Immunological Features in Patients With Pneumonitis Due to Influenza A H1N1 Infection

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Abstract

Background: Pneumonitis induced by pandemic influenza A H1N1 has a potential to cause respiratory failure, which is a risk factor for death. The underlying immunopathological mechanisms, however, have not yet been fully elucidated.

Patients and Methods: We investigated changes in plasma cytokines, T cell subsets, and C-reactive protein (CRP) in 16 hospitalized patients with pneumonia caused by 2009 H1N1 influenza infection. The patients were classified into a severe disease group and a mild disease group according to PaO2.

Results: Cytokine profiles showed no changes in interferon \(\gamma\) (IFN-\(\gamma\)), interleukin 6 (IL-6), IL-8, or transforming necrosis factor \(\alpha\) (TNF-\(\alpha\)) levels throughout the observation period. Transforming growth factor \(\beta\) (TGF-\(\beta\)) was overproduced in the severe group but not in the mild group. Accordingly, we also found some signs of pulmonary fibrosis during the recovery period. Elevated CRP levels and lymphopenia were common in both the severe and the mild group. After treatment, there was a significant elevation in lymphocytes in both groups, but a significant decrease in CRP in the mild group. Lymphocyte counts and CRP levels rapidly recovered to normal levels in all survivors posttreatment; otherwise it seemed to be related to poor prognosis.

Conclusions: Serial measurements of cytokines showed that only TGF-\(\beta\) was overproduced, possibly in relation to the early use of corticosteroids, which may have downregulated immune responses to H1N1 infection. Pretreatment TGF-\(\beta\) plasma concentrations and absolute lymphocyte counts were independent predictors of severity. However, the role of elevated TGF-\(\beta\) in H1N1 infection-associated pulmonary fibrosis requires further investigation.

Key words: Cytokines. Immunological FEATURES. Influenza A H1N1. Pneumonitis.

Resumen

Antecedentes: La neumonitis inducida por la gripe A pandémica, H1N1, puede causar insuficiencia respiratoria, un factor de riesgo de muerte. Sin embargo, todavía no están claros los mecanismos inmunopatológicos subyacentes.

Pacientes y métodos: Se estudiaron los cambios en las citocinas plasmáticas, los subconjuntos de linfocitos T y la proteína C-reactiva (PCR) en 16 pacientes hospitalizados con neumonía causada por una infección por gripe H1N1 en 2009. Los pacientes se clasificaron en un grupo de enfermedad grave y un grupo de enfermedad leve en función de la PaO2.

Resultados: Los perfiles de citocinas no mostraron cambios en los niveles de interferón \(\gamma\) (IFN-\(\gamma\)), interleucina 6 (IL-6), IL-8 o factor de necrosis tumoral \(\alpha\) (TNF-\(\alpha\)) a lo largo del período de observación. Se observó una sobreproducción de factor transformador de crecimiento \(\beta\) (TGF-\(\beta\)) en el grupo de enfermedad grave pero no en el de enfermedad leve. En consecuencia, también se detectaron algunos signos de fibrosis pulmonar durante el período de recuperación. Los niveles elevados de PCR y la linfopenia fueron frecuentes tanto en el grupo de enfermedad grave como en el de enfermedad leve. Tras el tratamiento, se produjo un aumento significativo de los linfocitos en ambos grupos y una reducción significativa de la PCR en el grupo de enfermedad leve. Los recuentos linfocitarios y los niveles de PCR volvieron rápidamente a la normalidad en todos los supervivientes después del tratamiento; en caso contrario, se asoció con un pronóstico desfavorable.

Conclusiones: Las mediciones serials de citocinas únicamente mostraron sobreproducción de TGF-\(\beta\), posiblemente debido al uso temprano de corticosteroides, que pueden reducir las respuestas inmunitarias frente a la infección por H1N1. Las concentraciones plasmáticas de TGF-\(\beta\) y los recuentos linfocitarios absolutos previos al tratamiento fueron factores predictivos de gravedad independientes. No obstante, se requieren más estudios para determinar la función de los niveles elevados de TGF-\(\beta\) en la fibrosis pulmonar asociada a infección por H1N1.

Introduction

Pneumonitis caused by the pandemic H1N1 2009 influenza virus infection may develop into life-threatening acute lung injury and even acute respiratory distress syndrome (ARDS). The underlying molecular mechanisms are very complex. Some patients have a stronger host response to virus infection than others with similar viral replication levels [1]. In addition, patients continue to manifest lung injury when the viral load falls after antivirus therapy, providing further support of the immune nature of the lung damage that occurs in such cases. However, virus-specific antibody(Ab) does not seem to play a role in the early stages of disease because it has not been detected 2 to 3 weeks after severe acute respiratory syndrome-coronavirus (SARS-CoV) infection [2]. Cytokine activation, which usually occurs much earlier than clinically observable responses, may be an early event. We wished to investigate cytokine induction in this context as we believed it might play an important role in stimulating an immune response to the H1N1 virus and subsequently be responsible for extensive parenchymal damage. We also wished to investigate changes in C-reactive protein (CRP) and lymphocytes counts, which are vital predictors for prognosis in SARS [3,4]. The main aim of this study was to characterize the immunological features of hospitalized pneumonia patients with 2009 H1N1 influenza.

Methods

Patients

From November 9, 2009 to December 31, 2009 nasopharyngeal swab samples from suspected cases were tested for 2009 H1N1 influenza virus infection by reverse transcription polymerase chain reaction. Sixteen adults, diagnosed with H1N1 pneumonitis and admitted to the First Affiliated Hospital of China Medical University with profound hypoxemia, were included in the study. According to the World Health Organization, the main characteristics of H1N1 pneumonitis include a history of close contact with a person diagnosed with H1N1, persistent high fever (temperature >38°C), dry cough or sputum production, dyspnea, and a new atypical form of pneumonia verified by multiple spot films of chest computed tomography (CT) scans. Patients were classified into 2 disease severity groups according to pretreatment PaO2 levels: a severe disease group (PaO2 <60 mm Hg, n=9) and a mild disease group (PaO2 >60 mm Hg, n=7). A control group was formed by healthy volunteer doctors and nurses (n=8). The study protocol was approved by the Clinical Research Ethics Committee of the Chinese University of China, and informed consent was obtained from all participants.

Demographics

The age of the patients, sex, body mass index (BMI), and underlying diseases were recorded. According to the criteria established by the Working Group On Obesity In China in 2005, patients were classified as overweight if their BMI was 24 to 27.9 kg/m² and obese if their BMI was ≥28 kg/m².

Results

Demographics

No pregnant woman or children were enrolled in the study. The median age of the patients was 47 years (range, 19-66 years). Fourteen (87.5%) of the 16 patients were aged between 33 and 59 years. There was just 1 patient (6.25%) over 60 years old and another under 30 years old. The male to female ratio was 1.67 to 1 (10:6). Based on BMI calculations, 3 patients (18.75%) were overweight and 8 (50%) were obese (P=.1). Seven patients (43.75%) had an underlying medical condition and 1 (6.25%) had 2 underlying conditions. The conditions included diabetes, nephrotic syndrome, hypertension, hyperthyroidism, pituitary tumor, fatty liver, and old tuberculosis.

Treatment and Prognosis

All 16 patients received oseltamivir treatment and oxygen supplementation. The median±SEM time from symptom onset to the initiation of antiviral therapy was 7 days (range, 2-13 days). The corresponding figures for the severe disease group and the mild disease group were 8±3 days and 5.71±2.93
days, respectively \((P=0.149)\). Only 2 patients (12.5%) were treated with oseltamivir within 48 hours of symptom onset; the remaining 14 (87.5%) were treated after 48 hours. All patients received preventive antibiotic therapy, with 14 (87.5%) receiving more than 1 antibiotic. The median duration of antibiotic treatment was 7 days. Commonly used antibiotics were cefminox and levoflaxcin. Of the 16 patients, 14 (87.5%) received corticosteroids by means of an intravenous injection. The average dose of methylprednisolone was 1 mg·kg⁻¹·d⁻¹. Fourteen patients (87.5%) received extrasin alpha1 by means of a subcutaneous injection. The number of patients who survived was also 14. The median length of hospitalization in the survival group was 14.43±6.27 days. Two patients were admitted to an intensive care unit (ICU) and died on days 17 and 15 after disease onset. Perplexingly, neither had serious underlying disease and both had undergone the process of virus positive-negative transformation. Most of the survivors showed nearly complete resolution of thoracic CT abnormalities within 30 days. However, several continued to have shortness of breath and there were persistent radiologic images of ground glass opacities and reticular opacities.

**Measurement of Plasma Cytokines**

The variable trends of plasma cytokines in the 16 patients are illustrated in Figures 1A and 1B.

**TNF-α, IL-6, IL-8, and IFN-γ Levels**

As shown in Figure 1A, there were no significant increases in TNF-α, IL-6, IL-8, or IFN-γ levels in any of the patients. The serum levels of these 4 cytokines varied more greatly than those of TGF-β1 in both healthy controls and patients. In

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**Figure 1.** MA, Plasma cytokine levels. There were no significant differences in tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), IL-8, or interferon γ (IFN-γ) levels in patients with either severe disease \((\text{PaO}_2 <60 \text{ mm Hg})\) or mild disease \((\text{PaO}_2 >60 \text{ mm Hg})\). Pretreatment and posttreatment differences were also insignificant in both groups \((P>.05)\). B, Transforming growth factor β1 (TGF-β1) levels. Pretreatment TGF-β1 levels were lower in the mild disease group and the control group than in the severe group \((^{*}P<.001 \text{ and } ^{*}P=.027, \text{ respectively})\). There were no significant changes in TGF-β1 levels with treatment \((P>.05)\).

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**Figure 2.** Analysis of T cell subsets and lymphocytes. Using 2 independent \(t\)-tests, pretreatment lymphocyte counts were seen to be much lower in the severe disease group \((\text{PaO}_2 >60 \text{ mm Hg})\) than in the mild disease group \((\text{PaO}_2 <60 \text{ mm Hg})\) \((P=.002)\). Using the paired-samples \(t\)-test, the levels were seen to increase significantly with treatment in both the severe group \((^{*}P=.014)\) and the mild group \((P=.02)\). CD4⁺ T cell counts also increased significantly in the severe group after treatment \((^{*}P=.034, \text{ paired-samples } t\text{-test})\).
contrast to TGF-ß1, plasma concentrations of the 4 cytokines were lower than or within the normal range. Pretreatment IL-6 and TNF-α levels were slightly lower in the mild disease group than in the control group ($P=.083$ and $P=.094$, respectively). Furthermore, IL-6 levels were seen to increase after treatment ($P=.085$). In general, the levels of the 4 cytokines remained highly stable throughout the observation period.

**TGF-ß1 Levels**

As summarized in Figure 1B, TGF-ß1 levels were significantly higher in the severe group than in both the control group ($P=.027$) and the mild group ($P=.001$); the differences between the control group and the mild group were insignificant (Figure 1B). After 1 week of treatment, the levels increased in the mild group ($P=.088$) but remained high in the severe group. However, there were no significant differences in TGF-ß1 levels between the 2 patients who died and the survivors from the severe group.

**Depletion of Lymphocytes**

As shown in Figure 2, lymphocyte levels determined by routine blood testing were lower than the lower limit of normal (approximately 800 cells/µL) in 62.5% (10/16) of the patients. Levels were much lower in the severe group than in the mild group before treatment ($P=.002$) but not after treatment ($P>.05$). Nonetheless, they increased significantly after treatment in both the severe group ($P=.014$) and the mild group ($P=.02$). Pretreatment and posttreatment CD4+ and CD8+ T cell counts, measured by flow cytometry, did not differ significantly between the groups ($P>.05$). Analysis by the paired-samples $t$ test showed a significant increase in posttreatment CD4+ T cells in the severe group only ($P=.034$). CD8+ T cell levels also increased with treatment, albeit insignificantly ($P=.053$). Lymphocyte, CD4+, and CD8+ T cell counts in the 2 patients who died decreased after treatment.

**CRP Levels**

CRP levels were higher than 2mg/dL in 75% (12/16) of the patients, with higher levels detected in the severe group than in the mild group ($P=.08$). Furthermore, levels showed no significant decrease after treatment in the severe group ($P>.05$) and they actually increased in the 2 patients who died. In the mild disease group, the levels returned rapidly to normal levels with treatment ($P=.045$).

**Correlation Analysis**

Only pretreatment lymphocyte count was correlated negatively with TGF-ß1 levels. Both pretreatment and posttreatment levels were correlated with PaO$_2$ levels ($r=-.621$, $P=.010$; $r=.771$, $P<.001$; and $r=-.688$, $P=.003$, respectively) (Figure 3). No correlation was found between

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**Figure 3.** Correlation analysis. Only pretreatment lymphocyte counts were correlated negatively with TGF-ß1 concentrations ($P=.010$, $r=-.621$) (Figure 3A). Both pretreatment and posttreatment counts were correlated with PaO$_2$ ($P=.000$, $r=0.771$, Figure 3B; $P=.003$, $r=-.688$, Figure 3C).
TGF-β1 levels and the time of antiviral therapy initiation, length of hospital stay, or CRP levels ($P > 0.05$).

**Discussion**

We report on a series of 16 hospitalized patients with pneumonia caused by 2009 H1N1 influenza infection in Shenyang, China, from November to December 2009. In total, 2 patients (12.5%) were admitted to the ICU and both died, findings which are similar to the majority of reports from other countries [5–7]. Obesity and preexisting chronic diseases have also been found to be associated with death in such cases [8–10]. In our study, 50% of the patients were obese and 43.75% patients had an underlying medical condition. However, these factors were not predictive of either ICU admission or death, possibly due to the small sample size. Serum samples were not collected at the onset of disease. Before blood collection, immunomodulators such as steroids had been used. The plasma samples were collected on days 7 and 14 after symptom onset. The collection time of the second serum samples was close to the remission phase in the mild group, but approached the peak stage in the severe group.

Viral infection can induce lymphopenia. An inverse correlation between lymphocyte count and viral load in both nasopharyngeal and endotracheal specimens has been observed in SARS-COV and H5N1 infection [11,12]. Lymphopenia can result in a decrease in virus-specific Ab response and the production of cytokines. The mechanism of lymphopenia is complicated. IFN-γ and TNF-α via the Fas or TNF-α pathway might induce apoptosis of activated T cells [13–15]. In a molecular pathology study, direct virus infection of T lymphocytes of the lymph node has been implicated in the pathogenesis of lymphopenia in H5N1 infection [16]. In the present study, lymphopenia was also common in patients with severe disease. We concluded that lymphocyte counts determined by routine blood tests were more sensitive and convenient than CD4+ or CD8+ T cell counts from T cell subsets. It is a pity that we did not perform a lung pathology study to check whether regionally intense lymphocytic filtration was also involved in lymphopenia. Although the initiation of oseltamivir treatment was delayed in our series of patients, lymphocyte counts rapidly returned to normal levels in all survivors. Noteworthily, the 2 patients who died had persistent lymphopenia. We can therefore conclude that lymphopenia that did not resolve with treatment was a risk factor for death. Oseltamivir resistance-related death can be excluded considering that all the patients (even the 2 who died from ARDS) underwent the process of nasopharyngeal viral load positive-negative transformation. Our results are not consistent with those from a study in Taiwan which found that an initial lymphocyte count of less than 800 cells/µL and initiation of oseltamivir treatment more than 48 hours after symptom onset were associated with the development of respiratory failure [17]. To et al [18] also observed a slower decline in viral shedding in patients with severe disease than in those with mild disease. We speculate that virus replication is an important fatal factor but not the only one.

Initial CRP levels have been found to be predictive of death in SARS-COV infection [3,4]. In our study, CRP was neither a sensitive nor a specific predictor of disease severity. However, irrespective of initial levels, it rapidly returned to normal levels with treatment in all survivors. In the 2 patients who died, levels increased, possibly due to bacterial coinfection. However, all the patients had negative blood or sputum cultures, but it should be borne in mind that most patients had received antibiotics close to the time of culture collection, possibly reducing diagnostic sensitivity.

Various cells in the lung, such as macrophages, epithelial cells, endothelial cells, pneumocytes, and fibroblasts, are involved in the overproduction of cytokines [19]. Several papers have reported a significant increase in levels of proinflammatory cytokines (eg, TNF-α and IL-6), antiviral cytokines (eg, IFN-γ), and neutrophil chemotactic factors (eg, IL-8) in SARS-COV infection or ALI/ARDS [20–23]. Nonetheless, there have also been reports of no increase in classic cytokines such as TNF-α during SARS-COV infection [19]. In our study, none of these cytokines were overproduced. We are unable to explain this but it is possibly related to the use of corticosteroids, which may have inhibited the activation of lymphocytes or macrophages and prevented the production of cytokines. Our findings do not support the therapeutic use of these cytokine antibodies for H1N1 infection.

To et al [18] assayed the profiles of 25 cytokines and chemokines in patients with H1N1 infection, but they did not analyze TGF-β1 [18]. TGF-β1 plays a pivotal role in pulmonary fibrosis [24]. It increases the production of extracellular matrix proteins, enhances the secretion of protease inhibitors, and reduces the secretion of proteases, leading to the deposition of extracellular matrix proteins. It can also induce pulmonary fibrosis directly through stimulation of fibroblast chemotactic migration and proliferation as well as fibroblast-myofibroblast transition. Several viral proteins have been reported to modulate TGF-β1 signaling, which could induce the proliferation of fibroblasts [25]. Pang et al [27] found higher serum concentrations of TGF-β1 in SARS patients compared to controls for all clinical courses, including initial, peak, remission, and recovery stages [26,27]. During short-term follow-up, persistent ground glass opacities, reticular opacities, and pathologic findings of fibrosis have been found in some SARS survivors [28,29]. However, long-term follow-up showed a gradual decrease in ground glass opacities and reticulation. Willis et al [30] pointed out that TGF-β1 was necessary but not sufficient to induce the formation of pulmonary fibrosis [30]. In our study, TGF-β1 levels increased significantly and remained high after treatment in the severe group, coinciding with reports of patients with SARS-CoV infection [23,26]. This phenomenon could well be explained by the existence of imminent pulmonary fibrosis. The role of elevated TGF-β1 levels in H1N1 infection, however, requires further investigation as some survivors have also shown signs of pulmonary fibrosis during short-term follow-up.

Our study has certain limitations. The retrospective nature of the study yields inherent selection bias; no pregnant woman or children were included; the small sample size might have rendered some valuable indicators insignificant; it would have been desirable to perform long-term follow-up, with chest imaging and pulmonary function tests, of survivors.
Fortunately, the mortality of the current 2009 influenza A (H1N1) pandemic is not as high as that of the avian influenza A (H5N1). Persistent lymphopenia and high CRP levels, which are indicators of immune dysregulation, seem to be correlated with mortality. Virus replication is vital but it is not the only cause of death or of ARDS. Although an exaggerated activation of cytokines has been proposed as a factor of adverse outcome in SARS, only TGF-ß1 was an independent predictor in our study, possibly due to the early use of corticosteroids. Although the impact of corticosteroids on outcomes is controversial, based on our findings, it should be recommended in selected patients. The beneficial effects of immunoregulators are probably the interruption of cytokine storms induced by virus infection. The mechanisms of pulmonary fibrosis are complicated, and therapy targeting TGF-ß1 designed to block lung fibrosis requires further study.

References


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