

Platelet-activating factor enhances interleukin-6 production by alveolar macrophages

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The production of the cytokine interleukin-6 (IL-6) by rat alveolar macrophages (AMs) was analyzed after their stimulation with muramyl dipeptide (1 µg/ml), in the presence of graded concentrations of platelet-activating factor (PAF). Significantly enhanced production of IL-6 was observed at 10^{-10} to 10^{-8} mol/L PAF, with peak effect at 10^{-10} mol/L. This enhancement was blocked by three structurally unrelated specific PAF receptor antagonists BN 52021, WEB 2170, and CV 3988. The biologically inactive PAF precursor/metabolite, lyso-PAF, and the enantiomer enantio-PAF failed to induce significant enhancement in IL-6 production. In parallel, addition of PAF to AM triggered leukotriene B_4 (LTB_4) release. Inhibition of 5-lipoxygenase pathway by AA-861 or MK 886 inhibited the PAF-induced augmentation of both IL-6 and LTB_4 production, suggesting an implication of endogenous leukotrienes in this mechanism. Furthermore, addition of exogenous LTB_4 to AMs could augment their IL-6 production, with peak activity at 10^{-12} mol/L LTB_4 , and reverse the inhibitory effects of 5-lipoxygenase inhibitors. Taken together, these observations suggest that PAF can modulate lung immune and inflammatory responses by enhancing IL-6 production and that this activity may be dependent on secondary 5-lipoxygenase metabolites. This may have clinical relevance in PAF-mediated events in the lung, such as the cellular components of late-phase asthma. (J ALLERGY CLIN IMMUNOL 1992;90:796-802.)

Key words: Cytokines, PAF, lipid mediators, lipoxygenase products, inflammation, LTB_4 , IL-6, immune regulation, alveolar macrophages, receptor antagonists, five lipoxygenase activating protein

In the lung, the alveolar macrophages (AMs) are the first cells of the host defense system to interact with foreign substances and to modulate the ensuing inflammatory response by secreting chemotactic factors, various enzymes and cytokines, and a variety of lipid mediators such as the arachidonic acid metabolites, prostaglandins and leukotrienes, and the phospholipid platelet-activating factor (PAF).¹⁻⁶ Recent attention has been directed to the role of PAF in the mediation of allergic inflammation and in the pathogenesis of asthma.⁷⁻¹¹ The precise role of PAF in asthma, however, remains unknown. In common with

Abbreviations used

AM:	Alveolar macrophage
LTB_4 :	Leukotriene B_4
MDP:	N-acetyl-muramyl-L-alanyl-D-isoglutamine
PAF:	Platelet-activating factor, 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine
5-LOX:	5-lipoxygenase
IL:	Interleukin
TNF:	Tumor necrosis factor
FBS:	Fetal bovine serum

other inflammatory mediators, PAF is capable of reproducing many of the functional features seen in asthma both in vivo and in vitro, including bronchoconstriction and liberation of bronchoconstrictor compounds such as thromboxane A_2 and leukotrienes,¹²⁻¹⁵ increase in vascular permeability,¹⁶ stimulation of bronchial mucus secretion, and activation of inflammatory cells.^{17, 18} In addition, PAF has potent chemotactic activity for eosinophils and neutrophils,¹⁸ and instillation of PAF into rabbit airways can cause an inflammatory reaction, with an accumulation of polymorphonuclear leukocytes and macrophages.¹⁹

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The role of cytokines in inflammatory responses is the focus of intense research both at the local and systemic level. It has become increasingly evident that the cytokine interleukin-6 (IL-6) is an important modulator in inflammatory processes.²⁰ Some of the effector functions of IL-6 are similar to those of the pleiotropic cytokines IL-1 and tumor necrosis factor- α (TNF- α),²¹ but IL-6 has also been reported to regulate IL-1 and TNF production, and may thus act as an antiinflammatory modulator.^{22, 23} More specifically, lipopolysaccharide-induced lung inflammation is inhibited by IL-6.²⁴ Monocytes/macrophages, endothelial cells, and fibroblasts are believed to be the major IL-6-producing cells in mammals.^{20, 25}

Eicosanoids have been considered to be responsible for some actions of PAF and have also been found to modulate the effects of PAF itself.^{26, 27} Several cell populations, such as mast cells, AMs, and eosinophils can release PAF after appropriate stimulation. Recently we have shown that PAF-induced augmentation of TNF α production by AMs and monocytes is dependent on endogenous leukotriene production.^{28, 29} Little is known, however, about the potential modulatory role of lipid mediators on IL-6 production. Since the lung is a site of inflammation in several disease states, we investigated the action of PAF on the modulation of IL-6 production by rat AMs and the participation of 5-lipoxygenase (5-LOX) metabolites in this regulation.

MATERIAL AND METHODS

Rats. Male Wistar rats weighing 200 to 250 gm were purchased from Charles River Canada, Inc. (St. Constant, Quebec City, Canada). These animals were derived from a pathogen-free colony and shipped behind filter barriers.

Chemicals. Muramyl dipeptide (MDP; N-acetyl-muramyl-L-alanyl-D-isoglutamine) was purchased from Calbiochem (La Jolla, Calif.). PAF, lyso-PAF, and enantio-PAF (Novabiochem, Switzerland) were dissolved in ethanol and resuspended in RPMI 1640 medium containing bovine serum albumin (0.25%). PAF antagonist BN 52021, provided by Dr. P. Braquet (IHB-IPSEN, Le Plessis Robinson, France), was first dissolved in NaOH 0.5N (100 μ l), followed by HCl 0.1N (100 μ l), and diluted in RPMI 1640. WEB 2170, provided by Dr. H. Heuer (Boehringer Ingelheim, Germany), was resuspended in RPMI medium. Leukotriene B₄ (LTB₄) and the leukotriene synthesis inhibitors, MK 886³⁰ (gift from Dr. A. W. Ford-Hutchinson, Merck-Frosst, Dorval, QC, Canada) and AA-861 (provided by Dr. Masao Nishikawa, Takeda Chemical Industries, Osaka, Japan) were dissolved in ethanol and further diluted in RPMI 1640 medium. Final ethanol concentrations were less than 0.1%. Controls contained vehicle only. Bovine serum albumin, zymosan A, calcium ionophore A23187, and MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Alveolar macrophage preparation. Rat AMs were obtained by bronchoalveolar lavage. Rats anesthetized with ketamine-hydrochloride were exsanguinated, the thoracic cavity was opened, and the trachea was cannulated. The lungs were lavaged with a total volume of 60 ml phosphate-buffered saline (PBS) in 10 ml aliquots. After recovery, the bronchoalveolar cells were centrifuged, washed twice in PBS, and resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS). Cells were counted in a hemocytometer chamber, and viability was determined by trypan blue exclusion. The cells (>98% AMs, determined by nonspecific esterase and Wright-Giemsa staining) were incubated overnight at 1×10^6 cells/ml in RPMI 1640 supplemented with 5% FBS in polystyrene tubes to allow them to return to baseline activity before stimulation. No removal of AMs was necessary before supernatant production.

Supernatant production. After overnight incubation, AMs were washed once in their original tubes with RPMI 1640 medium. They were then treated with or without MDP (1 μ g/ml) and graded concentrations of PAF, PAF antagonists, LTB₄, or leukotriene synthesis inhibitors, either alone or in combination. After 18 hours of culture, the tubes containing AMs were centrifuged at 400g for 10 minutes, and the cell-free supernatants were harvested and stored at -80°C until used for the IL-6 assay. Control cultures contained corresponding vehicle only and were otherwise treated as experimental cultures. Final concentrations of ethanol or methanol were less than 0.08% and had no effect by themselves in the experiments.

Interleukin-6 assay. Bioactivity of IL-6 was assayed with use of the IL-6-sensitive B9 hybridoma cell line (gift from Dr. J. Gauldie, Hamilton, Ontario, Canada) according to Aarden et al.³¹ In brief, 5×10^3 cells were seeded into flat-bottomed 96-well plates in RPMI 1640 plus 5% FBS together with serial dilutions of AM supernatants in duplicates. Test cultures were incubated at 37°C for 72 hours before the addition of 20 μ l of MTT (5 mg/ml). After an additional 4 to 6 hours of incubation the cells were solubilized with 100 μ l of sodium dodecylsulfate 10%, and absorbance was read at 590 nm on a microplate reader model 3550 (BioRad Laboratories, Richmond, Calif.). A standard curve with recombinant human IL-6 (Endogen, Boston, Mass.) was run in each assay. Units were calculated by probit analysis of the sample dilution curves in comparison with the IL-6 standard. A neutralizing polyclonal rabbit anti rat IL-6 antiserum (gift from Dr. J. Gauldie, McMaster University Hamilton, Ontario) was used to identify the bioactive cytokine measured in the B9 cell assay as IL 6.

LTB₄ assay. AMs (1×10^6 cells/ml) were incubated in RPMI medium in presence or absence of PAF, leukotriene synthesis inhibitors, zymosan A, or ionophore A 23187 either alone or in combination. After 30 minutes and 3 hours of incubation, supernatants were collected and assayed for LTB₄ content by RIA (Amersham, Oakville, Ontario). LTB₄ values are expressed as pg/ 1×10^6 cells.

Statistical analysis. Statistical significance of differences between groups of experiments was assessed by anal-

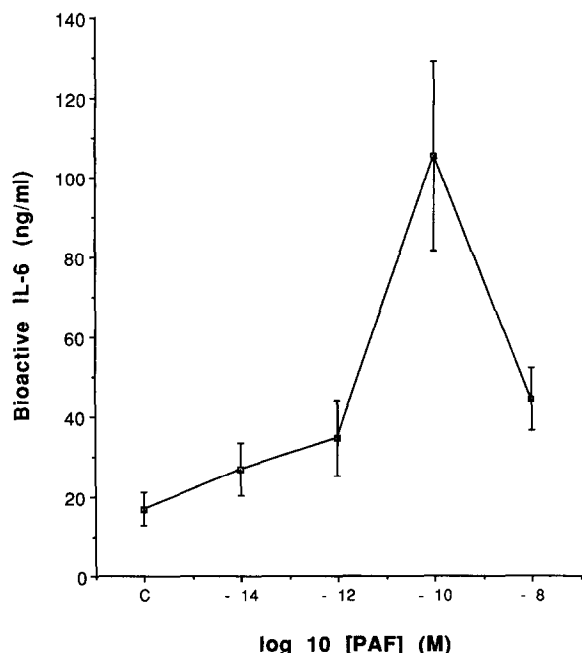


FIG. 1. Effect of PAF on IL-6 production by rat AMs. Macrophages ($10^6/\text{ml}$) stimulated with MDP ($1 \mu\text{g}/\text{ml}$) were cultured in the absence (vehicle control: c) or presence of graded concentrations of PAF for 18 hours. Cell-free supernatants were then analyzed for IL-6 bioactivity with use of the B9 cell proliferation assay (Materials and Methods). Data represent mean $\pm$ SE of 11 separate experiments. Statistically significant (ANOVA, Scheffé test) effects were noted for PAF concentrations of $10^{-10} \text{ mol}/\text{L}$ ($p < 0.01$) and $10^{-8} \text{ mol}/\text{L}$ ($p < 0.05$).

ysis of variance (ANOVA, Scheffé test). Confidence level was at $p < 0.05$.

RESULTS

Enhanced production of IL-6 by PAF-stimulated AMs

IL-6 production by unstimulated and PAF-stimulated lung macrophages was assessed by use of the B9 hybridoma proliferation assay. AMs stimulated or not with the macrophage activator MDP ($1 \mu\text{g}/\text{ml}$) were cultured overnight with graded concentrations of PAF ($10^{-14} \text{ mol}/\text{L}$ to $10^{-8} \text{ mol}/\text{L}$). In the absence of MDP, PAF alone had no significant effect on IL-6 production by AMs (data not shown). However, the combined addition of PAF and MDP to AM cultures markedly enhanced IL-6 production compared with AM cultures with MDP alone. As shown in Fig. 1, PAF concentrations of 10^{-8} and $10^{-10} \text{ mol}/\text{L}$ induced a twofold to fivefold increase in IL-6 production by MDP-stimulated AMs with peak effect observed at $10^{-10} \text{ mol}/\text{L}$. Anti-IL-6 antiserum (90 to 900 $\mu\text{g}/\text{ml}$) totally abrogated the PAF-induced AM supernatant

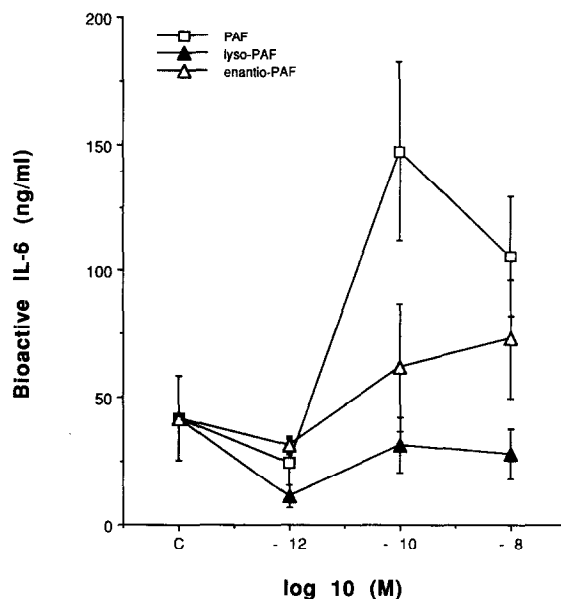


FIG. 2. Effect of PAF, lyso-PAF, and enantio-PAF on IL-6 production by rat AMs, measured in supernatants of 18-hour cultures stimulated with MDP ($1 \mu\text{g}/\text{ml}$). Data represent mean $\pm$ SE of four separate experiments. Statistically significant (ANOVA, Scheffé test) effect was noted only for PAF ($p < 0.01$).

activity on B9 cells, indicating that IL-6 bioactivity was indeed being measured.

Compared with the active form of PAF, the biologically inactive PAF precursor/metabolite, lyso-PAF, and the enantiomer enantio-PAF failed to induce any significant IL-6 production (Fig. 2).

Blocking of PAF-induced IL-6 production by PAF antagonists

Three structurally unrelated PAF receptor antagonists were used in studies designed to verify the specificity of PAF-induced IL-6 production by AMs and its mediation through the PAF receptor. For these experiments, AMs were pretreated for 15 to 30 minutes with or without a PAF antagonist before the stimulation with PAF. Fig. 3, A shows that the ginkgolide BN 52021, as a structural analog of PAF, completely blocked the PAF-induced increase in IL-6 production.

Effect of lipoxygenase inhibitors on PAF-stimulated IL-6 and LTB_4 production

We next investigated the possible role of endogenous 5-LOX in PAF-stimulated IL-6 production, since PAF has been shown to elicit leukotriene production in certain cell types.³² For these experiments, we used AA-861, a specific 5-LOX inhibitor and MK 886, a new leukotriene synthesis inhibitor that prevents 5-LOX activation by binding to the 5-LOX activating

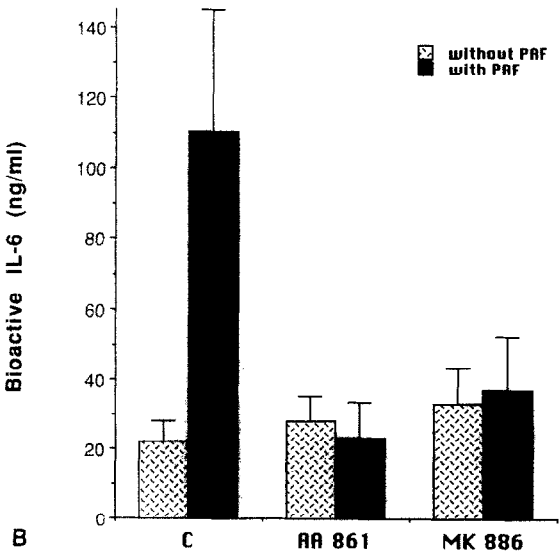
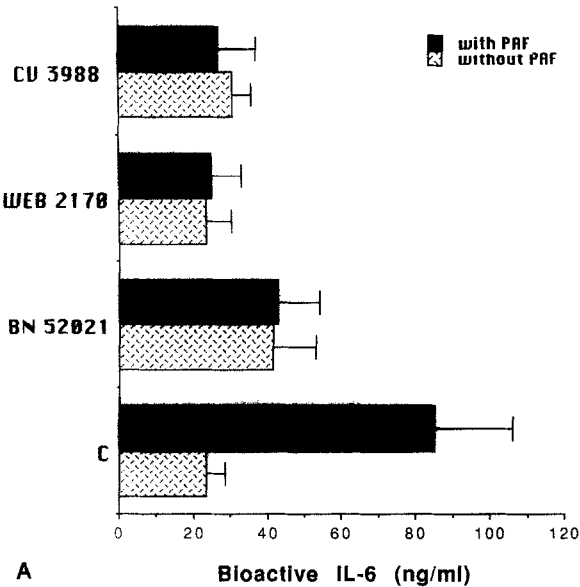


FIG. 3. Effects of blocking PAF receptors or inhibiting 5-LOX activation on IL-6 production by AMs stimulated by PAF. AMs were incubated with (A) the PAF receptor antagonists BN 52021 (100 μ mol/L), WEB 2170 (10 μ mol/L) or CV 3988 (10 μ mol/L) or (B) the inhibitors of 5-LOX pathway, AA 861 (1×10^{-5} mol/L) or MK 886 (2.2×10^{-6} mol/L) 30 minutes before addition of MDP and PAF. After 18 hours of incubation, supernatants were collected and IL-6 was measured by the B9 cell proliferation. Data represent mean $\pm$ SE of four (A) and nine (B) separate experiments.

protein, FLAP. AMs were preincubated for 15 minutes at 37 $^{\circ}$ C with these inhibitors before stimulation with PAF. Data shown in Fig. 3, B indicate that AA-861 (1×10^{-5} mol/L) and MK 886 (2.2×10^{-6} mol/L) completely abolished PAF-stimulated IL-6 ac-

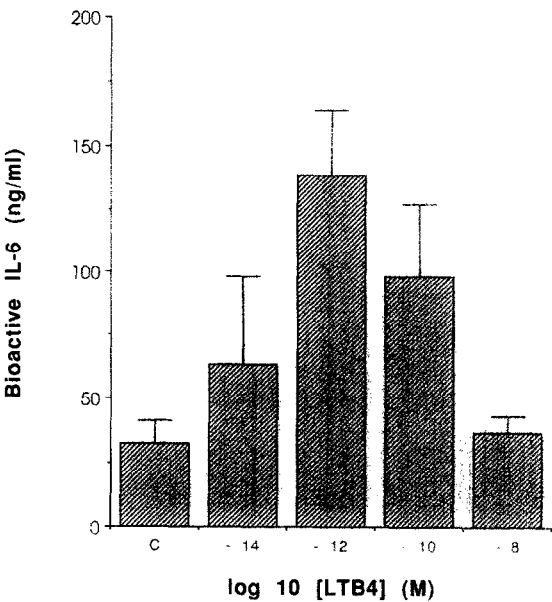


FIG. 4. Effect of exogenous LTB₄ on IL-6 production by AMs. Macrophages stimulated with MDP (1 μ g/ml) were incubated in the absence (vehicle control: c) or presence of graded concentrations of LTB₄ for 18 hours. IL-6 activity was tested by use of the B9 cell proliferation assay. Data represent mean $\pm$ SE of four separate experiments. Statistically significant enhancement of IL-6 production was noted for LTB₄ concentration of 10^{-12} mol/L ($p < 0.05$).

TABLE I. Effect of leukotriene synthesis inhibitors on LTB₄ production by rat AMs stimulated with PAF

Culture conditions	LTB ₄ (30 min)*†	LTB ₄ (3 hr)
Control	6	500
PAF 10 ⁻¹⁰ mol/L	10	>800
AA861 1 $\times$ 10 ⁻⁵ mol/L	1	350
AA861 + PAF	0	62
MK886 2.2 $\times$ 10 ⁻⁶ mol/L	7	80
MK886 + PAF	0	30
A23187 1 $\times$ 10 ⁻⁶ mol/L	520	>800
Zymosan (50 particles/AM)	600	>800

*AMs stimulated with MDP (1 μ g/ml) were incubated 30 minutes or 3 hours with or without inhibitors and PAF. Data are from a representative experiment.

†LTB₄ detected by RIA and expressed as pg/1.0 $\times 10^6$ cells.

tivity. In parallel experiments, we verified the production of LTB₄ by PAF-activated AMs and the capacity of the leukotriene synthesis inhibitors to block this effect. Table I shows that, at the same concentrations found to inhibit IL-6 production, both inhibitors effectively abolished PAF-stimulated LTB₄ production.

TABLE II. Reversal by exogenous LTB₄ of the inhibitory effects of 5-LOX inhibitors on IL-6 release induced by PAF

Culture conditions	Experiment 1*†	Experiment 2
Control	17.2	14.0
PAF 10 ⁻¹⁰ mol/L	57.0	49.0
LTB ₄ 10 ⁻¹² mol/L	30.2	21.0
MK 886 2.2 × 10 ⁻⁶ mol/L	15.1	16.2
MK 886 + PAF	9.6	11.0
MK 886 + PAF + LTB ₄	36.9	28.1

*AMs stimulated with MDP (1 µg/ml) were preincubated for 15 minutes in the presence or absence of MK 886 and then incubated for 18 hours with PAF and/or LTB₄.

†Data are from two separate experiments and are expressed as ng/ml of bioactive IL-6.

Effect of exogenous LTB₄ on PAF-induced IL-6 production

Because endogenous 5-LOX metabolites appeared to be implicated in the PAF-induced enhancement of IL-6 production, we assessed whether a 5-LOX metabolite, such as LTB₄, could reproduce and mimic the effect of PAF. AMs were incubated for 18 hours with graded concentrations of LTB₄ (10⁻¹⁴ to 10⁻⁸ mol/L). As can be seen in Fig. 4, exogenous LTB₄ significantly augmented IL-6 production with a maximal action at 10⁻¹² mol/L. Furthermore, LTB₄ could reverse the inhibitory effects of 5-LOX inhibitors on IL-6 release induced by PAF (Table II).

DISCUSSION

AMs play a central role in the lung defense system, and they constitute the main link between primary inflammatory events and the ensuing immune response. Several macrophage functions are mediated through their ability to release potent mediators. Among them are lipid compounds, derived from membrane phospholipids, and cytokines, such as IL-1, TNF, and IL-6. Although the modulation of TNF and IL-1 production by PAF has been established in rat AMs,²⁸ in human monocytes, and in rat spleen macrophages,³³⁻³⁵ no data exist on the effects of PAF on IL-6 production in any cell type.

In the present study we provided first evidence that (1) PAF can enhance IL-6 production by AMs, (2) that endogenous 5-LOX metabolites can act as secondary mediators in PAF-induced IL-6 production, and (3) that exogenous LTB₄ can mimic the effect of PAF and stimulate IL-6 production by AMs.

PAF alone failed to stimulate IL-6 production in rat AMs, suggesting that a double signal, provided by MDP + PAF, was needed to trigger the effect. This may correlate with the findings of Glaser et al.³⁶ who showed that both lipopolysaccharide and PAF were needed to trigger phospholipase A₂ synthesis and activity in the macrophage-like cell line, P388D₁.

Neutrophils and monocyte-macrophages constitute

a major source of leukotrienes, when adequately stimulated by inflammatory or phagocytic stimuli. The finding that IL-6 production is associated with inflammatory states suggests that its production may be modulated by inflammatory mediators. The 5-LOX metabolites of arachidonic acid, and in particular LTB₄, have been recognized as powerful activators of several phagocyte functions and modulators of cytokine production. Exogenous and endogenous LTB₄ was found to enhance IL-1, IL-2, and interferon gamma production.³⁷⁻³⁹ Our data indicated that exogenous LTB₄ could enhance IL-6 production by AMs. Picomolar concentrations were sufficient to induce significant augmentation of release of this cytokine. Furthermore, our data also suggest a possible implication of endogenous leukotriene in the PAF-induced IL-6 production by AMs. We noted the concomitant inhibition of PAF-enhanced IL-6 production as well as LTB₄ generation by the 5-LOX inhibitors AA-861 and MK 886. Our findings are consistent with the possibility that the action of PAF on AMs can be mediated by the generation of endogenous lipoxygenase metabolites. We have previously reported this kind of regulation in terms of TNF production by rat AMs.²⁸ Furthermore, we recently reported that LTB₄ could stimulate IL-6 production by human monocytes and that this appeared to involve both transcriptional and post-transcriptional mechanisms.⁴⁰ Horiguchi et al.⁴¹ and Mohri et al.⁴² have also reported that TNF production by HL-60 cells stimulated with phorbol esters or lipopolysaccharide, respectively, was sensitive to lipoxygenase inhibitors.

Over the past few years, PAF has been shown to play a crucial role in the pathogenesis of allergic and inflammatory diseases such as asthma.⁷⁻⁹ Studies have demonstrated that asthma is a complex airway inflammatory process associated with bronchoconstriction, mucosal edema, increased vascular permeability, epithelial damage, and, particularly in the late phase, the recruitment of inflammatory cells.^{10, 11}

PAF is chemotactic for a number of inflammatory

cells,¹⁸ and one possible mechanism of PAF-induced bronchoconstriction is through the recruitment of a secondary cell type (or types) to the airway. PAF is also known to augment TNF- α , IL-1, and IL-6 production (by the present study) by monocytes/macrophages. The ability of PAF to induce the production of cytokines may be one of the several possible mechanisms by which this mediator can induce delayed or persistent inflammatory reactions. Not only does PAF stimulate synthesis of cytokines and itself,^{43, 44} but cytokines can induce the synthesis of more PAF,^{45, 46} thereby completing a cycle of positive feedback loops. These loops could be important for the amplification of the immune response, but they could, if they escape local regulation, lead to uncontrolled, sustained release of these potent factors and thereby result in diseases of inflammation.

Numerous studies have indicated a role for PAF in lung disease, namely asthma, as evidenced by inhibition with use of PAF receptor antagonists.⁴⁷ On the other hand, direct measurements of PAF concentrations at the alveolar level during specific inflammatory conditions are lacking and would be important to make a correlation between the *in vitro* "effects" of PAF on AMs, as shown in this article and an *in vivo* "role" for PAF in AM-derived cytokine production.

In conclusion, our findings showed that PAF could positively modulate IL-6 production by rat AMs and that this effect occurred via endogenous generation of 5-LOX metabolites, such as LTB₄. Since cellular sources of PAF and stimuli for its production are present in many inflammatory reactions, the effects of PAF and LTB₄ on the production of cytokines such as IL-1, TNF, and IL-6 may constitute a powerful regulatory mechanism in inflammation and immunomodulation. Our findings should help elucidate the underlying mechanisms of pathophysiology in conditions such as asthma and provide elements of clinical importance for therapy.

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