G-CSF and IL-8 but not GM-CSF correlate with severity of pulmonary neutrophilia in acute respiratory distress syndrome

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ABSTRACT: Activated neutrophils play a major role in the pathogenesis of acute respiratory distress syndrome (ARDS), and persistence of pulmonary neutrophilia is related to poor survival. Interleukin (IL)-8 is implicated in recruiting neutrophils to the lungs but it has been postulated that granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), which can promote the survival of neutrophils by delaying apoptosis, may prolong the inflammatory response. The aim of this study was to investigate the levels of GM-CSF and G-CSF in the lungs of patients with ARDS and determine their relationship relative to IL-8 with levels of neutrophils and clinical outcome.

The lungs of 31 patients with ARDS were sampled by means of bronchoalveolar lavage (BAL) and assays of the three cytokines were conducted via enzyme-linked immunosorbent assay.

GM-CSF, G-CSF and IL-8 were all increased in the patients compared to healthy controls but concentrations of GM-CSF were much lower than those of G-CSF and IL-8 (GM-CSF < G-CSF < IL-8). Levels of G-CSF and IL-8, but not GM-CSF, correlated strongly with each other (r = 0.86, p < 0.001) and with BAL neutrophil counts, and only levels of G-CSF were significantly higher in nonsurvivors than survivors (p < 0.05).

This evidence indicates that granulocyte colony-stimulating factor as well as interleukin-8 plays a role in the mechanisms of pulmonary neutrophilia in acute respiratory distress syndrome, whereas the role of granulocyte-macrophage colony-stimulating factor remains unclear. The higher levels of granulocyte colony-stimulating factor in nonsurvivors, together with previous reports that recombinant granulocyte colony-stimulating factor occasionally induce acute lung injury, emphasize that the role of these mediators in pathogenesis needs to be elucidated.


Acute respiratory distress syndrome (ARDS) can develop in many serious conditions including sepsis, pneumonia, traumatic injury and major surgery [1, 2]. Mortality is >40% and there is no effective therapy apart from supportive measures [2]. The initiating factors trigger an acute systemic inflammatory response which causes microvascular damage leading to increased pulmonary vascular permeability, interstitial and alveolar oedema, and hypoxaemic respiratory failure [1, 2]. Activated neutrophils play a major role in mediating the microvascular damage and also contribute to lung tissue damage [3]. They infiltrate the lungs in large numbers and their persistence in the lungs is an important determinant of poor survival [4, 5]. Elucidation of the mechanisms that maintain pulmonary neutrophilia in ARDS may therefore be of considerable prognostic and therapeutic significance.

The main chemotactic factor for neutrophils in the blood and bronchoalveolar lavage (BAL) fluids of patients with ARDS has been shown to be the cytokine interleukin (IL)-8, and other products of inflammation make a lesser contribution [6]. Higher levels of IL-8 are present in BAL samples from nonsurvivors than survivors with ARDS [7], and elevations of IL-8 concentration in BAL fluid predict progression to ARDS in at-risk populations [8]. Assays of chemotactic function indicate that IL-8 accounts for >50% of the neutrophil chemotactic activity in BAL fluids of patients with ARDS [9]. However, evidence is emerging that factors that have the capacity to prolong neutrophil survival may also be present in the lungs of patients with ARDS. Circulating neutrophils have a short life span of 6–8 h due to programmed cell death, “apoptosis”, which normally ensures that the effete cells are recognized and cleared by phagocytes and other cells without spilling their potentially toxic contents [10]. Recent morphological evidence suggests that apoptosis of neutrophils is suppressed during the course of ARDS [11]. Furthermore, BAL fluid from the same patients prolonged the life span of normal neutrophils in vitro. This effect was prevented by preincubation of the fluids with antibodies directed against the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF).
In addition to their ability to promote increased release of granulocytes from the bone marrow, both of these cytokines can prolong the life span of neutrophils in vitro by delaying their apoptosis [12]. However, little is known about in vivo levels of GM-CSF or G-CSF in the lungs of patients with ARDS, or their potential role in the pathogenesis of this syndrome. The main aims of this study, therefore, were to: 1) evaluate levels of GM-CSF and G-CSF in the lungs of patients with ARDS at different stages in the injury by studying samples of alveolar epithelial lining fluid obtained by means of BAL; 2) determine their relationship to numbers of BAL neutrophils and to IL-8; and 3) investigate these mediators in regard to clinical outcome.

**Methods**

**Study groups**

Thirty-one patients with ARDS (19 male, 12 female; median age 37 yrs, range 18–66 yrs, 14 never, nine current and eight exsmokers; 22 survivors and nine nonsurvivors) who met the diagnostic criteria of the American/European Consensus [1], and had severe lung injury according to the Murray lung injury score [2] were investigated. The onset of injury was associated with bacterial or viral pneumonia in 10 patients (four of whom also had sepsis), sepsis alone in three, cardiopulmonary bypass surgery in six (one of whom also had sepsis), other major surgery in four (one of whom also had sepsis), major trauma due to road traffic accidents in five, pancreatitis in one, aspiration pneumonia in one and smoke inhalation in one. All had bilateral diffuse parenchymal shadows on their chest radiographs, had developed acute-onset severe hypoxaemia shortly after encountering the risk factor and had an arterial oxygen tension ($P_{aO_2}$) (in kPa)/inspiratory oxygen fraction ($F_{I,O_2}$) ratio of $\leq$21.6 at some stage during the injury. All had a pulmonary artery wedge pressure of $<18$ mmHg. Table 1 shows the clinical details. The study protocol was approved by the Ethics Committee of the Royal Brompton Hospital Trust. The control group for the cytokine studies comprised seven healthy nonsmoking volunteers without evidence of lung or heart disease (median age 34 yrs, range 29–42 yrs).

**Bronchoalveolar lavage and study protocol**

BAL was performed via a fibreoptic bronchoscope wedged into the lateral segment of the lower lobe of the right lung. The bronchoscope was inserted through the endotracheal tube in the patients and transnasally under local anaesthetic in the healthy volunteers. The standard protocol at the authors' centre accords with European guidelines [13]. Briefly, a standard volume of four aliquots of 60 mL normal saline, buffered to pH 7.0 using 8.4% sodium bicarbonate, was sequentially introduced into the trachea and aspirated into a sterile siliconized glass bottle and immediately transferred on ice to the laboratory. The total standard input was achieved in the controls, but in the severely ill ARDS patients instillation was aborted if falls in oxygenation caused clinical concern; thus the introduction volumes were lower in most cases (table 1). The proportions of the fluid recovered were also significantly lower in the patient group compared to the healthy controls (table 1), presumably due to the substantial increase in lung permeability that is characteristic of this syndrome [1, 2]. The BAL fluid was centrifuged at $160 \times g$ for 10 min at 4'C to sediment the cells, and the supernatant divided into aliquots, frozen and stored at -70'C in the presence of 1.8 mg·mL$^{-1}$ dipotassium ethylene diamine tetra acetic acid (EDTA). It was planned to obtain a BAL sample from each patient during the first week on ventilation and again at weekly intervals until successfully weaned in survivors or until death. However, the high mortality rate and other clinical constraints prevented sampling every patient at all follow-up intervals. In total, 15 patients were sampled during the first week "earlier phase" of injury (nine survivors and six nonsurvivors), 12 in the second or third week "mid phase" (10 survivors and two nonsurvivors) and eight survivors during the "later stages" in weeks 4–9 (seven survivors and one nonsurvivor). During the first week, enough samples were available to undertake statistical analysis to determine whether any differences could be identified between the survivors and nonsurvivors. The prognostic value of the

![Data are presented as absolute values or median (range). *: pulmonary artery occlusion pressure (PAOP) of $<18$ mmHg conformed with the diagnostic criteria for ARDS [1, 2]; **: patient's lowest value for arterial oxygen tension ($P_{aO_2}$) in kPa/inspiratory oxygen fraction ($F_{I,O_2}$). ARDS is defined by a $P_{aO_2}/F_{I,O_2}$ of $\geq21.6$ according to MURRAY et al. [2] or $\leq26.7$ according to BERNARD et al. [1]. All patients fulfilled one of these criteria at some time during their illness. BAL: bronchoalveolar lavage; MV: mechanical ventilation. **: p<0.001 compared to healthy controls (Mann-Whitney U-test).]
initial BAL findings for all 31 patients was also investigated. Survival was defined as successful weaning from ventilation and discharge from intensive care. A single BAL sample was obtained from each of the controls.

**Bronchoalveolar lavage cell counts**

Total counts of nucleated cells were carried out using Kimura stain and an improved Neubauer counting chamber (Hawksley and Sons Ltd., London, UK). Results were expressed as the numbers of cells per millilitre of BAL fluid. Differential counts to identify the cells were performed by making cytocentrifuge slide preparations containing $1.5 \times 10^5$ cells stained with May-Grünewald-Giemsa. At least 300 cells were counted in random fields and the results expressed for each cell type as a percentage of the total cell population.

**Granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor and interleukin-8 assays**

Levels were determined in BAL fluid supernatants containing EDTA using sandwich enzyme-linked immunosorbent assay kits (R&D Systems, Abingdon, UK). All standards and samples were assayed in duplicate and concentrations were determined by extrapolation from standard curves. G-CSF was determined using the Quantikine™ human G-CSF immunoassay kit (Catalogue No. DCS50), validated against international reference preparation National Institute for Biological Standards and Control (NIBSC) 88/502. GM-CSF was measured using the high-sensitivity Quantikine™ human GM-CSF immunoassay kit (Catalogue No. HSGM0). IL-8 was evaluated using the standard Quantikine™ human IL-8 immunoassay kit (Catalogue No. D8050), validated against international reference preparation NIBSC/World Health Organization 89/520.

**Statistical analysis**

Data distributions were nonparametric; thus comparisons between independent groups were made using the Mann-Whitney U-test. Correlations were carried out using the Spearman rank correlation coefficient.

**Results**

**Bronchoalveolar lavage neutrophil counts and cytokine levels**

The clinical characteristics of the 31 patients with ARDS and their BAL cell counts at the time of initial lavage are shown in table 1. All the patients showed increased percentages of neutrophils and highly significant increases in neutrophil numbers per millilitre of BAL fluid recovered compared to healthy controls ($p<0.001$). The results of cytokine analysis in the same initial lavage samples are shown in figure 1. These confirm that patients with ARDS have highly significant increases in IL-8 level compared to controls (median (range) 1,350 (1.00–49,631) versus 9.00 pg·mL$^{-1}$ (0.24–50) $p<0.001$), and also show that these patients exhibit highly significant increases in G-CSF (175 (0–1,000) versus 4.55 pg·mL$^{-1}$ (0.06–50) $p<0.0025$) and GM-CSF levels (2.20 (0–8.90) versus 0.39 pg·mL$^{-1}$ (0.32–0.71) $p<0.001$). There was no significant difference in the cytokine levels or neutrophil counts between the 15 patients with infection (primary or secondary) and the 16 without infection. However, G-CSF levels were significantly higher in the nine patients who failed to survive than in the 22 survivors ($p<0.05$; table 2). Levels of IL-8 and neutrophils in the same samples also tended to be higher in the nonsurvivors. There was no relationship between GM-CSF levels and survival.

**Correlation between levels of cytokines and neutrophil counts**

The correlation between numbers of neutrophils and concentrations of each cytokine in the same BAL samples from the patients is shown in table 3. There were significant correlations between both concentrations of IL-8 and G-CSF and neutrophil percentages and neutrophil numbers per millilitre. By contrast, there was no correlation between concentrations of GM-CSF and neutrophil counts. Consistent with these findings, IL-8 and G-CSF levels correlated closely with each other ($r=0.86$, $p<0.001$) but less closely with GM-CSF concentrations in the same lavage samples ($r=0.449$, $p<0.025$ and $r=0.394$, $p<0.05$, respectively).
Table 2. – Bronchoalveolar lavage (BAL) findings in nonsurvivors compared with survivors in the acute respiratory distress syndrome group at initial lavage

<table>
<thead>
<tr>
<th>BAL marker</th>
<th>Nonsurvivors</th>
<th>Survivors</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients n</td>
<td>9</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>(32.85–94.90)</td>
<td>(14.01–98.40)</td>
<td></td>
</tr>
<tr>
<td>Neutrophils $\times 10^4$ cells·mL$^{-1}$</td>
<td>(4.93–203.45)</td>
<td>(0.82–141.30)</td>
<td></td>
</tr>
<tr>
<td>IL-8 pg·mL$^{-1}$</td>
<td>2617.60</td>
<td>1170.00</td>
<td></td>
</tr>
<tr>
<td>G-CSF pg·mL$^{-1}$</td>
<td>(133–12352)</td>
<td>(1–49631)</td>
<td></td>
</tr>
<tr>
<td>GM-CSF pg·mL$^{-1}$</td>
<td>457</td>
<td>80</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(4.34–1000)</td>
<td>(0–866)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.49–8.90)</td>
<td>(0–8.60)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as median (range). IL-8: interleukin-8; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor.

Influence of stage in injury on the findings

Due to clinical constraints, the initial lavage samples from the 31 patients were not all obtained during the first week of injury; therefore, a further analysis was undertaken to better define the influence of time of sampling on the findings. Fifteen patients were lavaged during their first week on mechanical ventilation, 12 during weeks 2 or 3 and eight patients during weeks 4–9, while still receiving ventilatory support. The results are shown in figures 2 and 3. Compared to controls, there were significant increases in neutrophil counts and IL-8 and GM-CSF concentrations at all three time phases, although the levels tended to be lower during the last phase. There were also significant increases in G-CSF concentrations during the first and second time phases but, unlike the results for the other markers, levels of this cytokine were not significantly increased during the last phase, by which time most of the patients remaining were survivors (seven survivors, one nonsurvivor). During the first week, samples were available from nine survivors and six nonsurvivors. At this earlier stage, trends of prognostic differences were identified in those who did not survive towards higher concentrations of G-CSF (nonsurvivors median (range) 48 pg·mL$^{-1}$ (42–1,000); survivors 174 pg·mL$^{-1}$ (10.55–866) p=0.08) and neutrophil counts (nonsurvivors median (range) 32.48 $\times 10^4$ cell·mL$^{-1}$ (18.32–203.45); survivors 18.57 $\times 10^4$ cell·mL$^{-1}$ (1.43–85.91) p=0.09).

Table 3. – Correlation between levels of cytokines and neutrophils in the 31 patients at initial lavage

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Neutrophils %</th>
<th>Neutrophils $\times 10^4$ cells·mL$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs p-value</td>
<td>rs p-value</td>
</tr>
<tr>
<td>IL-8 pg·mL$^{-1}$</td>
<td>0.62 &lt;0.001</td>
<td>0.43 &lt;0.025</td>
</tr>
<tr>
<td>G-CSF pg·mL$^{-1}$</td>
<td>0.54 &lt;0.0025</td>
<td>0.42 &lt;0.025</td>
</tr>
<tr>
<td>GM-CSF pg·mL$^{-1}$</td>
<td>0.22 NS</td>
<td>0.23 NS</td>
</tr>
</tbody>
</table>

IL-8: interleukin-8; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor.

Discussion

This study confirms previous reports that IL-8, a potent neutrophil chemokine, is elevated in BAL fluid from patients with ARDS and correlates with the numbers of neutrophils present, indicating that it plays a major role in promoting the massive pulmonary neutrophilia that mediates lung damage [6–9]. It also confirms that neutrophil counts and IL-8 levels tend to be higher in nonsurvivors [5, 7, 14, 15]. However, this study shows that these patients also have increased concentrations of G-CSF in BAL, which correlate closely with the levels of neutrophils and IL-8. Moreover, the levels of G-CSF were a stronger predictor of poor outcome than that of either neutrophils or IL-8. This provides the first direct in vivo evidence in patients with ARDS that this factor, which has the ability to prolong the survival of neutrophils by delaying apoptosis [12], may also play a role in the pathogenesis of ARDS. There has been one previous study of G-CSF levels in BAL fluid from patients with ARDS [11]. They were on average 10-fold higher than those in BAL from normal volunteers (median (range) 238 pg·mL$^{-1}$ (19.5–5,241) versus mean±SEM 25±20 pg·mL$^{-1}$), but were measured only on the first day after onset of ARDS, and the
relationship with neutrophil counts and clinical outcome was not investigated. The main aim of the study was to seek evidence that apoptosis of neutrophils might be inhibited in the lungs of patients with ARDS. In support of this hypothesis, the authors showed that the proportion of apoptotic neutrophils in BAL fluid was low (median \( \leq 3\% \)) throughout the course of ARDS (days 1, 3, 7, 14 and 21), and similarly low in patients at risk. These findings alone are difficult to interpret because apoptotic cells are rarely detected \( \text{in vivo} \) due to their very rapid clearance by phagocytes [16]. More importantly, BAL fluid from their patients with ARDS reduced the rate of apoptosis of blood neutrophils \( \text{in vitro} \) [11]. Furthermore, the anti-apoptotic effect could be blocked by preincubating BAL fluids with polyclonal antibodies directed against G-CSF and GM-CSF, indicating that both cytokines may prolong the survival of neutrophils in the lungs of patients with ARDS. The present results extend these findings by documenting the levels of both these cytokines \( \text{in vivo} \) in the lungs of patients with ARDS at different stages in the progression of the syndrome, and by defining their relationship with the neutrophilic response and patient outcome.

The evidence presented here also supports a role for G-CSF \( \text{in vivo} \) in the mechanisms that promote the severe pulmonary neutrophilia that is associated with poor survival in ARDS [4, 5]. The concentrations of G-CSF in BAL fluid during the first week of injury (median (range) 240 pg·mL\(^{-1}\) (11–1,000)) are similar to those reported in the previous study on the first day after the onset of injury [11]. Initial levels for the total group of patients were significantly higher in nonsurvivors than survivors (p<0.05). At later stages in the injury, the levels were lower and were within the normal range in nearly all patients by weeks 4–9. At this stage most of the patients were survivors who were subsequently weaned from ventilation, indicating that levels of G-CSF return to normal with recovery. Counts of neutrophils and levels of IL-8 were also lower during the recovery phase.

By contrast, no significant relationship between concentrations of GM-CSF in BAL fluid and levels of neutrophils, nor prognosis, was found in patients with ARDS in the present study. Its role in the pathogenesis is therefore less clear. The increases in GM-CSF levels in BAL were much lower (median (range) 2.2 pg·mL\(^{-1}\) (0–8.9) than those of G-CSF, and, although \( \text{in vivo} \) concentrations may be up to 180-fold higher because of the dilution due to BAL, it is not known whether such low concentrations of GM-CSF within the lungs would have any pathophysiological effect. The median tolerated dose of intravenous recombinant GM-CSF is 15 \( \mu \text{g} \cdot \text{kg body weight}^{-1} \cdot \text{day}^{-1} \) [17]. The present findings suggest that the numbers of neutrophils in the lungs in ARDS are influenced more by intrapulmonary levels of the mediators G-CSF and IL-8 than by GM-CSF.

Unlike G-CSF, the colony-stimulating and anti-apoptotic effects of GM-CSF are less specific to neutrophils. It prolongs the survival of eosinophils as well as neutrophils from the blood of normal volunteers \( \text{in vitro} \) by inhibiting apoptosis [18], promotes the release of monocytes, neutrophils and eosinophils from bone marrow [19] and enhances many of the biological functions of these cells [18]. However, ARDS is a disorder that specifically involves increases only in neutrophil numbers. The absence of increases in numbers of eosinophils and monocytes in ARDS suggests that the low levels of GM-CSF detected in the lungs \( \text{in vivo} \) may not have a significant colony-stimulating effect. The levels were on average five-fold higher than normal, whereas those of G-CSF were 38-fold higher and those of IL-8 150-fold higher. Other functions of GM-CSF may be more relevant to its role in the mechanism of ARDS, which remains to be elucidated.

Experimental studies have provided evidence that G-CSF plays a more important role than GM-CSF in the regulation of blood neutrophil numbers. Dogs depleted of G-CSF by a
neutralizing antibody develop profound and selective neutropenia [20], but dogs similarly depleted of GM-CSF do not become neutropenic [21]. Furthermore, disruption of the G-CSF gene in knock-out mice results in chronic neutropenia [22], whereas GM-CSF knock-out mice do not become neutropenic [23]. Thus G-CSF appears more important than GM-CSF in the normal regulation of neutrophil homeostasis. There is very little information on levels of G-CSF in the lungs of healthy subjects, but alveolar macrophages recovered by BAL from healthy controls can produce G-CSF after stimulation with endotoxin [24]. The sources of G-CSF in the lungs of patients with ARDS are unknown, but G-CSF can be produced by monocytes, macrophages, endothelial cells and fibroblasts in vitro in response to stimulation by IL-1 and tumour necrosis factor-α, which are key inflammatory mediators in ARDS [25, 26]. In addition, bronchial epithelial cells containing G-CSF messenger ribonucleic acid have been seen in a rat model of acute lung injury [26]. Further studies are now needed to identify the main sources of G-CSF in the lungs of patients with ARDS and to clarify its role in pathogenesis.

The potential of G-CSF to contribute to the pathogenesis of ARDS is supported by reports of development of acute lung injury in patients treated with recombinant G-CSF [28–30]. In addition, experimental models show that intravenous instillation of recombinant G-CSF can exacerbate pre-existing acute lung injury [31–33]. Furthermore, it has recently been reported that G-CSF alone can induce acute lung injury when introduced directly into the lungs of rats [34]. Doses of 300–3,000 ng recombinant human G-CSF introduced intratracheally caused hypoxia, marked pulmonary oedema and pulmonary neutrophilia. The maximum level of G-CSF found in the present study in BAL fluid from patients with ARDS was 2.5 ng·mL⁻¹, but, since the dilution factor due to BAL could reach 180-fold, in vivo concentrations of G-CSF in lung lining fluid may be up to 450 ng·mL⁻¹, sufficient to have a pathophysiological effect.

In conclusion, this study shows that increases in granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor levels occur in the lungs of patients with acute respiratory distress syndrome. Their role in the pathogenesis and whether such increases also occur in patients at risk, now needs to be clarified.

References

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