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Increased levels of cytokines and high-mobility group box 1 are associated with the development of severe pneumonia, but not acute encephalopathy, in 2009 H1N1 influenza-infected children

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ABSTRACT

Background: The 2009 A(H1N1) influenza virus has caused a large outbreak, and resulted in major complications of severe pneumonia and acute encephalopathy in the pediatric population in Japan.
Methods: This study examined six patients with acute encephalopathy, 34 patients with severe pneumonia, five patients with both pneumonia and encephalopathy, and 46 patients without severe complications. The concentrations of 27 cytokines were examined in the cerebrospinal fluid of patients with encephalopathy, and the levels of these cytokines, Cytochrome c, high-mobility group box 1 (HMGB1) were measured in the serum of all patients.
Results: Patients with severe pneumonia had higher serum concentrations of 16 cytokines, including Th1 cytokines, Th2 cytokines, chemokines, and growth factors, than patients with uncomplicated influenza. The distribution of 27 cytokines in the CSF did not parallel the serum levels in 11 patients with acute encephalopathy. HMGB1 concentrations in the serum were significantly higher in pneumonia patients with or without encephalopathy than in uncomplicated influenza patients, and were significantly associated with the upregulation of 10 cytokines.
Conclusions: Elevated levels of Th2 cytokines appear to be unique to influenza caused by 2009 H1N1 influenza virus and HMGB1 could play an important role in the pathogenesis of severe pneumonia. There appear to be different pathologic processes for encephalopathy and pneumonia.

1. Introduction

A novel influenza virus, 2009 A(H1N1), emerged last year and rapidly spread worldwide. Clinical investigations revealed that the 2009 H1N1 influenza virus causes a self-limiting, uncomplicated infection in the majority of patients with clinical symptoms that are similar to those of seasonal A(H1N1) and A(H3N2). By contrast, some demographic groups appear to be at a higher risk for more complicated or severe illness following infection with the 2009 H1N1 influenza virus, including pregnant women, young children, and people of any age with certain chronic lung or other medical conditions [1]. Many severe cases have been caused by viral pneumonia, which is more difficult to treat than the bacterial pneumonias that are usually associated with seasonal influenza [2–4]. In Japan, the 2009 H1N1 influenza virus has also caused a large outbreak with more than 17 million estimated influenza cases (published exclusively in Japanese). Surveillance data showed that most of the reported influenza cases and hospitalizations have occurred in individuals aged 5–14 years [5]. By December 2009, 361 severe pneumonia and 94 acute encephalopathy cases were reported in the pediatric population. Acute seasonal influenza-associated

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encephalopathy has been described extensively in Japan, where the incidence has been higher than in other countries [6,7]. A higher incidence of viral pneumonia was observed in 2009 H1N1 influenza, while the incidence of acute encephalopathy was similar to that associated with seasonal influenza (published exclusively in Japanese). One-third of encephalopathy patients also suffered from pneumonia (published exclusively in Japanese). The incidence of acute respiratory distress syndrome (ARDS) was very low in 2009 H1N1 influenza (published exclusively in Japanese).

Studies in mammals revealed that the 2009 H1N1 influenza virus has a unique pathogenicity and replicates efficiently in lung tissue, unlike the seasonal influenza virus [8–10]. This is consistent with the fact that the 2009 H1N1 influenza virus is associated with a higher incidence of severe pneumonia. Virus invasion into the lung tissue increases the production of pro-inflammatory cytokines in the lungs of mammals, resulting in the development of pneumonia [10]. Pro-inflammatory cytokines were also associated with seasonal influenza encephalopathy [11–13]. Thus, increased cytokine production upon infection with the 2009 H1N1 influenza virus could play a role in pathogenesis in patients with encephalopathy. High mobility group box 1 (HMGB1) is a ubiquitous nuclear protein that can elicit the production of pro-inflammatory cytokines that induce inflammatory responses through several immune receptors, including Toll-like receptors [14–16]. HMGB1 is thought to play a crucial role in the development of various inflammatory diseases, such as sepsis [14–16]. Hypercytokinemia was observed in seasonal influenza-infected mice and the role of HMGB1 was examined in previous reports [17,18]. The HMGB1 concentrations were higher in the bronchoalveolar lavage fluid [18], whereas the serum HMGB1 levels were not increased in mice infected with the influenza virus [17]. Some pro-inflammatory cytokines have the potential to enhance apoptosis in tissues and endothelial cells, and apoptosis plays a role in the development of multiple organ failure during infection-induced hypercytokinemia [19]. Upon release from the mitochondria, Cytochrome c activates apoptosis and therefore is a good marker of apoptosis [20].

In the present study, we measured the concentrations of 27 cytokines/chemokines, including pro-inflammatory cytokines, in the serum of 2009 H1N1 influenza patients with or without severe complications (severe pneumonia and encephalopathy). The levels of these cytokines in the cerebrospinal fluid (CSF) of patients with encephalopathy were also measured. In addition, the serum levels of HMGB1 and Cytochrome c in 2009 H1N1 influenza patients with or without severe complications were examined. The results were compared between 2009 H1N1 influenza patients with severe complications and those without severe complications to investigate the pathologic processes of severe complications that are associated with the 2009 H1N1 influenza virus.

2. Materials and methods

2.1. Patients

Ninety-one influenza patients who were admitted to hospitals between October 2009 and January 2010 were enrolled in this study. The patients consisted of 56 males and 35 females with a mean age of 6.8 years (range 1–14 years). None of the patients had underlying disease. Six patients had encephalopathy, 34 patients had severe pneumonia, five patients had both encephalopathy and severe pneumonia, and 46 patients had no severe complications (uncomplicated influenza). Patients were considered to be infected with influenza virus if they had a positive viral antigen test from a nasopharyngeal swab. During the study period, the majority of influenza cases that were diagnosed by a viral antigen test were caused by the 2009 H1N1 influenza virus based on surveillance in Japan [5]. Infection with the 2009 H1N1 influenza was confirmed by RT-PCR analysis of nasopharyngeal swabs in all patients with severe pneumonia and/or acute encephalopathy. Patients were defined as having influenza-associated encephalopathy if they showed clinical symptoms and signs compatible with acute encephalopathy, such as altered consciousness (i.e., delirium, confusion, and cognitive impairment) or loss of consciousness (i.e., deep coma, coma, semicoma, stupor, and somnolence) that persisted for more than 24 h. Patients with meningitis, myelitis, and febrile convulsions without prolonged unconsciousness were excluded. Severe pneumonia patients had dyspnea and required oxygen administration and/or mechanical ventilation. The CSF and serum samples during the acute phase of disease were taken from patients within 4 days of disease onset (day of fever onset was considered the first day of disease). Unfortunately, uninfected healthy children were not enrolled as a control group in the present study. The study design and purpose were approved by the institutional review board of Nagoya University and fully explained to all patients and/or their guardians. Informed consent was obtained from all patients.

2.2. Measurement of cytokines/chemokines, Cytochrome c, and HMGB1 concentrations

The concentrations of 27 cytokines/chemokines (interleukin (IL)-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, IFN-γ-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated upon activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF)) were measured in the serum of all patients and in the CSF of 11 patients with influenza encephalopathy using a multiplex bead-based assay (Bio-Plex Pro Human Cytokine 27-plex Assay, BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, serum and CSF samples were diluted 1:4 and incubated with antibody-coupled beads. The complexes were washed and incubated with the biotinylated detection antibody followed by streptavidin–phycoerythrin and then the cytokine concentrations were measured. Standards ranging from less than 10 pg/mL to more than 5050 pg/mL were used to generate broad-range standard curves. Cytokine levels were determined using the Bio-Plex array reader (BioRad) and the concentrations of the samples were calculated using the software provided by the manufacturer. The concentrations of Cytochrome c and HMGB1 in the serum samples of all patients were determined using Cytochrome c and HMGB1 ELISA kits (Cytochrome c, PromoKine, Heidelberg, Germany; HMGB1, Shino-Test, Tokyo, Japan). The dynamic range was 0.08–5 ng/mL and 2.5–80 ng/mL for Cytochrome c and HMGB1, respectively.

The normal ranges for the serum concentration of these molecules in healthy volunteers are as follows: IL-1β < 10 pg/mL; IL-1ra 85.6–660 pg/mL; IL-2 < 15.6 pg/mL; IL-4 < 3.02 pg/mL; IL-5 < 7.8 pg/mL; IL-6 < 4.0 pg/mL; IL-8 < 2.0 pg/mL; IL-10 5.0 pg/mL; IL-12 p70 < 3.15 pg/mL; IL-13 < 28.6 pg/mL; G-CSF < 5.78–27.5 pg/mL; GM-CSF < 2.0 pg/mL; IFN-γ < 0.1 pg/mL; TNF-α, 0.6-2.8 pg/mL; MCP-1 < 149 pg/mL; and HMGB1, not detected (unpublished data). The normal serum levels for the other molecules and the normal CSF concentrations for all of the examined molecules were not available.

2.3. Statistical analysis

The characteristics among the patient groups were compared using the Chi square test and Kruskal–Wallis test. The cytokines,