

Cytokine levels in bronchoalveolar lavage and serum in 3 patients with 2009 Influenza A(H1N1)v severe pneumonia

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Abstract

Introduction: Pandemic Influenza A (H₁N₁)v pneumonia has led to a notable increase of admissions to intensive care units. A cytokine-mediated inflammatory response has been well documented in pneumonia and acute respiratory distress syndrome. However, few studies have focused on the role of these inflammatory mediators in infections caused by the Influenza A (H₁N₁)v. In this study, we assess the inflammatory response mediated by cytokines at the local and systemic levels in three cases of severe pneumonia caused by Influenza A (H₁N₁) virus.

Methodology: Serum and bronchoalveolar lavage samples were obtained from three mechanically ventilated patients diagnosed with Influenza A (H1N1) virus pneumonia by bronchoscopic bronchoalveolar lavage. Levels of interleukin 6 (IL-6), interleukin 8 (IL-8), tumour necrosis factor alpha (TNF α) and interleukin 1 beta (IL-1 β) were measured in these samples by enzyme-linked immunosorbent assay (ELISA).

Results: High levels of C Reactive Protein, Procalcitonin below 1 ng/ml and absence of leukocytosis were common findings in all patients. TNF α and IL-1 β were not detected in the serum. IL-6 levels in serum were (94, pg/ml, 77 pg/ml and 84 pg/ml) respectively in the three patients, while IL-8 levels were (30,2 pg/ml, 128 pg/ml and 40,5 pg/ml). In the BAL samples, only one of the analysed cytokines, IL-1 β was present at detectable levels in two patients (21 pg/ml and 11 pg/ml respectively).

Conclusions: Our results support previous findings which suggest that high levels of IL-6 and IL-8 in serum somehow participate in the inflammatory response in severe cases of pandemic influenza pneumonia.

Key words: Cytokine; pandemic influenza; severe pneumonia; ICU

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Introduction

Pulmonary involvement is the most common severe manifestation of infection by the Influenza A (H₁N₁) virus. Pneumonia caused by the pandemic Influenza A virus has led to a notable increase in the number of admissions to intensive care units, and has been associated with high morbidity and considerable mortality rates. Severe cases have been described even in people with no known risk factors or underlying diseases [1]. In recent months, many studies have been published regarding the epidemiologic and clinical characteristics of this disease [2,3].

Among the complications described, the development of acute respiratory distress syndrome has been common in cases of severe primary viral pneumonia due to Influenza A (H₁N₁) virus [4]. A cytokine-mediated inflammatory response has been well documented in cases of pneumonia and acute respiratory distress syndrome [5,6,7]. However, few

studies have focused on the role of these inflammatory mediators in infection by the Influenza A (H₁N₁) virus [8]. In this study, we assess the inflammatory response mediated by cytokines at the local and systemic levels in three cases of severe pneumonia, caused by Influenza A (H₁N₁) virus, who required respiratory support and underwent bronchoscopy-guided bronchoalveolar lavage.

Methodology

Patients

An observational study was conducted involving three patients admitted between October 2009 and January 2010 due to pneumonia caused by the Influenza A (H1N1) virus who required mechanical ventilation and whose diagnosis was made by bronchoscopy-guided bronchoalveolar lavage. For each case, the following variables were registered: age; sex; comorbidities; APACHE II score (severity of disease classification system on admission to the

Table 1. Clinical characteristics of the patients

	<i>Patient 1</i>	<i>Patient 2</i>	<i>Patient 3</i>
Age	43	55	43
Sex	female	Male	male
Apache II score on admission	6	11	15
Comorbidities	Morbid obesity	Morbid obesity COPD, Diabetes.	Dilated cardiomyopathy
Days from onset of symptoms to ICU admission	5	4	8
Mechanical ventilation (days)	9	16	11
Days at the ICU	11	38	17
Vasoactive medication	No	Yes	No
Radiological findings	Bilateral infiltrates	Bilateral infiltrates	Bilateral infiltrates
Day of bronchoscopy during ICU hospitalization (day of BAL after onset of symptoms)	4 th day after admission (9 th)	8 th day after admission (12 th)	5 th day after admission (13 th)

intensive care unit [ICU]); need for vasoactive medication; laboratory test results, including white blood cell and platelet counts, and levels of C-reactive protein (CRP), procalcitonin (PCT), transaminases (GOT and GPT), creatinine (CTN) and urea; length of mechanical ventilation and stay in the ICU (days); time interval between the onset of symptoms and ICU admission; timing of bronchoscopy relative to date of admission in ICU; radiological findings and microbiological results.

Measurement of cytokine levels

Flexible bronchoscopy was performed, and blood samples were taken for measuring the cytokine levels in serum. Samples were collected in sterile non-toxic pyrogen-free tubes, with no anticoagulant, centrifuged at 3,000 rpm for 10 minutes and stored at -70°C until processed. The samples were sent to an external laboratory (Balagué Center L'Hospitalet de Llobregat) for *in vitro* quantification of the following cytokines: interleukin 6 (IL-6), interleukin 8 (IL-8), tumour necrosis factor alpha (TNF α) and interleukin 1 beta (IL-1 β), using enzyme-linked immunosorbent assay (ELISA).

Bronchoscopy-guided bronchoalveolar lavage

Written informed consent was obtained from a relative or representative of the patient prior to the bronchoalveolar lavage (BAL) procedure. A total of 150 ml of physiological saline solution, divided into

three aliquots, was instilled into the right middle lobe given that all cases showed bilateral lung infiltration. The first 20 ml of the BAL were discarded and a sample of the remaining fluid was collected for microbiological analysis. In the laboratory, samples were stained using a direct method (Gram staining) for mycobacteria and fungi, and the BAL sample was processed for a microbiologic quantitative culture. In addition, RT-PCR for the Influenza A (H₁N₁) virus was performed using the MagNApure Compact kit (Real Time Ready Influenza A H1N1 Detection Set, Roche Applied Science, Mannheim, Germany).

Finally, a further ~25 ml of the BAL fluid was sent to the laboratory to analyse levels of the same cytokines as those measured in the serum samples.

Results and discussion

The three patients were positive for 2009 Influenza A (H1N1)_v in the the bronchoalveolar lavage samples. Blood cultures, bacterial culture of respiratory samples and pneumococcal antigen in urine were all negative. Regardless of the results obtained, treatment with Oseltamivir was initiated on admission to the ICU. The clinical characteristics of the patients are shown in table 1.

Blood test results were qualitatively similar in the three patients, all showing high levels of CRP along with PCT levels below 1 ng/ml and absence of leukocytosis (table 2).

Table 2. Laboratory test results at admission to the ICU

	Patient 1	Patient 2	Patient 3
White blood cells	3500	8200	6800
/ul PMN %	60.9%	70%	88%
Lymphocytes %	28.3%	14%	6%
CRP (mg/dl)	7.08	8.76	40
PCT (ng/ml)	0.1	0.1	0.83
GOT/GPT (U/l)	18/18	39/53	228/77
Platelets (/µl)	194000	167000	101000
CTN (mg/dl)	0.64	0.57	1.31
Urea (mg/dl)	19.5	27	113

Regarding cytokine measurements, TNF α and IL-1 β were not detected in the serum of any of the three patients studied. IL-6 levels were 94 pg/ml, 77 pg/ml and 84 pg/ml respectively. IL-8 levels in serum were 30.2 pg/ml, 128 pg/ml and 40.5 pg/ml. In the BAL samples, only one of the analysed cytokines, IL-1 β , was present at detectable levels in two patients (21 pg/ml and 11 pg/ml respectively).

During the recent 2009 pandemics, pneumonia caused by the Influenza A (H₁N₁) virus has represented a considerable challenge for intensive care units due to the peculiarities of this disease. The rapid progression to acute respiratory distress syndrome, the affected population including those with risks factors such as obesity and pregnancy (which have not been described in other types of pneumonia), and the appearance of severe complications even in patients with no known comorbidities make it particularly important to investigate the physiopathology of this disease. In relation to these factors, the first study on critically ill patients that described the role of the immune response and cytokines in these patients, published by Bermejo-Martin *et al.*, reported an increase of a group of cytokines participating of the T helper-1 and T helper-17 immune response in the early phase of the disease in plasma [8]. Recently Arankalle *et al.* analysed the cytokine patterns and viral load of subjects in a study that included 15 critically ill Indian patients. In accordance with our results, the two above-mentioned studies also documented high systemic levels of IL-6 [9].

In critically ill patients, cytokines are involved in the pathogenesis of several diseases [10]. Respiratory infections are not an exception and are a stimulus for the activation of the inflammatory response at both the local and systemic levels [11]. The association between high levels of cytokines, such as IL-6, and acute respiratory distress syndrome is well

documented [12-14]. This cytokine has been identified as a marker of the severity of pneumonia caused by the Influenza A (H₁N₁) virus. In accordance with the findings of Bermejo-Martin *et al.*, we found higher levels of IL-6 and IL-8 in serum in our patients in the ICU than those reported for the non-critically ill, non-hospitalized patients and healthy controls in their study. Interleukin 1 β in particular has been identified as an early marker for bacterial pneumonia in patients under mechanical ventilation [15]. In two patients in our study, interleukin 1 β in BAL could be detected.

In bacterial infections, levels of this cytokine in BAL are even higher than those found in our study. In mechanically ventilated patients, the role of this cytokine in BAL has been scarcely studied and usually in the context of bacterial pneumonia. On the other hand, the role of IL 1 β in local host defense in viral pneumonia remains unclear. When we compared the levels of cytokines in the BAL fluid with those reported in a study of 59 patients with bacterial pneumonia [16], the levels of IL-6, IL-8 and TNF α were much higher than those observed in these three patients. Moreover, there are descriptions in the literature of higher levels of these mediators in cases of infection due to other subtypes of Influenza virus such as Influenza A (H₅N₁) [17].

A major limitation of our study was the small number of patients included. Another notable limitation was the moment when samples were taken: the bronchoscopy was conducted after the early phase, an average of ~10 days from the onset of symptoms. Hayden *et al.* [18], using healthy volunteers, described the kinetics of these mediators in the infection caused by the Influenza A virus, reporting that the levels of the various cytokines peaked, in general, between the second and fourth day after the onset of symptoms. Consequently, the cytokines which were below the levels of detection in our analysis may have been at higher levels at earlier stages of the disease. However, BAL fluids are valuable samples that are difficult to obtain, since severe hypoxemia makes their collection impossible in many occasions; also, the potential formation of aerosols during the procedure of BAL implies a risk of infection for ICU health-care workers. Nevertheless, its usefulness for the diagnosis of this type of pneumonia has been recognised [19].

Our findings regarding levels of serum IL-6 and IL-8 in severe pandemic influenza are in accordance with those described by Bermejo-Martin *et al.* and indicate that even in the most advanced stages of the

disease the levels of these interleukins remain high. Although these high levels in serum have been associated with degree of severity and may be used as a marker for prognosis [20], their role in disease pathogenesis still remains unclear. Further studies are necessary to clarify the role of cytokines in the systemic and local inflammatory immune response during an infection by the Influenza A (H₁N₁) virus.

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