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Enhanced Eosinophil Luminol-Dependent Chemiluminescence and Complement Receptor Expression by Platelet-Activating Factor and Interleukin-5

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SUMMARY **The cytokine interleukin-5 (IL-5) and the lipid mediator platelet-activating factor (PAF) have both been shown to be involved in eosinophil differentiation and activation. We have measured and compared the effect of PAF and IL-5 on human eosinophils in terms of their luminol-dependent chemiluminescence (CL) response and their expression of complement receptors, CR1 and CR3. Both IL-5 and PAF enhanced the eosinophil CL response. The optimal concentrations were 40 U/ml for IL-5, and 10^{-6} M for PAF. The priming effect of IL-5 was slow and reached a maximal response after 90 minutes incubation. In contrast, the effect of PAF peaked early and declined during incubation. In the complement receptor study, only PAF was able to enhance CR3 expression ($p < 0.05$) while the effect of IL-5 on eosinophil complement receptor expression was negligible. These results provide evidence that both inflammatory mediator (PAF) and cytokine (IL-5) can activate eosinophils but the effects of IL-5 and PAF on eosinophil CL response appear to be distinct. The activation of eosinophils by PAF and IL-5 may occur through different mechanisms.**

Eosinophilia is associated with a wide spectrum of clinical disorders. These include immediate-type hypersensitivity and helminthic infections.[1] Eosinophils share many biological properties with neutrophils but also possess very distinct features. These include their content of large granules such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN).[1] Eosinophils are also able to release membrane derived sulphidopeptide leukotriene LTC_4 and platelet-activating factor (PAF).[2,3] Eosinophil-derived products probably play an important role in the destruction of helminthic larvae whereas in some situations, eg chronic bronchial asthma, the cell may be responsible for considerable tissue damage.[4] Knowledge of eosinophil and eosinophil-associated events is growing rapidly since a number of factors have been shown to selectively activate eosinophils.

Interleukin-5 (IL-5) is a potent eosinophil differentiation factor which induces the selective differentiation and proliferation of eosinophils from mononuclear cells (MNCs) of human umbilical cord blood[5] and bone marrow cells.[6] IL-5 has also been reported to be selectively chemotactic for eosinophils with no effect on neutrophils and monocytes.[7] PAF is a potent pro-inflammatory mediator, which causes PMN chemotaxis and degranulation *in vitro*.[9] and when injected into human skin causes PMN and MNC cell accumulation.[9] PAF also appears to be a potent eosinophilotactic agent[10] and is able to enhance bronchial hyperresponsiveness after inhalation in man.[11]

It has recently been reported that peripheral blood eosinophils during exacerbation of myalgia symptoms displayed characteristics of "activation", including hypodense phenotype and increased responsiveness to PAF *in vitro* with respect to expression of CD11b surface adherence proteins.[12] It has also been demonstrated that IL-5 mRNA can

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be elaborated by cells in the bronchial mucosa of a majority of mild asthmatics but not normal control.[13] The amount of mRNA detected also correlated with the number of eosinophils and activated T-lymphocytes in biopsies. Both PAF and IL-5 may be of relevance to eosinophil activation.

In order to compare and contrast the difference in eosinophil activation by PAF and IL-5, we have investigated the effect of IL-5 and PAF on eosinophil activation by looking at their enhancement of (a) complement receptor (CR1 and CR3) expression and (b) phagocytotic activity as determined by luminol-dependent chemiluminescence.

MATERIALS AND METHODS

PAF, L-α-phosphotidylcholine, β-acetyl, γ-o-octadec-9-cis-enyl), (Sigma Chemical Co., St. Louis, MO, USA) was dissolved at 0.1 mM in RPMI-1640 (Gibco), aliquoted and stored at -70° C. IL-5, murine recombinant (Genzyme Corp., Boston MA, USA), was dissolved at 400 U/ml in RPMI-1640, aliquoted and stored at -70°C. BN52021, PAF antagonist, was kindly given by Dr P Braquat. Anti-CR1 and anti-CR3 MAb were purchased from Becton Dickinson, Sunnyvale Inc., CA., USA. Metrizamide, fMLP, luminol, zymosan and FITC-conjugated F(ab) $'_2$ fragments of rabbit antibody to mouse Ig were purchased from Sigma Chemical Co.

Opsonization of zymosan

One hundred microgram of zymosan was washed twice with PBS for 10 minutes (1,600×g, 20°C). The washed-zymosan was resuspended in 30 ml PBS and swelling in the boiling water bath for 60 minutes. After swelling, the zymosan was washed with PBS at 1,600×g for 10 minutes, then adjusted to 2×10^8/ml and stored at -70°C. For opsonization, fresh human serum was mixed with equal volume of zymosan and incubated at 37°C on a rock-and-roller for 1 hour. After incubation, the zymosan was washed twice with PBS (1,600×g, 10 minutes). The opsonized zymosan was resuspended to the original volume with PBS and stored at -70°C.

Separation of eosinophils

Human eosinophils were isolated from peripheral blood according to the method of Vadas *et al.*[14] Blood was obtained from patients attending a routine allergy clinic with either allergic rhinitis or bronchial asthma, who were found to have an eosinophilia between 5% to 20% and total eosinophil count > 500/mm^3, or from in-patients with hypereosinophilia admitted to our medical ward, Veterans General Hospital, Taipei. Blood with 10 U/ml of preservative-free heparin was mixed with 0.2 vol of 6% dextran 110. The plasma/leukocyte fraction was pipetted off after 30 minutes incubation at 37°C, and the cells were harvested by centrifugation at 250×g for 10 minutes at 4°C and washed twice with RPMI-1640 containing 20 μg/ml of deoxyribonuclease I. The cell pellet was resuspended in the same buffer at approximately 5 to 7×10^7 cells per ml and layered onto discontinuous gradients of metrizamide (18%, 20%, 22%, 23%, 24% and 25% metrizamide in Tyrode buffer containing 0.1% gelatin). Gradients were centrifuged at 1,200×g for 40 minutes at 20°C, and most eosinophils were recovered from the 23%/24% interface (metrizamide densities 1.123 to 1.129 g/ml) with a purity over 85%. In the low-density population, the contamination of neutrophil was as high as 70% to 86% with smaller numbers of mononuclear cells. In the cases of patients with the hypereosinophilic syndrome an additional low-density eosinophil population was recovered from the 20% and 22% metrizamide layers (metrizamide 1.107 and 1.118 g/ml).

Chemiluminescence (CL) assay

Measurement of the CL response was adapted from the method of Prendergast and Proctor.[15] Eosinophils (2×10^6 cells/ml) were incubated with different concentrations of PAF or IL-5 for varying lengths of time in air with 5% CO_2 at 37°C and 100% humidity. Cell mixtures (100 μl) were placed in the counting vials and maintained at 37°C in a Lumacounter 1251 (LKB Wallac, Turku, Finland). To activate eosinophils, opsonized zymosan (2×10^8/ml, 100 μl) and luminol (10^{-4}M, 100 μl) were added to the cell mixture. CL was recorded as millivolts (mV) every 6 seconds in summation mode and the peak of CL response was regarded as the maximum of eosinophil activation. RPMI-1640 was used as the baseline CL response. For the inhibition test, BN52021 was added to eosinophils prior to the addition of PAF, luminol and opsonized zymosan. The eosinophil mixure was rapidly assayed for the CL response.

Immunofluorescent staining and flow cytometry analysis

Eosinophils were treated under the following conditions: (1) IL-5 (40 U/ml), (2) PAF (10^{-6}M) and (3) buffer control for 90 minutes at 37°C before staining for CR1 or CR3. IgG1 and IgG2a myeloma proteins were used for baseline controls of CR1 and CR3. The details of immunofluorescent staining and FACS analysis have been published previously.[16] Briefly, eosinophils (50 μl, 5×10^5 cells), after treatment with IL-5 or PAF, were incubated with anti-CR1 or anti-CR3 MAb for 15 minutes at 4°C. Cells were washed once and fixed with FITC-conjugated F(ab)$'_2$ fragments of rabbit antibodies to mouse Ig for 15 minutes. Thereafter cells were washed once, fixed with 1% paraformaldehyde, resuspended in 0.5 ml of PBS containing 0.1% w/v sodium azide, and stored at 4°C

before analysis. Mean fluorescence was measured by EPICS C flow cytometry (Coulter Electronic, Hialeah, FL, USA). A total of 10,000 cells was analysed in each sample.

Statistical analysis

A paired *t*-test was used to compare eosinophil CL response induced by PAF, IL-5 and buffer control. The data were expressed as the enhancement percentage of luminal-dependent chemiluminescence by eosinophils following PAF or IL-5 stimulation, calculated using the following formula:

$$\frac{\text{CL experimental - CL control}}{\text{CL control}} \times 100\%$$

CL experimental is the peak of chemiluminescence in the PAF-induced eosinophil CL response, CL control is the peak of chemiluminescence in the buffer-induced eosinophil CL resposne.

RESULTS

Time course and dose response of PAF and IL-5 on eosinophil CL response

The time course of CL response in eosinophils induced by PAF and IL-5 was different. Following PAF stimulation, the CL response immediately reached a maximal degree and declined rapidly to baseline within 30 minutes (Fig. 1). Following IL-5 stimulation, the reaction decreased rapidly after 30 minutes incubation, then increased gradually and reach a plateau up to 90 minutes (Fig. 1).

The dose responses of CL induced by PAF and IL-5 were tested over different incubation periods, 0 minutes for PAF and 90 minutes for IL-5. Both PAF and IL-5 were able to enhance eosinophil CL response in a dose-related manner. The optimal dose of PAF and IL-5 to enhance CL response were 10^{-6} M and 40 U/ml, respectively (Fig. 2). Both normal density and hypodense eosinophils from the same individual were investigated in this study; the results showed that hypodense eosinophils gave higher CL responses than those of normal density eosinophils induced by PAF (1,856 mV, 1,323 mV *vs* 83.4 mV, 121 mV, n = 2).

The effect of PAF and IL-5 on eosinophil CL response

Eosinophils were incubated with PAF (10^{-6} M) or IL-5 (40 U/ml) for 0 minutes and 90 minutes, res-

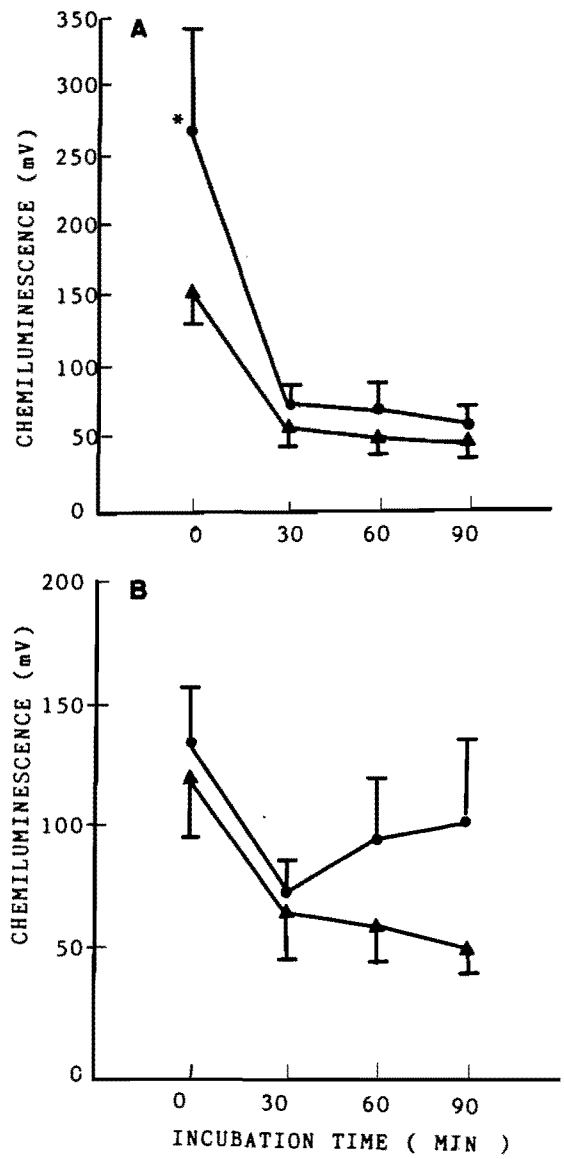

Fig. 1 Time course of the effect of PAF and IL-5 on eosinophil CL response. A: Eosinophils were incubated with 10^{-6}M PAF (•) or RPMI (▲) for varying length of time. Luminol and opsonized zymosan were added to facilitate CL response. Results were expressed as mean ± SEM of mV of seven experiments (* $p < 0.05$). B: Eosinophils were incubated with 40 U/ml IL-5 (•) or RPMI (▲) for varying length of time. Luminol and opsonized zymosan were added to facilitate CL response. Results were expressed as mean ± SEM of mV of 5 experiments.

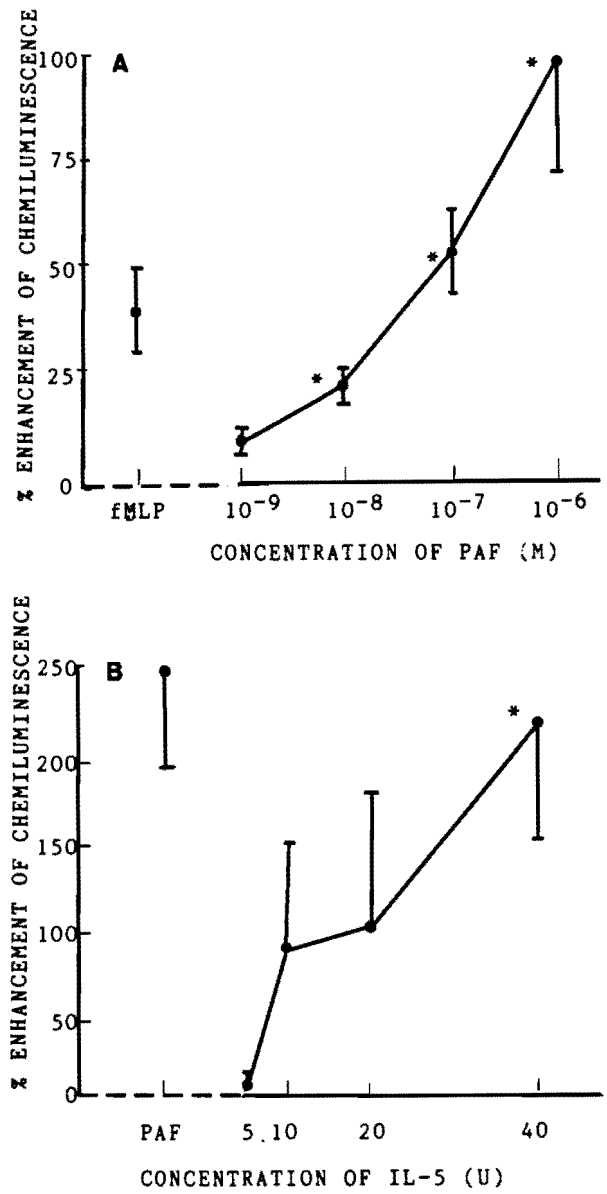

Fig. 2 Dose response of the effect of PAF and IL-5 on eosinophil CL response. A: Eosinophils were incubated with various concentrations of PAF (10^{-6} to 10^{-9} M). fmLP (10^{-7} M) and RPMI were used as controls. Luminol and opsonized zymosan were added to facilitate CL response. Results were expressed as mean ± SEM of percent enhancement of 5 experiments (* $p < 0.05$). B: Eosinophils were incubated with various concentrations of IL-5 (5 to 40 U/ml) for 90 min. Eosinophils, preincubated with RPMI for 90 min, were stimulated with PAF (10^{-6} M) as a positive control. Luminol and opsonized zymosan were also added to facilitate CL response. Results were expressed as mean ± SEM of percent enhancement of 4 experiments (* $p < 0.05$).

pectively, followed by stimulation with luminol and opsonized zymosan; RPMI was used as baseline control. In the 0 minutes incubation, PAF stimulation resulted in a great enhancement of CL response, which was four times as high as that obtained by IL-5 stimulation or in buffer (RPMI) control (Fig. 3). In the 90 minutes incubation, IL-5 stimulation resulted in a great enhancement of CL response; when eosinophils were incubated with PAF for 90 minutes, only mild enhancement of CL response was noted when compared with buffer control (Fig. 3). Sequential treatment of eosinophils with IL-5 and PAF was also evaluated. Results obtained from three separate experiments showed that eosinophils preincubated with IL-5 for 90 minutes, followed by stimulation with PAF obtained 752 ± 251 mV, repeated activation by IL-5 (IL-5 90 minutes, IL-5 0 minuts) gave 466 ± 247 mV, buffer control (IL-5 90 minutes, RPMI 0 minutes) gave 414 ± 249 mV.

Effect of BN52021 on PAF-induced eosinophil CL response

BN52021, a specific PAF antagonist, inhibited the PAF-induced eosinophil CL response in a dose-dependent manner (Fig. 4). Results showed BN52021 can significantly inhibit the PAF-induced CL-response at concentrations of 0.5 mM and 1 mM in 4 experiments.

Effects of PAF and IL-5 on the expression of complement receptors (CR1 and CR3) on eosinophils

PAF significantly enhanced CR3 expression on eosinophils. Its effect on CR1 expression was negligible. CR1 and CR3 expression on eosinophils were not enhanced by IL-5 (Table 1).

DISCUSSION

In this study, we demonstrated that both PAF and IL-5 can enhance eosinophil luminol-dependent CL

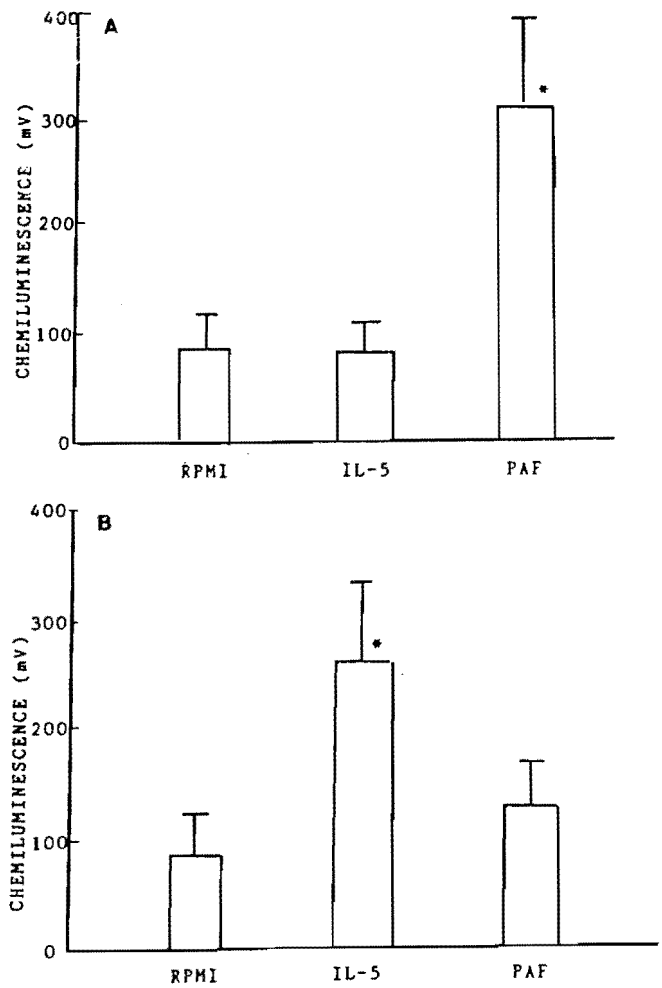

Fig. 3 The effect of PAF and IL-5 on eosinophil CL response. A: The immediate effect of PAF and IL-5 on eosinophil CL response. Eosinophils pretreated with PAF (10^{-6} M) or IL-5 (40 U/ml), then immediately stimulated with luminol and opsonized zymosan. Results were expressed as mean ± SEM of mV of 5 experiments ($^{*}p < 0.05$). B: The late effect of PAF and IL-5 on eosinophil CL response. Eosinophils, preincubated with PAF (10^{-6} M) or IL-5 (40 U/ml) for 90 min at 37°C, then stimulated with luminol and opsonized zymosan. Results were expressed as mean ± SEM of mV of 5 experiments ($^{*}p < 0.05$).

response. The duration of action was prompt and declined rapidly for PAF, however it was slow and increased gradually for IL-5. PAF, but not IL-5, was able to enhance the expression of complement receptor CR3 on eosinophils. There was also a further activation by PAF on IL-5-pretreated eosinophils.

In addition to stimulation of eosinophil proliferation, previous reports have indicated that IL-5 is an activator of human eosinophil but not neutrophil function. IL-5 can activate eosinophil phagocytosis of opsonized yeast, with production of reactive oxygen intermediates,[17] and also appears to be a selective eosinophil chemoattractant.[7] In this report, we have demonstrated that IL-5 could activate eosinophil phagocytosis of opsonized zymosan, with production of oxygen radicals as determined by Lumacounter. Neither CR1 nor CR3 complement receptor expression was enhanced by IL-5. These results suggest that IL-5-induced enhancement of eosinophil CL response was not mediated by complement receptors, CR1 or CR3.

It has been reported that the adhesion glycoprotein molecules (Mac-1, LFA-1, P150,95) play a role in a variety of leukocyte functions including adhesive-dependent phagocytosis. Although Mac-1 (CR3) may not be the sole factor responsible for phagocytosis, CR3 has been suggested to play a major role in granulocyte adherence.[18] In our results, PAF enhanced eosinophil CR3 expression by less than 10%, while PAF-enhanced CL response was as high as 293 ± 35%. It is unlikely that the trivial increase of CR3 receptor number on the eosinophil surface could account for the effect of PAF on CL response. The disparity between enhanced expression of CR3 receptor on the cell surface and the increased adherence following PAF stimulation was also noted by other investigators.[17,18] These results suggest that PAF enhancement of phagocytosis was only partially, if at all, due to an increase in CR3 expression. Mediator induced changes in receptor affinity may be more important but were not assessed in the present study.

PAF is a potent chemotactic agent for human eosinophils,[10] and is also able to enhance eosinophil cytotoxicity against schistosomula of *Schistosoma mansoni* pre-coated with C3b and specific antibody.[18,19] We have demonstrated that PAF can

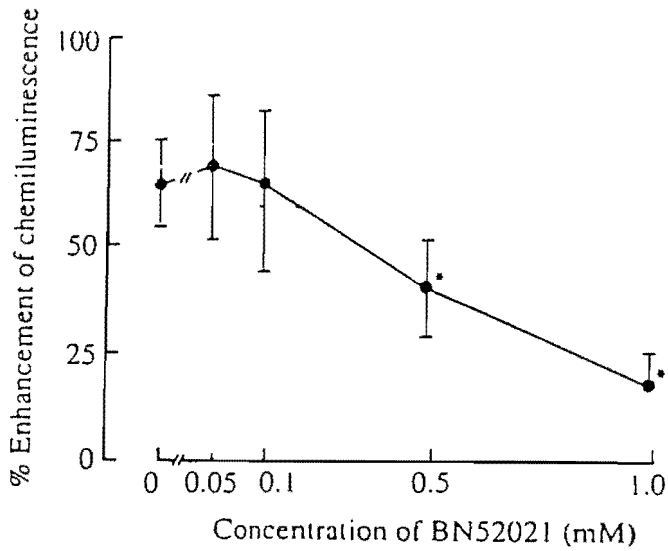

Fig. 4 Inhibition of the effect of PAF on eosinophil CL response by BN52021. BN52021, dissolved in DMSO, was added to the mixture of PAF (10^{-6} M), and eosinophil. Luminol and opsonized zymosan were added to facilitate the CL response. Results were expressed as mean ± SEM of percent enhancement of four experiments ($^{*}p < 0.05$).

greatly enhance the eosinophil CL response, an effect which is inhibited by specific PAF antagonist BN 52021, suggesting that PAF is a potent eosinophil activator and its effect might be through a PAF specific receptor on eosinophils. PAF-induced eosinophil CL response was short-lived and declined rapidly during incubation (Fig.1). The CL light remission represents a cellular "respiratory burst" which is dependent on peroxidase.[20,21] The rapid release of eosinophil peroxidase by eosinophils following PAF treatment has also been reported by Kroegel *et al.*[22] These results indicated that the rapid effect of PAF-induced eosinophil CL response might be partially due to the release of eosinophil peroxidase. In addition to its chemotactic and stimulatory activities on eosinophils, PAF itself can be produced in a considerable amount by eosinophils. This suggests that PAF may be a critical factor in the recruitment and activation of eosinophils in the human asthmatic airway.

IL-5 had an enhancing effect on eosinophil CL response without any increase in CR3 receptor expression. In contrast, PAF had enhancing effects on both CL response and CR3 receptor expression and further activation of IL-5 pretreated eosinophils. These result suggest that IL-5 and PAF activate eosinophils through separate mechanisms. More detailed investigation of the subcellular pathways of activation is needed to clarify the precise mechanisms.

It is now widely accepted that chronic mucosal inflammation plays an important role in the pathogenesis of asthma. Evidence from the report of Kay *et al* suggested that T-lymphocyte activation and lymphokine (IL-3, IL-4 and IL-5) production bring about local accumulation and activation of eosinophils. The release of inflammatory mediator (PAF and leukotrienes) from these cells results in tissue damage and may contribute to further inflammation.[24] Our results suggest that PAF and IL-5 might act sequentially on eosinophils. It has also been reported that IL-5 can prolong the survival of eosinophils[25] and prime eosinophils for further activation by PAF.[17] These results indicate that PAF and IL-5 could elaborate a wide variety of actions on eosinophils which might be important in the pathogenesis of eosinophil related diseases such as bronchial asthma.

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