
Analysis of bronchoalveolar lavage fluid in a mouse model of bronchial asthma and H1N1 2009 infection

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Running head: BAL fluid analysis in H1N1-infected asthmatic mice

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Abstract

Background: Bronchial asthma is known as a risk factor of admission to the intensive care unit. However, the mechanism by which pandemic 2009 H1N1 (A(H1N1)pdm09) infection increases the severity of symptoms in patients with bronchial asthma is unknown; therefore, we aimed at determining this mechanism.

Methods: Inflammatory cell levels in the bronchoalveolar lavage (BAL) fluid from the non-asthma/mock, non-asthma/A(H1N1)pdm09, asthma/mock, and asthma/A(H1N1)pdm09 groups were determined using BALB/c mice. Cell infiltration levels, cytokine levels, and viral titers were compared among the groups.

Results: Neutrophil, monocyte, interleukin (IL)-5, IL-6, IL-10, IL-13, and tumor necrosis factor (TNF)- α levels were significantly higher in the BAL fluid from the non-asthma/A(H1N1)pdm09 and asthma/A(H1N1)pdm09 groups than in the mock groups ($p < 0.05$ for neutrophils and monocytes; $p < 0.01$ for the rest). The number of eosinophils and CD8⁺ lymphocytes and the level of transforming growth factor beta 1 (TGF- β 1) in BAL fluid in the asthma/A(H1N1)pdm09 group were significantly higher among all groups ($p < 0.05$ for eosinophils and CD8⁺ lymphocytes; $p < 0.01$ for TGF- β 1). The levels of IL-6, IL-10, IL-13, and TNF- α were significantly higher in the asthma/A(H1N1)pdm09 group than in the non-asthma/A(H1N1)pdm09 group ($p < 0.05$ for IL-6 and IL-10; $p < 0.01$ for IL-13 and TNF- α). The level of IFN- γ in the asthma/A(H1N1)pdm09 group was significantly lower than that in the non-asthma/A(H1N1)pdm09 group ($p < 0.05$). The viral titers in the BAL fluids were higher in the asthma/A(H1N1)pdm09 group than in the non-asthma/A(H1N1)pdm09 group ($p < 0.05$). Histopathological examination showed more severe infiltration of inflammatory cells and destruction of lung tissue in the asthma/A(H1N1)pdm09 group than in the non-asthma/A(H1N1)pdm09 group.

Conclusions: Severe pulmonary inflammation induced by elevated levels of cytokines, combined with increased viral replication due to decreased IFN- γ levels, may contribute to

worsening respiratory symptoms in patients with bronchial asthma and A(H1N1)pdm09 infection.

Key words: 2009 pandemic H1N1 influenza, asthma, bronchoalveolar lavage fluid, cytokine

1. Introduction

The 2009 pandemic H1N1 (A(H1N1)pdm09) influenza virus originated in pigs and emerged among humans in Mexico during the spring of 2009 before spreading globally [1]. Most cases of A(H1N1)pdm09 infection involved mild symptoms, but some patients had severe respiratory problems such as severe pneumonia and acute respiratory distress syndrome (ARDS); these were particularly common in children and young adults [1-4]. In children, bronchial asthma increases the risk of admission to the hospital and intensive care unit [4-7]. A cytokine-mediated inflammatory response has been well documented in cases of pneumonia and ARDS [8-10], but to our knowledge, there are no published findings regarding the cytokine profile of bronchoalveolar lavage (BAL) fluid from patients who have bronchial asthma and are also infected by A(H1N1)pdm09. Thus, the mechanism by which A(H1N1)pdm09 infection increases the severity of symptoms in patients with bronchial asthma remains unclear.

In this study, we investigated the levels of cytokines and viral titers in BAL fluid in a mouse model of bronchial asthma with A(H1N1)pdm09 infection to determine the mechanism by which A(H1N1)pdm09 infection increases the severity of symptoms in mice with bronchial asthma.

2. Material and methods

2.1. Sensitization and allergen challenge of mice

BALB/c mice aged 6–8 weeks (Chiyoda Kaihatsu Co. Ltd. Tokyo, Japan) were sensitized and challenged with grade II ovalbumin (OVA; Sigma Chemical Co., St. Louis, MO, USA) or phosphate-buffered saline (PBS). For sensitization, mice were injected subcutaneously on days 0 and 14 with 50 µg OVA, which was dissolved in 300 µl PBS containing 2 mg aluminum hydroxide (alum; Wako Pure Chemical Industries, Ltd., Osaka, Japan). On days 22, 24, 26, and 28, the mice inhaled 1% (w/v) OVA in PBS for 30 min via a nebulizer (Fig. 1). We used PBS as a control in all procedures. Successful induction of the

mouse asthma model was assessed by determining the total number of cells and eosinophils in the BAL fluid. A total of 6 experiments using 5–8 mice in each group were performed. All the experimental protocols related to the mice were reviewed by the Committee for Ethics on Animal Experiments of Yamaguchi University Graduate School of Medicine.

2.2. Viruses and infection of mice

The mouse adapted A(H1N1)pdm09 virus (strain: A/Narita/1/09) was provided by the National Institute of Infectious Diseases (Tokyo, Japan). A/Narita/1/2009 was isolated from a patient, and propagated in Madin-Darby canine kidney (MDCK) cells. The isolate obtained was serially passaged 15 times in BALB/c mice. After intranasal inoculation of 20 μ L of viral suspension, 3 mice were sacrificed on day 3 after inoculation and their bronchoalveolar wash fluids were collected. These bronchoalveolar fluids were pooled and then used to inoculate 3 additional BALB/c mice, whose bronchoalveolar fluids on day 3 after inoculation were used to inoculate fresh mice.

On day 31, influenza virus was administered intranasally at a concentration of 1×10^6 pfu/20 μ l (non-asthma/A(H1N1)pdm09 and asthma/A(H1N1)pdm09 groups); uninfected groups were administered 20 μ l PBS (non-asthma/mock and asthma/mock groups). The mice were euthanized, and samples were collected on day 38 (post-infection day 7). A total of 6 experiments using 5–8 mice in each group were performed.

2.3. Preparation of BAL fluid

BAL fluid was collected on day 38 (post-infection day 7) by using 3 consecutive instillations of PBS (1 ml) at room temperature and was centrifuged at 1,500 rpm at 4°C for 5 min; supernatants were collected and stored at –80°C for measurement of cytokine levels. Cell pellets were resuspended in 500 μ l PBS and used for analysis of cellular infiltration. Cytospin samples were prepared using Auto Smear CF-12D (Sakura Co., Tokyo, Japan), and cellular

infiltration in BAL fluid was assessed on Wright-Giemsa-stained slides (Wako Pure Chemical Industries, Ltd).

2.4. Measurement of CD4⁺ and CD8⁺ lymphocytes in BAL fluid

Cell pellets were resuspended in PBS and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (BD Biosciences, San Jose, CA., USA) and allophycocyanin (APC)-conjugated anti-CD8 (BD Biosciences) antibodies; erythrocytes were lysed by the addition of FACS Lysing Solution (Becton Dickinson, San Diego, CA, USA). The cell suspensions were centrifuged, and the cell pellets were resuspended in PBS containing sodium azide and paraformaldehyde. The cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) equipped with the CellQuest software (Becton Dickinson). At least 10,000 cells were analyzed for each mouse in the flow cytometric studies.

2.5. Cytokine assays

The concentrations of cytokines in BAL fluid were measured. The levels of interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17A, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α were measured using a cytometric bead array (CBA) kit (BD Biosciences). The levels of IL-5, IL-12, IL-13, and transforming growth factor beta 1 (TGF- β 1) were measured using an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA). The CBA assay and ELISA were performed in accordance with the manufacturer's instructions. The lower detection limits for IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IFN- γ , TNF- α , and TGF- β 1 were 0.1, 0.03, 7, 1.4, 16.8, 2.5, 1.5, 0.8, 0.5, 0.9, and 4.6 pg/ml respectively. A total of 5 experiments using 5–8 mice in each group were performed for the cytokine assays.

2.6. Plaque assay

MDCK cells were maintained at 37°C in humidified 5% CO₂ in a stationary culture.

Six-well plates were seeded with 1×10^6 cells in each well 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 the cells were washed twice in serum-free Dulbecco's modified Eagle's medium (DMEM; GIBCO/Invitrogen), they were maintained in serum-free DMEM at 37°C for 1 h before being overlaid with 200 μ l/well of suitably diluted BAL and incubated at 37°C for 1 h. After the cells were washed once in serum-free DMEM, they were overlaid with serum-free DMEM containing 0.8% agarose (Becton Dickinson, Franklin Lakes, NJ, USA), 0.1% diethylaminoethyl-dextran (Sigma-Aldrich, Co., St Louis, MO, USA), and 7 μ g/ml trypsin (Sigma-Aldrich, Co.). The cells were further cultured for 72 h at 37°C, fixed in 10% formaldehyde (Wako Pure Chemical Industries, Ltd), and stained with 0.037% methylene blue (Wako Pure Chemical Industries, Ltd). Each experiment was performed in duplicate wells.

2.7. Histopathological examination of the lungs

Lung tissues were fixed in 10% formalin for 24 h at room temperature and embedded in paraffin. Three-micrometer-thick serial sections were cut and stained with hematoxylin and eosin (HE) (Muto Pure Chemicals Co. Ltd., Tokyo, Japan). The sections were observed using light microscopy to evaluate the degree of airway inflammation.

2.8. Statistical analysis

The results were analyzed using the Mann-Whitney *U*-test; *p*-values of <0.05 were considered significant. Analyses and calculations were performed using ystat2008.xls (Igakutosho-shuppan Ltd, Tokyo, Japan).

3. Results

3.1. Analysis of the cells in the BAL fluid

The number of inflammatory cells in the BAL fluid from the different groups is illustrated in Fig. 2. The number of neutrophils and monocytes in the non-asthma/A(H1N1)pdm09 and asthma/A(H1N1)pdm09 groups was significantly higher than that in the mock groups (all $p < 0.05$). The number of eosinophils in the asthma/mock group was significantly higher than that in the non-asthma/mock and non-asthma/A(H1N1)pdm09 groups (both $p < 0.05$), and the number of eosinophils in the asthma/A(H1N1)pdm09 groups was significantly higher than that in the 3 other groups (all $p < 0.05$). Furthermore, the number of lymphocytes in the asthma/mock, non-asthma/A(H1N1)pdm09, and asthma/A(H1N1)pdm09 groups was significantly higher than that in the non-asthma/mock group (all $p < 0.05$), and the number of lymphocytes in the asthma/A(H1N1)pdm09 group was significantly higher than that in the non-asthma/A(H1N1)pdm09 group ($p < 0.05$). Fig. 3 shows the number of CD4⁺ and CD8⁺ lymphocytes in the BAL fluid. The number of CD4⁺ lymphocytes in the asthma/mock and asthma/A(H1N1)pdm09 groups was significantly higher than that in the non-asthma/mock group (all $p < 0.05$). The number of CD8⁺ lymphocytes in the non-asthma/A(H1N1)pdm09 group was significantly higher than that in the non-asthma/mock and asthma/mock groups (both $p < 0.05$). Finally, the number of CD8⁺ lymphocytes in the asthma/A(H1N1)pdm09 group was significantly higher than that in the 3 other groups (all $p < 0.05$).

3.2. Cytokine concentrations in BAL fluid

The cytokine levels in BAL fluid are shown in Fig. 4. The levels of IL-5 (data not shown), IL-6, IL-10, IL-13, IFN- γ , and TNF- α in the non-asthma/A(H1N1)pdm09 and asthma/A(H1N1)pdm09 groups were significantly higher than those in the non-asthma/mock and asthma/mock groups (all $p < 0.01$). The levels of TGF- β 1 in the asthma/mock, non-asthma/A(H1N1)pdm09, and asthma/A(H1N1)pdm09 groups were significantly higher than those in the non-asthma/mock group (all $p < 0.01$). The levels of IL-6, IL-10, IL-13,

TNF- α , and TGF- β 1 in the asthma/A(H1N1)pdm09 group were significantly higher than those in the non-asthma/A(H1N1)pdm09 group ($p < 0.05$, $p < 0.05$, $p < 0.01$, $p < 0.01$, and $p < 0.01$, respectively). In contrast, the levels of IFN- γ in the asthma/A(H1N1)pdm09 group were significantly lower than those in the non-asthma/A(H1N1)pdm09 group ($p < 0.05$). There were no significant differences among the 4 groups with respect to the levels of IL-2, IL-4, IL-12 or IL-17A (data not shown).

3.3. Viral titers in BAL fluid

Fig. 5 shows the viral titers in BAL fluid on post-infection day 7. The viral titers in the asthma/A(H1N1)pdm09 group (mean \pm SEM; $2.2 \pm 5.1 \times 10^5$ pfu/mL) were significantly higher than those in the non-asthma/A(H1N1)pdm09 group (mean \pm SEM; $2.6 \pm 3.8 \times 10^4$ pfu/mL) ($p < 0.05$).

3.4. Histopathological findings for the lungs

Fig. 6 shows the lung tissues of mice stained with HE. The asthma/mock group had greater thickening of the basement membrane of bronchi and infiltration of inflammatory cells around vessels than the non-asthma/mock controls. Infiltration of inflammatory cells and tissue destruction in the non-asthma/A(H1N1)pdm09 and asthma/A(H1N1)pdm09 groups were greater than those in mock groups. Furthermore, the degree of inflammatory cell infiltration and tissue destruction were more severe in the asthma/A(H1N1)pdm09 group than in the non-asthma/A(H1N1)pdm09 group.

4. Discussion

To our knowledge, this is the first report of BAL fluid analysis in A(H1N1)pdm09-infected mice with bronchial asthma. The results show that A(H1N1)pdm09 infection induces high levels of pro-inflammatory cytokines such as IL-6 and TNF- α in BAL fluid and that this increase is enhanced by the presence of bronchial asthma.

IL-6 and TNF- α are known as pro-inflammatory cytokines [11-13]. The levels of these cytokines are elevated in the sputum and BAL fluid of patients with asthma and are thought to play a critical role in the initiation, maintenance, and progression of airway inflammation in asthma [13-15]. Administration of TNF- α leads to an increase in both airway hyper-responsiveness and the infiltration of neutrophils in the airway [16,17]; this is explained by the direct effect of TNF- α and the indirect effect of cysteinyl leukotriene release [18,19]. TNF- α is also known to contribute to acute lung injury (ALI) and ARDS [20-22]. The A(H1N1)pdm09 virus can infect both type I and II alveolar epithelial cells [23]; A(H1N1)pdm09 infection of type II alveolar epithelial cells may decrease the synthesis of pulmonary surfactant and lead to plastic bronchitis. In addition, the elevation in TNF- α during A(H1N1)pdm09 infection may exacerbate lung damage and bronchial asthma. The histopathological findings obtained for the lungs during this study are consistent with our hypothesis. The levels of IL-10 in the asthma/A(H1N1)pdm09 group were also significantly higher than those in the non-asthma/A(H1N1)pdm09 group, indicating significant elevation of IL-6 and TNF- α levels, as IL-10 inhibits the production of pro-inflammatory cytokines such as TNF- α , IL-1, IL-6, and IL-12 [24-29]. TGF- β 1 plays a key role in inflammation in asthma and has anti-inflammatory properties since it can regulate lymphocyte homeostasis by inhibiting Th-1 and Th-2 cell responses and promoting the differentiation of Treg cells [30]. In this study, the elevated levels observed for TGF- β 1 may indicate anti-inflammatory actions. On the other hand, TGF- β 1 also has pro-inflammatory properties [30]. Thus, it may have contributed to the exacerbation of bronchial asthma via its pro-inflammatory role in our model.

IL-13 is a Th-2 cytokine produced by T cells, natural killer cells, mast cells. It plays a pivotal role in the onset or exacerbation of human bronchial asthma [31]. In a previous report, the serum levels of IL-13 were found to be elevated in children with A(H1N1)pdm09 infection [32]. Our results indicated that an increased level of IL-13 in BAL fluid appears to contribute to worsening of respiratory symptoms in patients who have bronchial asthma and

are infected by A(H1N1)pdm09. IL-5 is also a Th-2 cytokine that plays an important role in the exacerbation of human bronchial asthma symptoms [33]. In this experimental system, the levels of IL-5 in the non-asthma/A(H1N1)pdm09 and asthma/A(H1N1)pdm09 groups were indeed significantly higher than those in the mock groups, although we do not believe that this difference is closely related to exacerbation, since the differences between the groups were slight. In contrast, the levels of IFN- γ in the asthma/A(H1N1)pdm09 group were significantly lower than those in the non-asthma/A(H1N1)pdm09 group. IFN- γ is produced by T cells, monocytes, macrophages, dendritic cells, and natural killer T cells, and its secretion is indicative of a Th-1-mediated inflammatory response [34]. The production of IFN- γ reduced in patients with bronchial asthma, indicating an alteration in the cytokine milieu, with an excess of Th-2 products and a decrease in Th-1 products [34,35]. In this regard, our data are consistent with those of previous reports. IFN- γ activates macrophages, which enhances its ability to kill intracellular pathogens [36]. Cytotoxic CD8⁺ T cells also produce IFN- γ , and this may represent an accessory mechanism by which they eliminate viruses from infected tissues [36]. In this study, the number of CD8⁺ T cells in the asthma/A(H1N1)pdm09 group was significantly higher but the levels of IFN- γ were significantly lower than those in the non-asthma/A(H1N1)pdm09 group. This discrepancy can be explained by focusing on natural killer (NK) cell activity. Kawakami et al. examined NK cell activity in a mouse model of eczema vaccinatum [37] and found that the number of NK and CD8⁺ T cells in the affected lesions was significantly higher in mice with eczema vaccinatum, while NK cell activity, including IFN- γ production, was significantly lower in these mice than in the control group [37]. The decrease in the activity of NK cells in bronchial asthma patients may be attributable to the fact that bronchial asthma is an atopic disease like atopic dermatitis. The viral titers in BAL fluid in the asthma/A(H1N1)pdm09 group were significantly higher than those in the non-asthma/A(H1N1)pdm09 group in our study. We believe that decreasing innate immunity-related activity, including NK cell activity and IFN- γ synthesis in the lungs, may contribute to the high replication rate of A(H1N1)pdm09.

Our present study has several limitations. The data shown in this report were only obtained at day 7 post-infection. Additionally, we did not investigate NK cell activity because NK activation does not occur in this model at day 7 post-infection, and we would need to obtain the results at different timepoints. Thus, further studies are required in order to clarify the relationship between A(H1N1)pdm09 infection and asthma.

In conclusion, our results demonstrate that A(H1N1)pdm09 infection induces severe pulmonary inflammation in a mouse model of asthma. This mechanism, combined with increased viral replication due to decreased IFN- γ levels, may increase the severity of symptoms in humans who have bronchial asthma and are also exposed to A(H1N1)pdm09.

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Figure legends

Figure 1. Study design. Ovalbumin (OVA)-sensitized mice were immunized with OVA plus alum subcutaneously (s.c.) on days 0 and 14. On days 22, 24, 26, and 28, the mice inhaled 1% (w/v) OVA/PBS or PBS for 30 min via a nebulizer. On day 31, the mice were infected with the pandemic H1N1 2009 (A(H1N1)pdm09) influenza virus. The mice were euthanized on day 38, and bronchoalveolar lavage (BAL) fluid was collected. Abbreviations: i.h., inhale; p.i.d., post-infection day

Figure 2. Differential cell counts in bronchoalveolar lavage (BAL) fluids collected from mice. The data shown represent the mean \pm SEM values. * $p < 0.05$ vs. non-asthma/mock. # $p < 0.05$ vs. asthma/mock. \$ $p < 0.05$ vs. non-asthma/A(H1N1)pdm09 (n = 5 per group).

Figure 3. Number of CD4⁺ T cells and CD8⁺ T cells in bronchoalveolar lavage (BAL) fluid collected from mice. The data shown represent the mean \pm SEM values. * $p < 0.05$ vs. non-asthma/mock. # $p < 0.05$ vs. asthma/mock. \$ $p < 0.05$ vs. non-asthma/A(H1N1)pdm09 (n = 5 per group).

Figure 4. Concentrations of interleukin (IL)-5, IL-6, IL-10, IL-13, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and transforming growth factor beta 1 (TGF- β 1) in bronchoalveolar lavage (BAL) fluid from mice. The data shown represent the mean \pm SEM values. The results shown are representative of 5 experiments using 5–8 mice in each group. ** $p < 0.01$ vs.

non-asthma/mock. ## $p < 0.01$ vs. asthma/mock. \$ $p < 0.05$ vs. non-asthma/A(H1N1)pdm09.

\$\$ $p < 0.01$ vs. non-asthma/A(H1N1)pdm09.

Figure 5. A plaque assay was performed to detect viral titers in the bronchoalveolar lavage (BAL) fluid. The data shown represent the mean \pm SEM values. The results shown are representative of 5 experiments using 5–8 mice in each group. * $p < 0.05$.

Figure 6. Photomicrographs of HE-stained lung tissue from each group: (a) non-asthma/mock group ($\times 40$), (b) asthma/mock group ($\times 40$), (c) non-asthma/A(H1N1)pdm09 group ($\times 40$), (d) asthma/A(H1N1)pdm09 group ($\times 40$), (e) non-asthma/mock group ($\times 100$), (f) asthma/mock group ($\times 100$), (g) non-asthma/A(H1N1)pdm09 group ($\times 100$), and (h) asthma/A(H1N1)pdm09 group ($\times 100$). Similar results were obtained in 3 independent mice from each group. Representative findings are shown.