Levels of IL-8 and myeloperoxidase in the lungs of pneumonia patients

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Abstract

Interleukin-8 (IL-8) is considered as the major polymorphonuclear neutrophils (PMNs) chemoattractant cytokine in lung diseases such as asthma and adult respiratory distress syndrome (ARDS). However, controversial results were obtained regarding the involvement of IL-8 in the pathogenesis of pneumonia. This study examines the role of IL-8 in the recruitment and activation of PMNs in the lung of pneumonia patients. The interesting aspect of this study is that it is a site-specific analysis of the infected and uninfected lungs of the same patient. The level of IL-8 mRNA, protein and myeloperoxidase present in the cells of the bronchioalveolar lavages (BALs) taken from the areas of known pneumonic consolidations on chest X-ray (infected lung) are compared with the BALs obtained from areas of no obvious infiltrate (non-infected lung). The results obtained from the infected and non-infected lungs of pneumonic patients were further compared with that of a control group of non-smoking patients. The level of IL-8 mRNA and protein were determined by RT-PCR and ELISA respectively. There was a significant increase in the level of IL-8 mRNA in the infected lung as compared to its level in the non-infected lung (p < 0.001). In correlation with the increase in mRNA, IL-8 protein concentrations in BAL fluids from the infected lung were 6 fold higher than those taken from the non-infected lung (p < 0.0001). This pattern was also consistent with MPO activity in the BALs (4.5 fold more MPO activity in the infected lung as compared to that of the non-infected lung), indicating that IL-8 is directly implicated in neutrophil accumulation that follows acute respiratory infection. The results of the present study, therefore, indicate the involvement of IL-8 in the pathogenesis of pneumonia. (Mol Cell Biochem 217: 107–112, 2001)

Key words: interleukin-8, bronchioalveolar lavage, polymerase chain reaction, IL-8 mRNA, myeloperoxidase, pneumonia

Introduction

Chemokines constitute a large family of regulatory cytokines that play a central role in immunological processes. The accumulation and appearance of polymorphonuclear neutrophils (PMNs) in the tissue may be considered as an initial marker of acute inflammatory reaction [1]. Neutrophils participate in the host response to a number of infectious and non-infectious diseases and in leukocyte migration [2–6]. They contain cytoplasmic granules that function in storage of bioactive neuromolecules (specific or secondary granules) or in fusion with phagosomes (azurophilic or primary granules). The azurophilic granules contain a variety of enzymes including myeloperoxidase, muramidase, cathepsin A, D, E, G, 5'-nucleotidase, β-galactosidase, elastase, collagenase, azurocidin and the defensins HNP-1, HNP-2, and HNP-3, arylsulfatase, α-mannosidase, N-acetyl-β-glucosaminidase, β-glucuronidase, acid β-glycerophosphatase and cationic peptides. The specific granules on the other hand contain vitamin-B12-binding protein, neutral proteases, lactoferrin, alkaline phosphatase, lysozyme, and probably collagenase [7]. Although the mechanisms that regulate the release of substances from both the granules are almost the same, there are certain specific stimuli such as IL-8 and zymosan that induce the release of substances from secondary granules [8]. Thus IL-8 functions as a potent chemotacting as well as degranulating agent.

Recently it has been shown that depletion of neutrophils using anti-rat neutrophil antiserum reduced subsequent development of chronic delayed type hypersensitivity reactions
This study clearly demonstrates the importance of neutrophil derived factors for monocyte and lymphocyte mobilization. Furthermore, it is shown that neutrophils produce a number of low molecular weight factors such as leukotriene B4 (LTB4) that attract more neutrophils and monocytes to the inflammatory site [10]. T-lymphocytes have also been shown to migrate in response to IL-8 both in vivo and in vitro [11, 12].

There is a clear involvement of IL-8 in the pathophysiology of various respiratory diseases [13–17]. In asthma, airway inflammation with eosinophils, lymphocytes and neutrophils is a characteristic feature [18–20]. In correlation with this cellular migration, there is an increase in the level of IL-8 in the serum, tissue and BAL of asthmatics. Similar to that in asthma, the involvement of IL-8 has been well investigated in adult respiratory distress syndrome (ARDS). However, the role of interleukin in the development of pneumonia is controversial [21]. Although, an increase in the level of IL-8 is a good indication of the inflammatory process, this information does not contribute much to clinical diagnosis. To our knowledge, no data is available on the production of IL-8 in BAL fluid from the same patient (i.e. infected and non-infected lung). Therefore this study is designed to measure the site-specific increase in the level of IL-8 in the lung of patients with bacterial pneumonia as compared to that of the non-smoking control group. The level of IL-8 mRNA and protein present in the BAL obtained from subsegmental bronchi of experimental and control group of patients were determined by RT-PCR assay and enzyme immunoassay respectively.

In this study we also determined the level of myeloperoxidase activity in the cells collected from 1 ml of BAL each from the infected and non-infected lung. Myeloperoxidase, a secreted heme protein, is an attractive candidate for monitoring phagocyte mediated cellular damage [22, 23].

## Materials and methods

### Patients

The study was performed on 36 patients with bacterial pneumonia who were admitted to the Chest Diseases Hospital in Kuwait. All patients underwent medical and laboratory examinations. The control group consisted of 17 non-smoking patients among which 9 patients were with chronic cough, 3 with hemoptysis and normal chest X-ray and 5 with old fibrotic shadows (Table 1).

### Bronchoalveolar lavage (BAL)

BALs were obtained first from the area of known pneumatic consolidations on chest X-ray (infected lung) followed by BALs from other areas with no obvious infiltrate (non-infected lung) of the same patient. BAL fluids were collected from the pneumatic patient and the control group after admission to the hospital, using sterile techniques and routine respiratory care. The bronchoscope was advanced into a subsegmental bronchus. Lavage was performed using 20 ml aliquots of warmed normal saline, introduced by a syringe through bronchoscopic aspiration port. A total volume of 100–120 ml saline was infused sequentially and the volume of the lavage fluid retrieval (approximately 60 ml) was pooled and transferred immediately into sterile pre-chilled polypropylene tubes. The pooled fluid was then filtered through one layer of sterile gauze and centrifuged at 1500 rpm for 15 min at 4°C. Following centrifugation, 5 ml of supernatant was taken into a sterile polypropylene tube and stored at −70°C until assayed.

### Isolation of cellular RNA

Total RNA was extracted from cells contained in 1 ml of the BAL obtained from the infected and non-infected lung, using the method of Chomczynski and Sacchi 1987 [24]. Briefly, the method is as follows. Cells were lysed in 0.5 ml of 4 M guanidinium isothiocyanate. The lysates were then acidified by adding 80 µl of 3 M sodium acetate at pH 4.0. Subsequently, 0.5 ml of water saturated phenol and 0.1 ml chloroform were added to the cellular lysate followed by shaking at 4°C for 20 min. Lysates were spun in the cold for 15 min and the supernatants were collected and extracted again with phenol-chloroform. The supernatants were finally extracted with chloroform and the aqueous layer was collected. RNA was precipitated with absolute ethanol [25]. The precipitate was further centrifuged and the pellet was air-dried. The RNA pellet was then dissolved in 100 µl diethylpyrocarbonate (DEPC) treated water. Concentration of RNA was determined at 260/280 nm optical absorbance.

### RT-PCR assay for IL-8 mRNA

Aliquots (2 µg) of total RNA were annealed with 250 ng of oligo dT primer by heating at 75°C for 10 min followed by its slow cooling to 37°C. Reverse transcription was carried out using 5 units of Avian Myeloma Virus (AMV) reverse transcriptase. The resulting cDNA was amplified by PCR using specific primers for IL-8 mRNA. The primers were designed to span an intron to eliminate the possibility of amplifying genomic DNA. The PCR products were separated by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining.
transcriptase and 20 units of RNA guard following the conditions described [26]. Reverse transcription reaction was carried out in 50 µl total volume and an aliquot of 5–10 µl from this cDNA was amplified for 35 cycles using the following PCR amplification parameters: denaturation 94°C × 30 sec, annealing 50°C × 30 sec and extension 74°C × 60 sec. The MgCl₂ was used at a concentration of 1.5 mmol/L.

The PCR amplification reaction was carried out in presence of 50 pmol each of upstream (5'-GGA ACC ATT CTC ACT GTG TG-3') and downstream (5'-CTC TTC AAA AAC TTC TCC ACA A-3') IL-8 specific primers using 1 unit of AmpliTaq enzyme in a thermocycler. These primers were synthesized based on human IL-8 cDNA sequence information [27]. PCR products were analyzed on 10% polyacrylamide gel electrophoretically [28], stained with ethidium bromide and photographed with a gel documentation system (Stratagene). All experiments were carried out under RNase free conditions and the solutions and glassware were made RNase free with DEPC treatment and or by autoclaving. Heat sensitive solutions were made in DEPC treated and autoclaved water followed by their filtration through 0.45 µ size Millipore filters.

Enzyme immuno assay for IL-8

The concentrations of IL-8 in plasma and BAL fluid supernatants were assayed in duplicate, using a quantitative immunometric, ‘sandwich’ enzyme immunoassay technique with a detection limit of 4.7 pg/ml (Amersham, UK).

Measurement of BAL myeloperoxidase activity

Level of myeloperoxidase activity was estimated in the cells collected from 1 ml of BAL from the infected and non-infected lung. The method was essentially the same as described earlier [29]. Cells were pelleted by centrifugation at 4°C and were homogenized in 1 ml of hexadecyltrimethylammonium bromide buffer, containing 14 mM hexadecyltrimethylammonium bromide and 50 mM KPO₄, pH 6.0. Samples were homogenized with polytron for 1 min and were kept cold on ice. The lysates were subsequently frozen in liquid nitrogen and thawed once. The lysates were then centrifuged for 2 min in cold at 14,000 rpm and the supernatants were used to estimate the level of MPO activity.

Aliquots of 20 µl supernatant were mixed with 980 µl of oditionisidine HCl (sigma) solution containing 16.5 mg of oditionisidine HCl, 90 ml of distilled water, 10 ml of KPO₄ buffer, pH 6.0, and 50 µl of 1% H₂O₂. Absorbance was recorded at 415 nm, every 15 sec for 1 min using Beckman DU700 spectrophotometer. The enzyme activity was calculated (units/min/ml) by dividing the rate of the change in the absorbance by the extinction coefficient, 1.13 × 10⁻². Enzyme unit is defined as the conversion of 1 µmol of H₂O₂ per min per ml of alveolar lavage at room temperature. Under these conditions, the residual activity in the pellet was < 10%.

Statistical analysis

Commutations were performed using the Statview 4.02 statistical package with Macintosh Centris 650 computer. Results are expressed as means ± S.E.M. The differences between groups were analyzed by Student’s t-test. The differences between the groups were considered significant if p < 0.05.

Results

In equal amounts of total cellular RNA, there was a significantly higher level of IL-8 mRNA in the infected lung as compared to that of the non-infected lung (p < 0.001; Fig. 1, lane 2). Before estimating the changes in the level of IL-8 mRNA, we characterized the identity of IL-8 PCR fragment (272 bp). For this purpose we employed HindIII restriction enzyme. This enzyme cut the IL-8 PCR fragment (272 bp)
into 193 and 81 bp fragments of expected size (data not shown), proving the identity of IL-8 PCR fragment (272 bp).

The concentrations of IL-8 in BAL fluids taken from patients with bacterial pneumonia were always high as compared to control group (p < 0.0001; Fig. 2). Furthermore, in all patients the levels of IL-8 in BAL obtained from the infected lung were 6 fold higher than those from the non-infected lung 130.6 ± 6 to 194.32 ± 54 pg/ml, n = 36 (p < 0.0001). This pattern of increased expression of IL-8 mRNA and protein was consistent with MPO activity in the lavages. In the cells from the equal amount (1 ml) of alveolar lavages there was 4.5 times more MPO activity (9.0 units/min/ml) in the right lung as compared to the level (2.0 units/min/ml) in the left lung (Fig. 3).

**Discussion and conclusion**

Recent reports have considered IL-8 as the most potent and major PMN chemoattractant factor in lung diseases [13–17], including ARDS and pneumonia [21], cystic fibrosis [30], human immunodeficiency virus (HIV)-infected patients with *Pneumocystis carinii* pneumonia, bacterial pneumonia, or tuberculosis [31]. Several studies have convincingly shown that IL-8 plays a key role in the pathobiology of asthma [18–20]. Presence of IL-8 has been demonstrated in the bronchoalveolar fluid (BAL) of the patients with asthma [13–15]. Furthermore, in asthmatics, there was an increase in the level of free and complex IL-8 in the blood as well as the bronchial mucosa [32], suggesting that free IL-8 may have a role in the activation of eosinophils. Various other convincing studies suggest that IL-8 is an eosinophil and neutrophil chemoattractant [33–36]. It has been shown that IL-8 plays a major role in adult respiratory syndrome (ARDS) [37–39].

On the other hand, certain studies could not find a correlation between the percentage of PMNs and the concentration of IL-8 in BAL fluid of patients with ARDS [21], suggesting that in addition to IL-8 there may be other chemoattractant agents that are involved in transendothelial migration of PMNs. Several investigators have demonstrated that BAL fluids obtained from patients with pulmonary infection, contain potent chemotactic factors, such as the complement peptide C5a and leukotriene-B4 (LTB4) [40].

In guinea pigs, exogenous IL-8 administration has been shown to recruit neutrophils in the airway lumen [41]. In addition, *in vivo* and *in vitro* studies have shown that IL-8 induces the release of T-lymphocyte chemoattractants from neutrophil [35]. *In vivo* studies in mice with a targeted deletion of IL-8 receptor homologue has shown that the total number of recruited cells to the airway lumen following a single antigen challenge was significantly low as compared to the wild type [42].

In consistent with the above studies, in the present study, high concentrations of IL-8 were found in BAL fluids taken from the infected lung of patients with bacterial pneumonia. These results are in agreement with other investigators [21, 43], who reported high levels of IL-8 in BAL fluids of patients with different lung diseases. Since alveolar macrophages are the major source of IL-8 in the lung, the local production of IL-8 by these cells may be responsible for the recruitment of PMNs into the pulmonary interstitial or air space in a variety of lung diseases. Further studies are required to determine the relationship between the severity of...
lungs diseases and the alteration of IL-8 in BAL fluids. Never-
theless, the data presented clearly show that the concentra-
tion of IL-8 in BAL fluid from pneumonic patients increased
in the infected lung.

In the lungs, IL-8 appears to be the primary chemoattractant
for neutrophils [43]. In addition to various cell types [44], IL-
8 is also synthesized and released by neutrophils [45]. Thus,
neutrophils contribute to the recruitment of additional neu-
trophils in an autocrine manner, by the synthesis and release
of IL-8. Several studies suggest that IL-8 also functions as a
chemoattractant for eosinophils [33–36]. It has been shown
that major basic protein (MBP), a 13.9 kD protein located in
the crystalloid core of eosinophil secondary granules, stimu-
lates the production of IL-8 through transcriptional and post-
transcriptional events [46, 47]. In neutrophils, MBP stimulated
IL-8 production occur post-transcriptionally through stabili-
zation of IL-8 mRNA [47].

In a recent study it has been noticed that type specific con-
sequences of lung infection may be due to the type specific
differences in the induction of cytokines by various infectious
agents [48]. In contrast to type 5 adenovirus, type 7 adenovi-
rus stimulated the production of IL-8 in human lung alveolar
epithelial cell line (A549 cells) and primary human fetal lung
fibroblasts (GM5387 cells). The regulation of IL-8 production,
in these cells, occurred at the transcriptional level and at the
level of message stability [48]. While adenovirus type 7 in-
creased endogenous IL-8 specific mRNA, both serotypes (type
7 and type 5) enhanced stabilization of IL-8 mRNA [48].

The data presented in our study shows that there was a sig-
ificant increase in the level of IL-8 mRNA in the infected
lung as compared to its level in the non-infected lung (p <
0.001). In correlation with the increase in mRNA, IL-8 pro-
tein concentrations in BAL fluids from the infected lung were
6 fold higher than those taken from the non-infected lung
(p < 0.0001). The mechanism of IL-8 specific mRNA increase
(transcriptional or post-transcriptional) in this study need to
be elucidated.

The techniques that are commonly used to quantitate in-
flammatory cells during the development of various dis-
ases in the lungs include histological analysis and ex vivo
radiolabeling of leucocytes and quantification of their accu-
mulation in the lungs by counting. These techniques, however,
are labor and time intensive and have practical limitations
[49–53]. To overcome the limitations of histological and
radiolabeling studies, in the present study, we have measured
the myelene peroxidase (MPO) activity, in order to quantify
the neutrophil accumulation.

The pattern of MPO activity in the BALs (4.5 fold more
MPO activity in the infected lung as compared to that of the
non-infected lung) was consistent with the level of IL-8
mRNA and protein. The results of the present study, there-
fore, indicate a site-specific involvement of IL-8 in the patho-
genesis of pneumonia.

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