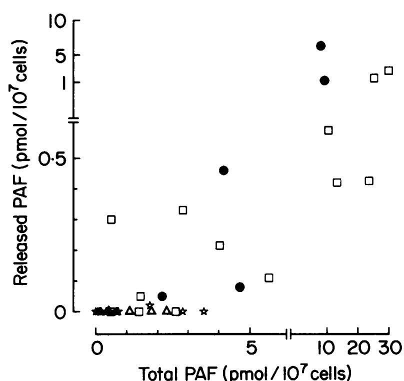

Figure 2. Release of PAF (supernatant) plotted against the total PAF formation (cell-associated+supernatant). Data are presented as individual observations and indicate PAF levels in non-primed or primed PMN subsequently stimulated with FMLP. In rhGM-CSF (squares) and TNF-α (filled circles) pretreated PMN 10/13 and 5/5 donors showed PAF release, respectively, whereas PAF levels were below the limit of detection (0·05–0·10 pmol/10^7 PMN) in IL-1α (triangles)-pretreated PMN from four donors. In non-primed PMN (control, stars), 12/13 donors failed to release detectable PAF levels upon stimulation with FMLP.

and qualitative changes in mediator generation and release took place in PMN primed with rhGM-CSF (1 nM), rhTNF-α (0·3 nM) or rhIL-α(60 pM). The basal generation of cell-associated PAF increased with each of these priming agents, as did the levels following FMLP stimulation (Fig. 1). Importantly, rhGM-CSF and rhTNF-α, which induced a quantitatively greater degree of priming than rhIL-1α, also induced PAF release in PMN stimulated with FMLP (Fig. 2), whereas levels remained undetectable in the supernatants of rhIL-1α-pretreated, FMLP-stimulated PMN. Furthermore, in rhGM-CSF-primed PMN, FMLP induced the release of detectable amounts of LTB_4 (Table 1).

The release of PAF showed considerable variability between donors (Fig. 2), but there were significant correlations between the amount released and the total amount formed for both GM-CSF ($r^2=0·72$, $n=14$, $P<0·05$) and TNF-α ($r^2=0·35$, $n=5$, $P<0·05$). PAF release ranged from 0% up to 60% for GM-CSF-primed, FMLP-treated PMN and from 2% up to 78% for those primed with TNF-α.

Table 1. Release of LTB_4 from non-primed and rh GM-CSF-primed PMN

		LTB_4 (pmol/10^6 PMN)	
Priming agent	Stimulation	Control	CGS 8515 3 μM
None	FMLP 100 nM	0	0
GM-CSF 1 nM	None	0	0
GM-CSF 1 nM	FMLP 100 nM	5·57±3·41*	1·79±1·68†

0=less than 0·50 pmol/10^6 PMN (limit of detection of the assay). Data are presented as the means and standard error of the means of five observations.

* $P<0·05$, Wilcoxon rank sum test, compared with level in non-primed PMN.

† $P<0·05$, Wilcoxon rank sum test, compared with the corresponding level in control PMN.

Priming of the respiratory burst

In parallel with changes in PAF and LTB_4 release, both rhTNF-α and rhGM-CSF primed PMN for an increased generation of O_2^- in response to FMLP (100 nM) (Table 2). rhIL-1α, which induced a smaller increase in the generation of PAF, did not consistently increase FMLP-induced O_2^- generation (Table 2).

Involvement of PAF in the enhanced respiratory burst

It is established that exogenous PAF acts as a priming agent, inducing an increase in the O_2^- generation in response to FMLP. Since we observed both PAF release and an enhanced O_2^- generation from rhTNF-α- and rhGM-CSF-primed and FMLP-stimulated PMN, the possibility that released PAF contributed to the priming was examined using the PAF receptor antagonist, WEB 2086. Pretreatment of PMN with PAF (0·1–1000 nM) for 5 min caused a concentration-dependent increase in FMLP-stimulated O_2^- generation, which was inhibited significantly ($P<0·05$) by preincubation of PMN with 1 μM WEB 2086 (Fig. 3). In contrast, preincubation with WEB 2086 had no effect ($P>0·05$) on priming induced by either rhGM-CSF or by rhTNF-α (Table 2). It is noteworthy that this concentration of WEB 2086 prevented completely the enhancing actions of PAF used at levels in the range detected in supernatants of primed and FMLP-stimulated PMN (0·1–1 nM).

Involvement of LTB_4 in the enhanced respiratory burst

The possible contribution of LTB_4 to priming was examined using the selective 5-lipo-oxygenase inhibitor, CGS 8515 (3 μM). The generation and release of LTB_4 in rhGM-CSF-primed, FMLP-stimulated PMN was prevented by pretreatment with CGS 8515 (Table 2). However, CGS 8515, either alone or in combination with WEB 2086, had no effect ($P>0·05$) on the enhancement of O_2^- generation in primed PMN. Nonetheless, CGS 8515 did reduce the respiratory burst in non-primed PMN.

Table 2. Effects of pretreatment with WEB 2086, CGS 8515 and the combination on the respiratory burst induced by FMLP in cytokine-primed PMN

	O_2^- (% FMLP)*			
Priming agent	Control	WEB 2086 1 μM	CGS 8515 3 μM	WEB 2086 and CGS 8515
None	100 10·54 ± 1·69†	102 ± 4 (6)	75 ± 7‡ (8)	83 ± 6‡ (7)
GM-CSF 1nM	174 ± 20§ (14) 15·31 ± 1·59†	184 ± 23§ (6)	150 ± 26§ (8)	121 ± 10§ (7)
TNF-α 0·3 nM	181 ± 22§ (14) 15·70 ± 1·65†	185 ± 28§ (6)	137 ± 18§ (8)	123 ± 13§ (7)
IL-1α 60 pM	108 ± 6 (10) 9·34 ± 1·24†	118 ± 9 (6)	85 ± 5 (4)	75 ± 4 (3)

* Data are presented as means and standard errors of means of (*n*) observations. Data have been expressed as a percentage of the response to FMLP in non-primed cells not treated with inhibitor (control).

† Absolute values of O_2^- generation (nmol/10^6 PMN).

‡ $P < 0·05$, Wilcoxon rank sum test, compared with value in control PMN.

§ $P < 0·05$, Wilcoxon rank sum test, compared with value in non-primed PMN.

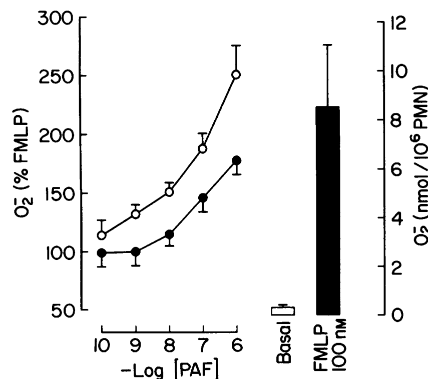

Figure 3. The enhancing effect of PAF (0·1–1000 nM, open circles) on the O_2^- generation in response to FMLP (100 nM). Inhibition of the PAF-induced enhancement by pretreatment with WEB 2086 (1 μM, filled circles) is also shown. Data are presented as the means and standard error of the mean of five observations (in duplicate). Data are expressed as percentages of the response to FMLP (100 nM) in the absence of PAF pretreatment. The basal and FMLP (100 nM)-induced O_2^- generation (nmol /10^6 PMN) are presented as histograms on the right hand side of the figure.

DISCUSSION

Our findings indicate that cytokine-priming of human PMN results in an increased generation of PAF which may be further increased by stimulation with the chemotactic peptide, FMLP. More importantly, primed PMN release detectable amounts of PAF upon stimulation. We examined the role of PAF and LTB_4 in the priming of PMN for increased O_2^- generation. Although released in increased amounts, neither of these lipid mediators accounts for the priming of O_2^- generation.

Previous studies have established that rhGM-CSF-primed PMN release increased amounts of LTB_4 in response to stimulation by a variety of neutrophil agonists, including FMLP and serum-treated zymosan.[4,5,24,37] Furthermore, LTB_4 is reported to prime human PMN for increased FMLP-induced O_2^- generation.[25] The selective inhibitor of 5-lipo-oxygenase, CGS 8515[30] reduced LTB_4 levels in rhGM-CSF-primed and FMLP-stimulated PMN supernatants by 70%, but failed to inhibit the priming of O_2^- generation. Thus, it seems unlikely that released LTB_4 plays a role in the priming phenomenon. Cellular retention of LTB_4 has been suggested[38] and several studies have provided evidence for second messenger functions of 5-lipo-oxygenase products in PMN.[39–42] Retention of LTB_4 is also unlikely to explain the priming phenomenon since the synthesis inhibitor would also have reduced these levels of LTB_4. Inhibition by CGS 8515 of the respiratory burst in non-primed PMN is consistent with previous suggestions, based on the inhibitory effects of relatively non-selective agents such as nordihydroguiaretic acid, that 5-lipo-oxygenase products act as second messengers in human PMN. However, the apparent contribution of products of the 5-lipo-oxygenase pathway to the transduction processes was not increased by priming, since there was no increase in the percentage inhibition of O_2^- generation by CGS 8515 in primed PMN.

Several studies report that FMLP is an ineffective stimulus for LTB_4 generation unless exogenous arachidonic acid is also added,[4,5,43] indicating that phospholipase activity is rate limiting for PMN leukotriene generation. The present study, and others on the influence of cytokines on PMN eicosanoid generation,[4,5] suggest that rhGM-CSF overcomes the requirement for exogenous arachidonic acid. Indeed, one of the biochemical responses to rhTNF-α and rhGM-CSF is the mobilization of arachidonic acid.[22] Activation of phospholipase A_2 is also likely to explain the enormous increase in PAF levels in cytokine-primed PMN, since this enzyme is the initial step in the remodelling pathway of PAF biosynthesis.[44] The failure of GM-CSF to stimulate LTB_4 release may be related to its

inability to stimulate phospholipase C and thereby increase intracellular Ca^{2+}, which is necessary for the activation of 5-lipo-oxygenase.

The release of PAF from primed and stimulated PMN and its established capacity to rapidly prime PMN (present study)[11,25–27] raised the possibility that PAF mediates the priming phenomenon, at least for O_2^- generation. This possibility was further supported by the failure of rhIL-1α to prime for PAF release or consistently increase FMLP-induced O_2^- generation. rhIL-1α did increase cell-associated PAF levels, as reported for human endothelial cells,[45] but to a lesser extent than either rhGM-CSF or rhTNF-α. In addition, a recent study indicated that PAF release during phagocytosis could increase intracellular calcium by an autocrine mechanism.[46] Unequivocal evidence that released PAF does not account for rhTNF-α- or rhGM-CSF-induced priming was provided by experiments using the competitive PAF receptor antagonist WEB 2086.[28] This compound completely prevented priming of PMN induced by 0·1–1 nM PAF, and reduced responses to higher concentrations of PAF (10–1000 nM), but had no effect on cytokine priming. In the study of Wirthmueller *et al.*[27] the potential role of PAF as a mediator of priming was proposed on the basis that total lipid extracts of primed and stimulated PMN could prime PMN for O_2^- generation. However, these studies took no account of the amount of PAF released and therefore available to cell-surface receptors. Our results provide clear evidence against an autocrine priming action of PAF released from cytokine-treated PMN, but it is not possible to exclude a role for cell-associated PAF in this phenomenon.

We have suggested that cell-associated PAF functions as a second messenger in phagocytes and endothelial cells.[33,35,47] At higher concentrations than that used in the present study, PAF receptor antagonists inhibit partially the non-primed respiratory burst in rabbit PMN[35] and in human PMN stimulated with FMLP (A. G. Stewart and T. Harris, unpublished observations)[26] or with rhTNF-α.[48] Further examination of the role of cell-associated PAF in the priming response awaits the availability of an effective and selective inhibitor of PAF biosynthesis.

PAF release was consistently observed in rhTNF-α- and rhGM-CSF-primed, FMLP-stimulated PMN, but not in non-primed PMN or in those exposed to rhIL-1α. Considerable variation in the values for PAF release from FMLP-stimulated PMN reported in the literature may be explained by the presence of trace quantities of endotoxin in the reagents.[31] Endotoxin induces priming of human PMN, and also by itself, as reported here for GM-CSF and TNF-α, markedly increases PAF levels.[11] The conditions of PMN incubation (cell number/extracellular pH; constant renewal of extracellular medium) may be critical in determining the proportion of PAF released by PMN.[49,50] The relationship, if any, of these findings to the ability of cytokines to increase PAF release is not clear. A recent study reported that FMLP stimulated PAF release from human PMN and that the release could be increased by pretreatment with GM-CSF, but cell-associated PAF levels were not measured.[51] The use of cytochalasin B, which disrupts the normal physiology of PMN by interfering with microtubule function, limits the conclusions that can be drawn from latter study. Moreover, these workers used a radiochemical method to determine PAF release which may not produce results indicative of changes in endogenous levels.

An important consideration is whether the release of PAF by cytokine-primed PMN is simply a result of over-loading the cellular storage capacity or whether a specific transport mechanism is involved. The latter is suggested by the observation that the ionophore, A23187, stimulates the generation of significantly more PAF than does FMLP but results in the release of a smaller proportion of the total amount of PAF.[52]

In conclusion, release of the phospholipid-derived mediators, LTB_4 and PAF, either singly or in combination, does not contribute to the rhTNF-α or rhGM-CSF priming of PMN for enhanced O_2^- generation. However, the release of these mediators by primed PMN is likely to be of importance for the pathogenesis of inflammatory conditions[44,48] involving PMN infiltration and in immune regulation.[53]

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