The effect of interleukin-8 and granulocyte macrophage colony stimulating factor on the response of neutrophils to formyl methionyl leucyl phenylalanine

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Abstract

Neutrophils isolated from patients with chronic bronchitis and emphysema have been shown to have enhanced responses to formyl peptides when assessed in vitro compared to age, sex matched controls. It is currently unclear whether the observed differences are due to a ‘priming’ effect by a second agent in vivo, or whether this is a primary difference in the neutrophils. We have studied the effects of interleukin-8, which is thought to be one of the major pro-inflammatory cytokines in chronic lung disease and granulocyte macrophage colony stimulating factor (GMCSF), in order to assess their effects on neutrophil chemotaxis and connective tissue degradation. In addition, we have assessed the effect of preincubation of these agents with neutrophils for 30 min followed by stimulation with F-Met-Leu-Phe (FMLP) to investigate any possible ‘priming’ effect that may be relevant to our clinical data. We report suppression of neutrophil chemotaxis to FMLP following incubation of the neutrophils with both IL-8 and GMCSF. However, we have observed an additive effect of IL-8 and FMLP for neutrophil degranulation leading to fibronectin degradation. The results suggest that IL-8 does not ‘prime’ neutrophils for subsequent FMLP stimulation as observed in vivo. Although the results for GMCSF were similar for the chemotactic response, the agent also had a synergistic effect on connective tissue degradation. However, it is concluded that neither agent could explain the enhanced neutrophil responses seen in our patients. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Neutrophils (PMN) play a key role in pulmonary defence, but have been implicated as a major cause of tissue destruction leading to the development and progression of chronic lung diseases [1]. It is thought that persistent and/or excessive recruitment of PMN to the airway followed by degranulation can also, in some instances, lead to amplification and perpetuation of the inflammatory process, resulting in chronic lung damage [2]. Studies in patients with chronic bronchitis and emphysema have indicated that PMN show an increased chemotactic response to a standard chemoattractant together with increased connective tissue degradation and upregulation by F-Met-Leu-Phe (FMLP), compared to age and smoking matched controls [3] and that these effects relate to increased receptor expression [4]. The implication of these observations is that in patients with
chronic lung diseases, the PMN would show an enhanced chemotactic response leading to increased recruitment to the lung and this, together with the greater potential for connective tissue degradation, could play a key role in the development and progression of the disease.

However, the reasons for the increased chemotactic response and destructive potential of the PMN from patients with COPD is largely unknown. Previous studies were carried out using the formyl peptide FMLP as the stimulating agent. Whereas this observation may relate to a primary difference in the neutrophils, it is possible that the PMN had been primed for their response to FMLP and that other mediators may play a key role in vivo. For instance, endotoxin has been shown to prime PMN for superoxide release in response to FMLP [5].

Another possible activating factor is interleukin-8 (IL-8) which is an important inflammatory mediator and may play a role in the pathogenesis of several lung diseases through its chemotactic activity for PMN [6]. In addition, IL-8 increases FMLP receptor numbers [7] which would be consistent with our clinical findings [4]. However, there is uncertainty concerning the potential of IL-8 to stimulate other PMN functions, including degradation of connective tissue and superoxide production. Some investigators demonstrated that IL-8 was unable to [7,8] or only weakly able to [9] stimulate superoxide production by PMN on its own. On the other hand, studies have shown that IL-8 enhances the response of other stimulants of superoxide production by PMN [7,8], although this effect was not confirmed by others [10]. There is also controversy regarding whether IL-8 has an effect on PMN degranulation with some studies failing to demonstrate an effect [11], while others succeeded [12]. Furthermore, it is degranulation of the primary granules that relate to connective tissue degradation by the release of proteases and this has yet to be studied.

The experiments reported here were performed to attempt to clarify some of these controversies. In particular, we wished to determine if IL-8 is able to prime PMN in a way which might explain the increased functional responses that were seen for PMN from patients with chronic destructive lung diseases. Finally, we wished to clarify whether IL-8 does affect these PMN functions alone. Additional studies were carried out with granulocyte macrophage colony stimulating factor (GMCSF) in a similar way, since this agent can also increase the number and affinity of FMLP receptors [13] which would be consistent with our finding in patients [4].

2. Materials and methods

2.1. Isolation of peripheral blood neutrophils

Venous blood was collected from normal healthy subjects into lithium heparin tubes and PMN isolated using the method of Jepson and Skottun [14]. Each sample was diluted with an equal volume of 0.15 M sodium chloride and layered onto a Percoll gradient (Sigma, Dorset, UK). The top layer consisted of 2 ml of 54% Percoll (density 1.075 g/ml) and the lower layer 3 ml of 78% Percoll (density 1.096 g/ml). The gradients were then centrifuged at 200 g for 25 min at room temperature. The PMN > 96% pure and > 98% viable (by exclusion of Trypan blue) were harvested from the interface between the 54 and 78% Percoll layers, washed twice in 0.15 M sodium chloride, counted and resuspended in the appropriate assay medium; RPMI 1640 (Flow Laboratories, Richmansworth, UK) for the fibronectin degradation assay or RPMI solution containing 2 mg/ml bovine serum albumin for the chemotaxis assay. All reagents were confirmed to contain less than 20 ng/l of endotoxin activity using the KabiVitrum Coat Test (Flow Laboratories, Richmansworth, UK).

2.2. PMN chemotaxis

The PMN chemotaxis assay was based on the method described by Falk et al. [15], using the 48-well microchemotaxis chamber. The chemotaxis chamber was assembled by pipetting 27 ml of the chemoattractants in the lower wells, a 2 μm pore polyvinylpyrrolidone (PVP)-free polycarbonate filter (Costar Nucleotide, Costar UK, High Wycombe, UK) was then carefully placed over the lower wells followed by a rubber gasket and the upper half of the chamber secured with screws. The PMN (50 μl suspended at 1.5 × 10⁶ cells/ml of assay medium) were then carefully pipetted into the upper wells and the
chemotaxis chamber incubated at 37°C for 20 min. The chamber was then disassembled and the filter removed, wiped across a wiper blade (to remove any cells that had not migrated through the pores) and fixed and stained using Diff-quick (Baxter, UK). The cells which had migrated through the 2 μm pores to the lower surface of the membranes were counted at ×400 magnification (five random fields per well). A mean value was obtained for each well and the average value for 3 replicate wells was taken as the result for that sample.

2.3. Experimental design

Firstly, PMN chemotaxis to a range of IL-8 (R&D Systems, Abingdon, Oxon) concentrations (0.01–100 nM) was performed to establish the dose response curve of PMN chemotaxis to IL-8. Once the dose response had been established, the effect of IL-8 on PMN chemotaxis to FMLP was investigated. This was carried out using two different concentrations of IL-8, firstly a non-chemotactic ‘low dose’ and secondly the optimal concentration for PMN chemotaxis (0.1 and 5 nM, respectively). The PMN were incubated with the IL-8 (0.1 and 5 nM) for 30 min at 37°C followed by washing twice with 0.15 M sodium chloride. The washed cells were then recounted and suspended in assay medium for the assessment of chemotaxis to FMLP at 0.1, 1, 10 and 100 nM.

Secondly, PMN chemotaxis to combinations of IL-8 and FMLP was investigated using non-chemotactic and optimal concentrations of both agents, i.e. low dose of both IL-8 and FMLP (0.1 and 0.2 nM, respectively), optimal concentrations of IL-8 and FMLP (5 and 10 nM, respectively), low dose IL-8 (0.1 nM) with an optimal FMLP concentration (10 nM) or lastly, optimal IL-8 (5 nM) with low dose FMLP (0.2 nM).

2.4. Fibronectin degradation

Degradation of fibronectin (FN) by PMN is due to the release of proteinases by degranulation of the primary granules and was assessed using the method of Campbell et al. [16] as modified by Burnett et al. [3]. Briefly, purified human FN (Sigma, Dorset, UK) was iodinated by the chloramine-T method with sodium 125I-iodine (ICN Flow, High Wycombe, UK). The radiolabelled FN was diluted with unlabelled FN in 0.05 M carbonate/bicarbonate buffer (pH 9.6) to give 2000 cpm/μg FN and then dispensed into the wells of microtitre plates as 30 μg FN/well in 200 μl buffer. The plates were allowed to dry at 37°C and then washed three times with phosphate buffered saline (pH 7.2) to remove any unbound iodine.
Freshly purified PMN suspended in RPMI medium (3×10^6 cells/ml) were preincubated with IL-8 at increasing concentrations (0.01–100 nM) for 30 min at 37°C as might be expected since it is likely that PMN within the pulmonary vasculature, extracellular fluid and lung secretions would be exposed to varying concentrations of IL-8 in vivo persistently rather than transiently. Following the preincubation, 200 μl of the cells with varying concentrations of IL-8 were dispensed into the wells of the microtitre plate coated with FN and the plate incubated in a humidified atmosphere of 5% CO₂/95% air for 3 h at 37°C. After 3 h incubation the supernatant was collected from the wells and the amount of FN degraded was assessed by counting the release of 125I using an LKB Multigamma counter. The 125I counts in the wells containing RPMI alone (blank wells) were deducted from the results for those wells containing PMN. The result for each assay was determined from the mean value for three replicate wells. In order to assess the effects of IL-8 alone and IL-8 in addition to FMLP (as more than one stimulating agent may be found in lung secretions), the experiments were performed with cells in the absence of IL-8 (control, resting cells), with cells preincubated with increasing concentrations of IL-8 with and without optimal concentrations of FMLP (1 μM) and with cells stimulated with FMLP, but without IL-8.

### 2.5. Effect of GMCSF

Studies were also performed to assess the effect of GMCSF on PMN chemotaxis and degradation of FN. Firstly, GMCSF (R&D Systems, Abingdon, Oxon) was confirmed to be non-chemotactic for PMN at concentrations up to 10 nM. Increasing concentrations of GMCSF were preincubated with PMN for 30 min at 37°C in order to assess the effect of this agent on PMN chemotaxis to the optimal concentration of FMLP (n = 4).

Secondly, the effect of GMCSF on PMN degradation of FN was assessed by preincubating PMN with increasing concentrations of GMCSF (0.001–10 nM) for 30 min at 37°C before performing the assay. Finally the effect of GMCSF preincubation, followed by subsequent stimulation with varying concentrations of FMLP, on PMN mediated degradation of FN was assessed using cells preincubated with GMCSF concentrations of 0.01 or 1 nM prior to performing the assay to determine whether this cytokine was able to prime neutrophils for these responses.

#### 2.6. Data analysis

Statistical analyses of the effects of IL-8 on PMN chemotaxis and FN degradation with or without FMLP stimulation were performed using Wilcoxon signed rank test for paired data.

### 3. Results

#### 3.1. PMN chemotaxis

The effect of IL-8 on PMN chemotaxis is shown in Fig. 1a, indicating a dose related response for PMN chemotaxis with a maximum effect at a concentration of 5 nM (23.6; S.E. ± 1.4 cells/HPF). When PMN chemotaxis to FMLP was measured after preincubation with a non chemotactic ‘low’ dose (0.1 nM) or an optimal dose (5 nM) of IL-8, a significant (P < 0.04) suppression was seen from a maximal control mean value of 32.5 ± 3.2 for 10 nM FMLP to 26.8 ± 2.9 cells/HPF in the presence of 0.1 nM IL-8.
and this was greater \( (P < 0.04) \) in the presence of 5 nM IL-8 (18.2 ± 3.2 cells/HPF). These results are summarised in Fig. 1b.

In the second series of experiments, non-chemo-
tactic (low dose) and ‘optimal concentrations’ of FMLP and IL-8 were mixed together in each combi-
nation and the PMN chemotactic response was as-
ased. When low doses of IL-8 (0.1 nM) and FMLP
(0.2 nM) were used together as the chemoattractant,
the PMN chemotactic response was only 2.9 ± 0.6
cells/HPF, which was similar to the result of both agents separately.
When optimal and low doses of IL-8 or FMLP were mixed the results showed a small increase in chemotactic response compared to each agent alone, although this was not statistically significant. However, the combination of optimal concentrations of IL-8 (5 nM) and FMLP (10 nM), although demonstrating a greater chemotactic response (30.2 ± 2.6 cells/HPF; *P* < 0.04) was lower than would be expected for an additive effect (≈ 48 cells/HPF). These results are summarised in Table 1.

### 3.2. Fibronectin degradation by PMN

Fibronectin degradation by PMN was increased slightly, but significantly, (*P* < 0.02) by IL-8 with a maximal effect at 10 nM as shown in Fig. 2a (from a control mean of 1.93 ± 0.31 µg FN to 2.85 ± 0.33). Fibronectin degradation by PMN stimulated with 1 µM FMLP (Fig. 2b) was also increased (*P* < 0.04) after preincubation of the cells with IL-8 in a dose dependent manner from a control value of 4.67 ± 0.61 to 6.20 ± 0.77 at 10 nM IL-8, although no change was seen at concentrations of IL-8 that had no effect alone (*n* = 6).

The average increase in fibronectin degradation by IL-8 alone (0.92 µg) appeared less than the increase induced in the presence of FMLP (1.53 µg). However, this failed to reach statistical significance. In view of this, the experiment was repeated with a further series of neutrophils (*n* = 6), comparing the effect of 10 nM IL-8 in the presence and absence of 1 µM FMLP. Again, the increase from control due to IL-8 alone (0.45 ± 0.03 µg) was not statistically different to that seen in the presence of FMLP compared to FMLP alone (0.85 ± 0.45 µg), suggesting the effect of the two agents is no more than additive, rather than demonstrating a synergistic effect.

### 3.3. Response to GMCSF

GMCSF itself was not chemotactic in the concentrations used. However, preincubation of PMN with increasing concentrations of GMCSF resulted in a dose related reduction in the chemotactic response of the PMN to 10 nM FMLP from a control value of 28.9 cells/HPF, S.E. ± 1.7 to 14.8 ± 0.38 with 10 nM GMCSF (Fig. 3).

On the other hand, GMCSF alone did produce an increase in fibronectin degradation at concentrations of 0.1 nM and above (control, 3.41 ± 0.85; 0.01 nM, 3.39 ± 0.76; 0.1 nM, 4.28 ± 1.0; 10 nM, 4.65 ± 0.88). Following preincubation of PMN with a non-effect-
tive concentration of GMCSF (0.01 nM) the subsequent response to FMLP showed no increase (1 μM FMLP, 6.68 ± 0.3; FMLP+GMCSF, 6.51 ± 0.4). However in the presence of 0.1 nM GMCSF which had a small effect on fibronectin degradation (control, 3.41 ± 0.85; 0.1 nM GMCSF, 4.28 ± 1.0), the response to FMLP was enhanced (see Fig. 4).

4. Discussion

PMN are considered as major cellular components in the defence of the lung against bacteria and fungal infection [17]. Although PMN chemotaxis, degranulation (leading to proteinase release) and superoxide anion production all play important roles in host defence, excessive and/or prolonged action would lead to increased tissue injury typical of chronic destructive lung diseases [1,2]. Previous studies have shown that PMN obtained from patients with chronic lung diseases show an increased chemotactic response and connective tissue degradation in response to FMLP, compared to age and smoking matched controls [3] and this was associated with increased FMLP receptor expression [4]. However, it was not certain whether this represented a primary difference in cell responses and hence was central to the pathogenesis of disease, or a secondary phenomenon due to cell activation or ‘priming’ as a result of established lung disease. The term ‘priming’ is used to describe the phenomenon that a non-effective dose of one agent alone enhances an effect of another when assessing PMN function. However, when the two agents interact at effective doses they may be ‘additive’ (the increase is greater than that expected for either agent individually) or ‘synergistic’ when the combined effect exceeds the additive response expected for both agents. The current study was carried out as part of a series of experiments to explore the possibility that partial activation or ‘priming’ of neutrophils in patients with lung disease due to exposure to a second agent in vivo was responsible for the enhanced responses noted previously [3].

There are a variety of stimulatory molecules for PMN that may originate in the lung of patients with chronic obstructive lung diseases [18–21]. Among them, IL-8 is thought to be a major cytokine responsible for PMN recruitment [19]. Not only pul-
showed a 170% increase in degranulation as assessed by fibronectin degradation compared to age and smoking matched controls. Furthermore, stimulation with FMLP led to a further increase (189%) in fibronectin degradation and this value remained 130% greater than control.

From the studies described here, although IL-8 alone produced an increase in fibronectin degradation by resting neutrophils there was no suggestion of ‘priming’ of the neutrophils with this chemokine since the increase in fibronectin degradation by FMLP was unaltered by the presence of IL-8 at concentrations below which IL-8 had its own effect. In addition, the increase in fibronectin degradation in the presence of both IL-8 and FMLP was only additive and less than that observed in our previous clinical study. The implications of these findings are that although IL-8 itself can have a modest ‘additive’ effect on fibronectin degradation, the data do not explain our previous findings in bronchitis and emphysema. Previous studies in vitro have, however, shown a priming effect for elastase release from PMN with TNFα, PAF and IL-1β when PMN are subsequently stimulated with IL-8 and/or FMLP [23,24].

Of most importance is the observations on the chemotactic response. In our previous studies, neutrophils from patients with chronic bronchitis and emphysema showed enhanced chemotaxis to FMLP compared to neutrophils from patients with other inflammatory lung diseases and those from age and smoking matched controls [3]. Although the current studies confirm that IL-8 is a major chemottractant and when preincubated with FMLP leads to an increased chemotactic response than to either agent alone, the opposite effect was seen when IL-8 was mixed with neutrophils. The experiments were performed in this way on the assumption that any IL-8 being released from the lung in patients with established lung disease would be in contact with the circulating neutrophils for a prolonged period of time prior to them being harvested and tested in the chemotactic assay described by us previously [3]. The exact mechanism for the reduction caused by IL-8 is uncertain, but may relate to the neutrophils being presented with two independent signals from each section of the chemotactic chamber. For instance, it is known that chemotactic response to chemotactants, such as IL-8, FMLP and LTB4, is reduced by placing the neutrophils in the chemotactic gradient during checkerboard experiments since this reduces the chemotactic gradient. Thus, although the cells were washed, receptor binding by IL-8 would activate the cells and thus influence cell migration in this assay compared to control cells. This phenomenon has been observed in cystic fibrosis where decreased chemotaxis of circulating neutrophils was thought to reflect previous exposure to chemotactants [20]. Thus, this form of priming/activation would give the opposite effect to that seen in vivo in our previous study in emphysema [3].

In a subsequent study, we have demonstrated that neutrophils from patients with chronic bronchitis and emphysema had increased numbers of FMLP receptors expressed on the surface of the cell [4]. The number of receptors was related to the chemotactic response and hence this was thought to explain the mechanism of increased chemotaxis. Although previous published work has indicated that the priming effect certainly with IL-1β is not associated with increased receptor expression, we decided to study a cytokine which would since this should be more relevant to our in vivo data. Thus, we investigated the effect of preincubating neutrophils with low concentrations of GMCSF that has been shown to increase surface expression of FMLP receptors [22], which reflects activation of neutrophils and degranulation of the specific granules. Our results show that although this cytokine is not chemotactic the overall effect was to reduce the chemotactic response to FMLP. Subsequent experiments (data not shown) indicated that incubation of neutrophils with GMCSF resulted in the release of IL-8 into the supernatant (consistent with previously published data [25]) and the supernatant was confirmed to be chemotactic. This would therefore have a similar effect to a checkerboard experiment by effectively exposing the neutrophils to a chemotactrant in the upper chamber and providing a reverse or reduced chemotactic gradient, thereby decreasing migration to the lower side of the membrane in the Boyden chamber assay. Thus, the effects of GMCSF may be indirect and related to the IL-8 effect discussed above. On the other hand, GMCSF had a synergistic effect on fibronectin degradation by PMN in response to FMLP. Thus, although the GMCSF effect could explain our previous results in patients for fi-
bronectin degradation, it would not explain the chemotactic results.

Overall, although the current study has confirmed that IL-8 and GMCSF activate some neutrophil functions and act additively or synergistically (respectively) with FMLP in fibronectin degradation, this activation process decreases chemotactic response as studied here. Thus, the experiments with IL-8 and GMCSF could not explain our previous findings of increased chemotactic and connective tissue degradation responses in patients with chronic bronchitis and emphysema. We have not investigated the effects of other cytokines (IL-1β, TNFα and PAF) which have previously been shown to have priming effects for PMN elastase release and superoxide production [23,24] and could also have priming effects for PMN chemotaxis. The previously reported data would not, however, be able to explain the increase in FMLP receptor numbers seen in our patient group [4]. This suggests that if the phenomenon is caused by the presence of lung disease, it is likely to be a mechanism that increases FMLP receptor expression on the cell surface without activation of the neutrophils. Alternatively, as suggested previously [4], the increased FMLP receptor expression may be a primary event and, therefore, central to the pathogenesis of disease.

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