



Cytokine profile of bronchoalveolar lavage fluid from a mouse model of bronchial asthma during seasonal H1N1 infection



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ARTICLE INFO

Article history:

Received 24 September 2013

Received in revised form 8 May 2014

Accepted 4 June 2014

Keywords:

Seasonal H1N1 influenza

Asthma

Bronchoalveolar lavage fluid

Cytokine

ABSTRACT

Background: Several studies support the role of viral infections in the pathogenesis of asthma exacerbation. However, several pediatricians believe that influenza virus infection does not exacerbate bronchial asthma, except for influenza A H1N1 2009 pandemic [A(H1N1)pdm09] virus infection. We previously reported that A(H1N1)pdm09 infection possibly induces severe pulmonary inflammation or severe asthmatic attack in a mouse model of bronchial asthma and in asthmatic children. However, the ability of seasonal H1N1 influenza (H1N1) infection to exacerbate asthmatic attacks in bronchial asthma patients has not been previously reported, and the differences in the pathogenicity profiles, such as cytokine profiles, remains unclear in bronchial asthma patients after A(H1N1)pdm09 and H1N1 infections.

Methods: The cytokine levels and viral titers in the bronchoalveolar lavage (BAL) fluid from mice with and without asthma after H1N1 infection (A/Yamagata and A/Puerto Rico strains) were compared.

Results: The interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)- α , IL-5, interferon (IFN)- α , IFN- β , and IFN- γ levels were significantly higher in the BAL fluids from the control/H1N1 mice than from the asthmatic/H1N1 mice. The viral titers in the BAL fluid were also significantly higher in the control/H1N1 mice than in the asthmatic/H1N1 mice infected with either A/Yamagata or A/Puerto Rico.

Conclusions: A(H1N1)pdm09 infection, but not H1N1 infection, can induce severe pulmonary inflammation through elevated cytokine levels in a mouse model of asthma.

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1. Introduction

Several studies have reported that viral infections play an important role in the pathogenesis of asthma exacerbation [1–4]. Also, many physicians believe that seasonal H1N1 influenza (H1N1) virus infection does not worsen bronchial asthma attack. The global 2009 H1N1 pandemic [A(H1N1)pdm09] resulted in significantly high infection rates in children. Approximately 80% of mortality due to A(H1N1)pdm09 infection occurred in individuals aged <65 years [5]. In contrast, H1N1-associated morbidity and mortality mainly occur in the elderly [6]. However, similar

illnesses are associated with both A(H1N1)pdm09 and H1N1 infections [7]. Children affected by the A(H1N1)pdm09 infection demonstrated milder symptoms than those affected by the H1N1 infection. In addition, severe respiratory issues such as severe pneumonia, severe asthma attack, and acute respiratory distress syndrome (ARDS) have been reported to be particularly common in children and young adults [8–11]. In children, bronchial asthma increases the risk of hospital admission and intensive care unit treatment [11–14]. We previously reported that A(H1N1)pdm09 infection induces severe pulmonary inflammation in a mouse model of asthma [15]. The levels of pro-inflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor (TNF)- α , and the anti-inflammatory cytokine IL-10 were significantly higher in the asthmatic/A(H1N1)pdm09 mice than in the control/

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A(H1N1)pdm09 mice [15]. In contrast, the levels of Th1 cytokine and interferon (IFN)- γ were significantly lower in the asthmatic/A(H1N1)pdm09 group than in the control/A(H1N1)pdm09 group [15]. In addition, the viral titer level was significantly higher in the asthmatic/A(H1N1)pdm09 group than in the control/A(H1N1)pdm09 group [15]. These results showed that the mechanism of infection, combined with increased viral replication and increased inflammatory cytokine levels due to decreased IFN- γ levels, might increase the severity of symptoms of bronchial asthma in patients exposed to A(H1N1)pdm09 virus [15]. However, there are few reports about the association of H1N1 infection with bronchial asthma exacerbation [16,17], and the differences of A(H1N1)pdm09 and H1N1 infection pathogenesis also remain unclear in bronchial asthma patients.

In this study, we investigated the levels of cytokines and the viral titers of the bronchoalveolar lavage (BAL) fluid between mouse models of bronchial asthma with H1N1 infection and those with H1N1 infection alone.

2. Materials and methods

2.1. Sensitization and allergen challenge of mice

BALB/c mice (age: 6–8 weeks) were obtained from Chiyoda Kaihatsu Co., Ltd. (Tokyo, Japan) and they were sensitized and challenged with grade II ovalbumin (OVA; Sigma–Aldrich, St. Louis, MO, USA), as previously described [15]. All animal experiments were conducted in accordance with the guidelines of the Institute of Laboratory Animals, Yamaguchi University (No. 29-S01).

2.2. Viruses, infection of mice, and preparation of the BAL fluid

Mouse-adapted H1N1 viruses (strains: A/Yamagata and A/Puerto Rico) were provided by the National Institute of Infectious Diseases (Musashimurayama, Tokyo, Japan). On day 31, influenza virus (concentration: 1×10^5 pfu/20 μ L) was administered intranasally in mice. The mice were euthanized and samples were collected 7 days after infection. The number, sex, and body weight of mice in each group were as follows: control/Yamagata: $n = 15$, male:female ratio = 9:6, mean body weight = 24.4 g; asthmatic/Yamagata: $n = 11$, male:female ratio = 5:6, mean body weight = 24.2 g; control/Puerto Rico: $n = 18$, male:female ratio = 9:9, mean body weight = 24.1 g; asthmatic/Puerto Rico: $n = 16$, male:female ratio = 8:8, mean body weight = 23.9 g.

The BAL fluid was collected on day 38 (post-infection day 7) by using three consecutive instillations of 1 mL phosphate-buffered saline (PBS) at room temperature. The collected BAL fluid was centrifuged at 1500 rpm at 4 °C for 5 min, and the supernatants were stored at -80 °C for estimation of cytokine levels and virus titers.

2.3. Cytokine assays

The concentrations of cytokines, IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- γ , and TNF- α in the BAL fluid were measured by using the Cytometric Bead Array (CBA) Kit (BD Biosciences, San Diego, CA, USA) in accordance with the manufacturer's instructions. The lower detection limits for IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- γ , and TNF- α were 0.1, 0.03, 1.4, 16.8, 0.8, 0.5, and 0.9 pg/mL, respectively. IL-5, IFN- α , IFN- β , and transforming growth factor (TGF)- β levels were measured by enzyme-linked immunosorbent assay (ELISA) (IL-5, TGF- β : R&D systems, Minneapolis, MN, USA; IFN- α , IFN- β : PBL Interferon Source, Piscataway, NJ, USA). The lower detection limits for IL-5, IFN- α , IFN- β , and TGF- β were 7.0, 12.5, 15.6, and 15.4 pg/mL, respectively.

2.4. Plaque assay

The Madin–Darby canine kidney (MDCK) cells were obtained from Lonza (Walkersville, MD, USA) and maintained at 37 °C in humidified 5% CO₂ chamber under stationary condition. Each well of a 6-well plate was seeded with 1×10^6 cells and cultured in α -minimum essential medium (MEM; GIBCO/Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin (GIBCO), and 100 μ g/mL streptomycin (GIBCO). After two washes with serum-free Dulbecco's modified Eagle's medium (DMEM; GIBCO/Invitrogen), the cells were maintained in serum-free DMEM at 37 °C for 1 h, followed by overlaying with 200 μ L/well of suitably diluted BAL (10^{-3} , 10^{-4} , and 10^{-5} dilutions) and incubation at 37 °C for 1 h. After one wash in serum-free DMEM, the cells were overlaid with serum-free DMEM containing 0.8% agarose (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 0.1% diethylaminoethyl-Dextran (Sigma–Aldrich), and 7 μ g/mL trypsin (Sigma–Aldrich). The cells were further cultured for 72 h at 37 °C, fixed in 10% formaldehyde (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and stained with 0.037% methylene blue (Wako Pure Chemical Industries, Ltd.). Each experiment was performed in duplicate.

2.5. Statistical analysis

The differences between the groups were analyzed using the Mann–Whitney U test. $p < 0.05$ was considered statistically significant. Analyses and calculations were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cytokine concentrations in the BAL fluid

The cytokine levels in the BAL fluid on post-infection day 7 are shown in Figs. 1–3. In A/Yamagata infection, the levels of IL-6

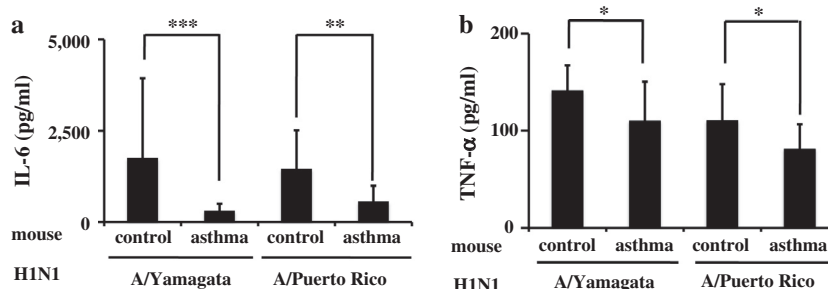


Fig. 1. The concentrations of interleukin (IL)-6 (a) and tumor necrosis factor (TNF)- α (b) in the bronchoalveolar lavage (BAL) fluid from the control/H1N1 and asthmatic/H1N1 mice infected with A/Yamagata and A/Puerto Rico strains. Data shown represent the mean $\pm$ SD. Three independent experiments were performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, control vs. asthmatic mice.

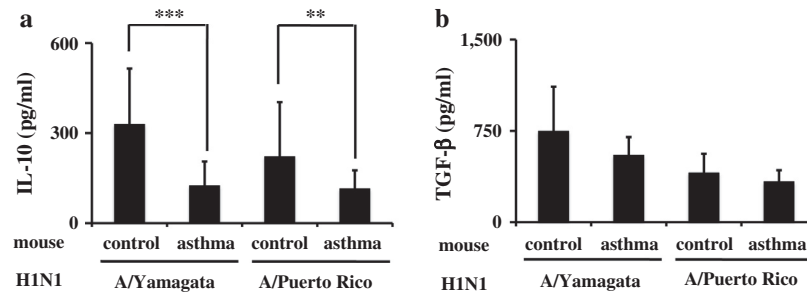


Fig. 2. The concentrations of IL-10 (a) and transforming growth factor (TGF)-β (b) in the BAL fluid from the control/H1N1 and asthmatic/H1N1 mice. Data shown represent the mean ± SD. Three independent experiments were performed. ** $p < 0.01$, *** $p < 0.001$, control vs. asthmatic mice.

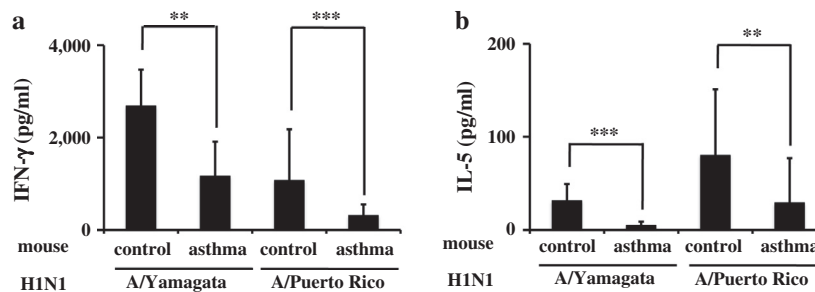


Fig. 3. The concentrations of interferon (IFN)-γ (a) and IL-5 (b) in the BAL fluid from the control/H1N1 and asthmatic/H1N1 mice. Data shown represent the mean ± SD. Three independent experiments were performed. ** $p < 0.01$, *** $p < 0.001$, control vs. asthmatic mice.

(Fig. 1a), TNF-α (Fig. 1b), IL-10 (Fig. 2a), IFN-γ (Fig. 3a), and IL-5 (Fig. 3b) were significantly higher in the control group than in the asthmatic group. No significant differences (IFN-α: $p = 0.568$, IFN-β: $p = 0.123$) were noted in IFN-α and IFN-β levels between the control (IFN-α: 24.7 ± 14.7 pg/mL, IFN-β: 8.2 ± 1.1 pg/mL, mean ± standard deviation [SD]) and asthmatic group (IFN-α: 20.7 ± 9.2 pg/mL, IFN-β: not detected).

In A/Puerto Rico infection, the levels of IL-6, TNF-α, IL-10, IFN-γ, and IL-5 were significantly higher in the control group than in the asthma group (Figs. 1–3). The IFN-α and IFN-β levels were also significantly higher (IFN-α: $p < 0.01$, IFN-β: $p < 0.01$) in the control group (IFN-α: 8.7 ± 3.1 pg/mL, IFN-β: 6.7 ± 5.0 pg/mL) than in the asthmatic group (IFN-α: 10.2 ± 6.0 pg/mL, IFN-β: not detected). However, no significant differences were observed between the two groups with respect to the levels of IL-2, IL-4, TGF-β (Fig. 2b), and IL-17A.

3.2. Viral titers in the BAL fluid

The viral titers in the BAL fluid on post-infection day 7 are shown in Fig. 4. The viral titers in the control/H1N1 group (A/Yamagata: $7.2 \pm 4.0 \times 10^4$ pfu/mL, A/Puerto Rico: $1.2 \pm 0.9 \times 10^6$ pfu/mL) were significantly higher (A/Yamagata: $p < 0.001$, A/Puerto Rico: $p < 0.01$) than those in the asthmatic/H1N1 group (A/Yamagata: $1.0 \pm 1.1 \times 10^4$ pfu/mL, A/Puerto Rico: $4.4 \pm 4.6 \times 10^5$ pfu/mL).

4. Discussion

We previously reported that A(H1N1)pdm09 infection induces high levels of pro-inflammatory cytokines and viral titers in the BAL fluid in a mouse model of bronchial asthma [15]. The levels of IL-6, IL-10, and TNF-α were significantly higher in the asthmatic/A(H1N1)pdm09 group than in the control/A(H1N1)pdm09 group [15]. In contrast, the levels of IFN-γ were significantly lower in the asthmatic/A(H1N1)pdm09 group than in the control/A(H1N1)pdm09 group [15]. In addition, the viral titer levels were

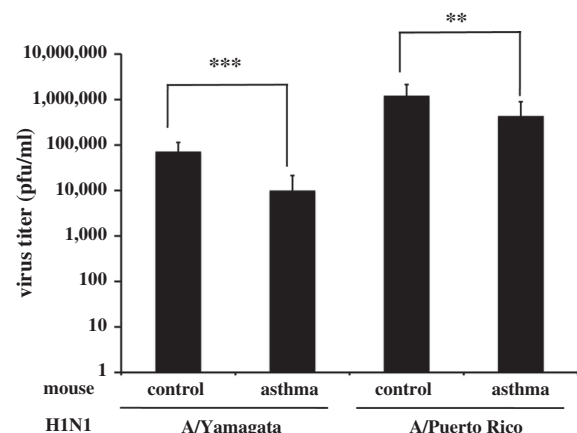


Fig. 4. The viral titers in the BAL fluid from the control/H1N1 and asthmatic/H1N1 mice determined by a plaque assay. Data shown represent the mean ± SD. Three independent experiments were performed. ** $p < 0.01$, *** $p < 0.001$, control vs. asthmatic mice.

significantly higher in the asthmatic/A(H1N1) pdm09 group than in the control/A(H1N1) pdm09 group [15]. These results differ from the measurements of cytokine levels, except IFN-γ, and viral titers in H1N1-infected mice, and suggest that H1N1 infection induces more severe pulmonary inflammation in non-asthmatic mice than in asthmatic mice. The IFN-γ level in the BAL fluid were significantly higher in the control/H1N1 groups (both A/Yamagata and A/Puerto Rico) than in the asthmatic/H1N1 groups, as well as in the A(H1N1)pdm09-infected group [15]. The levels of IFN-γ are reduced in bronchial asthmatic patients, indicating an alteration in the cytokine milieu, with an excess production of Th-2 products and a decreased production of Th-1 products [16,17]. Our results were consistent with clinical data reported elsewhere [14].

Previous studies have suggested a relationship between bronchial asthma and influenza infection [18,19]. In addition, it has

been reported that infection with influenza A H1N1 and H3N2 viruses increases the airway responsiveness and IgE production in asthmatic mice in comparison with those in non-asthmatic mice [18]. These results indicate that damage of the airway epithelium after influenza A infection may induce exacerbation of bronchial asthma. However, there have been no reports of influenza A infection inducing severe asthma attacks. In addition, many physicians do not believe that influenza A infection can induce severe asthma attacks, unlike respiratory syncytial virus (RSV), rhinovirus, and A(H1N1)pdm09.

In the present study, we used two strains of H1N1 virus—A/Yamagata and A/Puerto Rico. The BAL fluids obtained from control and asthmatic mice infected with both strains showed similar cytokine profiles and viral titers. The IFN- γ levels in both the control and asthmatic groups were higher after A/Yamagata infection than in those after A/Puerto Rico infection (Fig. 3a). On the other hand, the virus titer was lower in the A/Yamagata-infected mice than in the A/Puerto Rico-infected mice (Fig. 4). The A/Yamagata strain induced more cytokine production in the BAL fluids, and the A/Puerto Rico strain induced more viral replication. Our results suggested that the lower levels of IFN- γ were related to higher virus titers.

In this study, the levels of all cytokines in the BAL fluid of asthmatic/H1N1 mice were lower than those in the control/H1N1 mice; therefore, we concluded that pulmonary inflammation in the asthmatic/H1N1 group was milder than that in the control/H1N1 group, unlike in the case of A(H1N1)pdm09 groups. However, we cannot ascertain whether these differences also occur in patients. Nevertheless, we noted differences in the cytokine profiles between A(H1N1)pdm09 and H1N1 infections in bronchial asthma patients.

It has been reported that the IL-5 level in the BAL fluid is increased after influenza virus infection [20–23] and that the major source of IL-5 production may be group 2 innate lymphoid cells [20,24]. In the present study, we found that the IL-5 level in the BAL fluid of asthmatic/H1N1 mice was lower than that in the control/H1N1 mice. This result represents a discrepancy in the balance of Th1 and Th2 cells. In addition, IFN- γ is produced by Th1 cells, CD8⁺T (cytotoxic T) cells, natural killer (NK) cells, and NKT cells [25,26]. We previously demonstrated that the IFN- γ levels in the BAL fluid were significantly lower in the asthmatic/A(H1N1)pdm09 mice than in the control/A(H1N1)pdm09 mice [15]. In this study, the IFN- γ levels were lower in the asthmatic/H1N1 mice than in the control/H1N1 mice, as was also observed with A(H1N1)pdm09-infected mice. However, the levels of other cytokines and the viral titers showed an opposite trend after A(H1N1)pdm09 infection. In other studies, the production of IFN- γ was reduced in bronchial asthma patients, indicating an alteration in the cytokine milieu, with excess production of Th2 products and reduced production of Th1 products [16,17,27]. In this regard, our data are consistent with that of previous reports and this study [15]. IFN- γ activates macrophages, which enhances the ability of macrophages to kill intracellular pathogens [17]. It has been reported that bronchial asthma patients may have decreased innate immunity [28–31]. In our previous report, we speculated that decreasing innate immune activity, including NK cell activity and IFN- γ synthesis in the lungs, may contribute to the high replication rate of A(H1N1)pdm09 virus [15]. However, in the present study, the IFN- γ levels were significantly lower in the asthmatic/H1N1 mice than in the control/H1N1 mice, although the viral titer levels of both the mice groups were similar. Thus, there is a discrepancy in our hypothesis that A(H1N1)pdm09 infection can exacerbate pulmonary inflammation. We therefore conclude that the mechanisms through which A(H1N1)pdm09 infection can exacerbate pulmonary inflammation are very complex, for which further studies are needed to investigate the immune response to both A(H1N1)pdm09 and H1N1 virus infection using a mouse model of bronchial asthma.

5. Conclusion

In this study, we found that H1N1 infection did not exacerbate pulmonary inflammation in a mouse model of bronchial asthma, as has been observed with A(H1N1)pdm09 infection previously. Our results suggest that A(H1N1)pdm09 infection may induce severe pulmonary inflammation in bronchial asthma patients.

Acknowledgements

This study was supported by grants from the Ministry of Health, Labor, and Welfare (H24-Shinko-Ippan-002) and Howakai (H24 New Frontier Project).

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