Differences in Virus Detection and Cytokine Profiles between First Wheeze and Childhood Asthma

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Objective: To distinguish between first wheeze and asthma in early childhood, we investigated respiratory viruses and cytokine/chemokine profiles among patients with first wheeze and established asthma.
Methods: We enrolled children with acute exacerbations of wheezing (17 first wheeze and 32 asthma) and 11 controls (no wheezing) aged between 10 months and 6 years. Nasal aspirates were obtained, and virus detection was performed with antigenic assay kits and/or RT-PCR. Serum 27 cytokines/chemokines were assayed by a multi-cytokine detection system.
Results: Rhinovirus and respiratory syncytial (RS) virus were dominant in acute exacerbations of asthma. However, many types of viruses were isolated in first wheeze. Serum IL-8 and IL-12 values were significantly higher in first wheeze than in acute asthma or the controls. IL-5 and IP-10 levels in acute asthma and first wheeze cases were higher than in the controls. Both of them were significantly higher in cases of acute asthma than in convalescence stage of asthma cases. Only IP-10 was significantly higher in first wheeze than in convalescence stage of first wheeze cases.
Conclusions: Different profiles in virus detection and production of IL-8 and IL-12 might distinguish between first wheeze and childhood asthma.

Key words: chemokine, childhood asthma, cytokine, first wheeze, viral infection

INTRODUCTION

Epidemiological studies have shown that childhood wheezing consists of three phenotypes based on clinical manifestations of different conditions [1, 2]. The first is defined as transient wheezing in early childhood up to 3 years of age; the second is non-atopic wheezing, which is generally thought to be triggered by viral infections; and the third is atopic wheezing or IgE-associated wheezing/asthma, which is commonly associated with persistent asthma [2–4]. These definitions are based on studies showing that the majority of non-atopic preschool children with repeated episodes of wheezing do not continue have symptoms at school age. In contrast, children with atopic wheezing are likely to have chronic asthma, characterized by more severe symptoms, airway hyperresponsiveness, and impaired lung function [5]. Thus, the early identification of asthma enables appropriate treatment to be given and prevents unnecessary prescription of asthma drugs to the majority of patients with transient wheezing without asthma [6]. Attempts to differentiate asthma from transient wheezing in early infancy have identified several clinical factors such as gestational age at birth, parental atopy, and early sensitization that are associated with development of asthma [7, 8] and these have been used to develop asthma prediction tools and models [9]. However, these clinical prediction models are only moderately successful. In terms of cytokine and chemokine production, a recent report suggests that levels of macrophage inflammatory protein (MIP)-1α in nasopharyngeal aspirates obtained during the first episode of wheezing can predict recurrence [10].

Based on these findings, it is important to explore and compare the virus and cytokine profiles between cases of first wheeze and acute asthma induced by viral infections. Therefore, the aim of this study was to investigate the viruses isolated, the peripheral eosinophil and neutrophil counts, and the serum levels of cytokines and chemokines in cases of virus-induced acute exacerbations of first wheeze and asthma in children.

MATERIALS AND METHODS

Patients and study setting

We recruited both outpatients and hospitalized subjects with acute exacerbations of wheezing aged from 10 months to 6 years old at Tokai University Hospital, Hadano Red Cross Hospital, and Gunma Children's Medical Center between January 1, 2012 and December 31, 2015. Wheezing was defined as a high-pitched whistling sound during expiration and difficulty in breathing. Patients were examined by pe-

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diatricians who confirmed the diagnoses. First wheeze cases were treated with short-acting β agonists. These patients did not develop asthma at least two years after the first wheezing episode. Patients with asthma were diagnosed according to the guidelines of the Japanese Society of Pediatric Allergy and Clinical Immunology [11]. Briefly, a diagnosis of asthma was confirmed if the patient had a history of recurrent wheezing and dyspnea on at least three occasions, and the presence of reversible bronchoconstriction. These patients were prescribed short-acting β agonists and/or long-term controller medications; none were using systemic corticosteroids at the time of further examination. Children with obvious bacterial infection, congenital heart disease, or chronic lung disease, as well as those with demonstrable presence of a foreign body, had signs of severe infection, or were immunosuppressed were excluded, because these complications can interfere with the assessment of asthma-related outcome measures. Exclusion criteria for the controls included immunosuppression, the presence of other respiratory tract symptoms, or a history of wheezing and asthma. Controls and patient cases were age- and sex-matched. This study was approved by the Ethics Committees of Tokai University Hospital, Hadano Red Cross Hospital, and Gunma Children's Medical Center. Informed consent was obtained from the parents of patients and assent was obtained from children considered old enough (generally > 9 years old).

Virus detection

Nasal aspirates were obtained from patients during acute exacerbations of wheezing and/or asthma, and analyzed using antigen detection kits for respiratory syncytial (RS) virus (Becton Dickinson, Fukushima, Japan), influenza virus types A and B (Denka-Seiken, Gosen, Japan), and adenovirus (Tauns, Izunokuni, Japan). The remaining secretions were frozen at −80 °C until examination by reverse transcription-polymerase chain reaction (RT-PCR), followed by direct DNA sequencing analysis as previously reported [12, 13]. Several samples were tested by multiplex PCR (Seeplex RV15 OneStep ACE Detection kit, Seegene, Inc., Seoul, South Korea) for the presence of 15 human viral respiratory pathogens (adenovirus A/B/C/D/E, human metapneumovirus, enterovirus, human bocavirus 1/2/3/4, human coronavirus 229E/NL63 and OC43, human parainfluenza virus 1/2/3/4, influenza virus A/B, RS virus A/B, and rhinovirus A/B/C) [13]. PCR products were subsequently analyzed by automated electrophoresis (MCE-202 MultiNA; Shimadzu, Kyoto, Japan) [14].

Serum total IgE and allergen-specific IgE

Automated fluoroenzyme immunoassay (FEIAUnicap® 100, Phadia AB, Uppsala, Sweden) was used to measure serum levels of total IgE and allergen-specific IgE against inhalant allergens, including *Dermatophagoides farinae/pteronyssinus*, house dust, Japanese cedar, orchard grass, ragweed, cat and dog dander, *Alternaria, and Cladosporium*, as described previously [15]. Allergen-specific IgE values ≥ 0.35 U_A/mL (Class I) were considered positive, and the lower limit for detection of total IgE was 2.00 IU/mL. Allergic sensitization was defined as positivity to at least one allergen-specific IgE.

Serum cytokines/chemokines

We measured total IgE, peripheral eosinophil counts, and the serum concentrations of 27 cytokines/chemokines (IL-1β, IL-1 receptor antagonist [IL-1ra], IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, IFN-γ-induced protein [IP]-10, TNF-α, GM-CSF, granulocyte colony-stimulating factor, monocyte chemoattractant protein [MCP]-1, macrophage inflammatory protein [MIP]-1α, MIP-1β, eotaxin, regulated on activation normal T expressed and secreted chemokines [RANTES], platelet-derived growth factor BB, fibroblast growth factor basic, and VEGF) in samples from patients with acute exacerbations of 17 first wheeze and 32 asthma who showed signs of viral infection at the time of examination, and 11 control subjects. We also examined these parameters in convalescence stage of first wheeze and asthma cases. Serum cytokines/chemokines were measured with the Bio-Plex® multi-cytokine detection system, (Bio-Rad, Hercules, CA) and a Luminex System (Luminex Corporation, Austin, TX) according to the manufacturers' instructions; calculations were performed using Bio-Plex software (Bio-Rad), as previously reported [12].

Statistical analyses

Patient characteristics and frequency of virus type were evaluated by the Pearson x^2 test and Fisher's exact test for categorical variables. Unpaired data were analyzed using the Mann-Whitney U test. Differences between three or more groups were analyzed by the Kruskal-Wallis test, followed by Dunn's multiple comparison test. A P-value of < 0.05 (two-sided) was considered statistically significant. All statistical analyses were performed with IBM SPSS software for Windows (version 23.0, SPSS Japan Inc., Tokyo, Japan) or GraphPad Prism for Windows (version 6.0, GraphPad Software, San Diego, CA).

RESULTS

Virus detection

We compared several parameters between the 17 first wheeze and 32 asthma patients who showed signs of virus infection at the time of examination, and 11 controls. The patient's characteristics was shown in Table 1.

Among the 32 samples from patients with acute exacerbations of asthma, rhinovirus was detected in 13 (40.6%), RS virus in 6 (18.8%), enterovirus in 3 (9.4%), human coronavirus in 2 (6.3%), human bocavirus in 1 (3.1%), human parainfluenza virus type 3 in 1 (3.1%), and ≥ 2 viruses in 6 (18.8%), as shown in Fig. 1A. Among the 17 samples from patients with acute exacerbations of first wheeze, RS virus was detected in 5 (29.4%), human metapneumovirus in 3 (17.6%), human parainfluenza virus type 1 in 3 (17.6%), rhinovirus in 2 (11.8%), adenovirus in 1 (5.9%), human bocavirus in 1 (5.9%), and ≥ 2 viruses in 2 (11.8%), as shown in Fig. 1B. Among these type of viruses, the frequency of rhinovirus was only significantly higher in acute exacerbations of asthma than in first wheeze cases ($p = 0.037$).

Table 1 Patient characteristics

	Asthma	Wheeze	Control	Asthma vs Wheeze, p values	Asthma vs Control, p values	Wheeze vs Control, p values
Number	32	17	11			
Age, year, mean/ median (range)	2.6 2.5 (1.2–5.8)	2.6 1.9 (0.9–5.8)	3.2 3.3 (1.3–4.8)	0.345	0.243	0.348
Gender, % of male	65.6	64.7	72.7	0.831	0.759	0.657
Family history of asthma, % of positive	66.7	41.1	9.1	0.413	0.031	0.066
Atopic dermatitis, % of positive	33.3	38.4	18.2	0.830	0.482	0.221
Food allergy, % of positive	20.0	23.1	9.1	0.888	0.541	0.360
≥1 Positive aeroallergen, % of positive	65.6	41.7	0.0	0.150	0.000	0.016

Data were analyzed using Pearson x^2 test and Fisher's exact test for categorical variables. Unpaired data were analyzed using the Mann-Whitney U test.

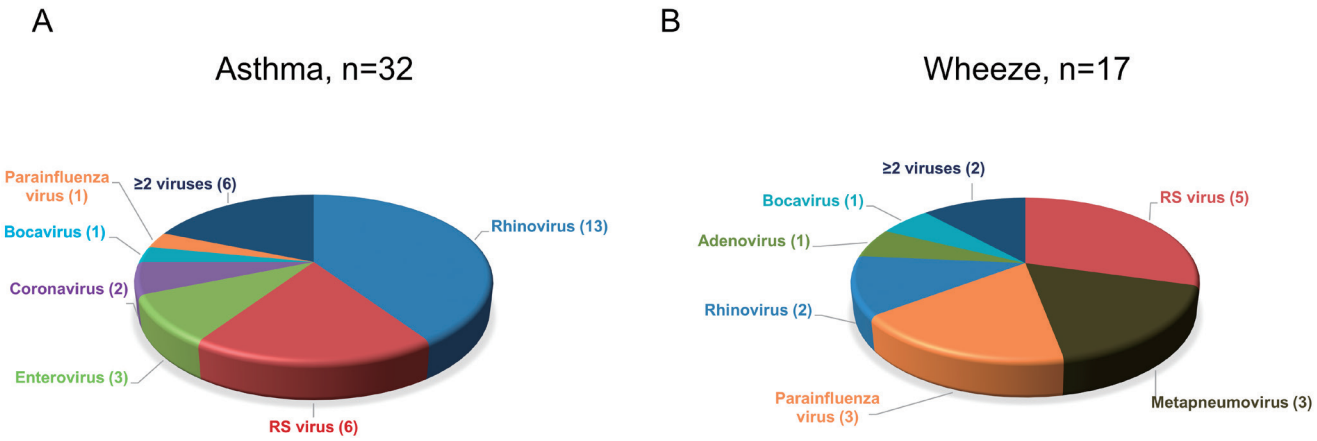

Fig. 1 Virus detection in acute exacerbations of asthma (Asthma, n = 32; A) or first wheeze (Wheeze, n = 17; B). Number of patients was shown in parentheses after each virus.

Peripheral eosinophil and neutrophil counts, serum total IgE, and cytokines/chemokines levels

The results of peripheral eosinophil and neutrophil counts, serum total IgE levels, and serum levels of 27 types of cytokines/chemokines are summarized in Table 2. There were no significant differences in peripheral eosinophil and neutrophil counts or serum IgE levels among the three groups (Fig. 2A–C). However, of the 27 cytokines/chemokines, serum IL-4, IL-6, and IL-9 levels were significantly elevated in cases of acute asthma compared with first wheeze, while theses cytokines in acute asthma were not significantly higher than in the controls (Fig. 3A–C). Conversely, IL-8 and IL-12 values were significantly higher in cases of first wheeze than in acute asthma or the controls (Fig. 3D, E). IL-5 and IP-10 levels in acute asthma and first wheeze cases were higher than in the controls (Fig. 3F, G). Next, we investigated the data from convalescence stage of first wheeze and asthma cases. Both of IL-5 and IP-10 levels were significantly higher in cases of acute asthma than in convalescence stage of asthma cases (Fig. 4A, C). In contrast, only IP-10 was significantly higher in first wheeze than in convalescence stage of first wheeze cases (Fig. 4B, D).

DISCUSSION

Rhinovirus and respiratory syncytial (RS) virus were the major viruses isolated in this study and were dominant in acute asthma cases. This result is compatible with previous reports [12–14]. RS virus, human metapneumovirus, parainfluenza virus, rhinovirus, adenovirus, and human bocavirus were isolated in cases of first wheeze. In a study on children hospitalized for wheezing, Heyman *et al.* reported that RS is the dominant virus in children younger than 3 years old, while rhinovirus is predominant in children 3 years and older [16]. However, our recent study demonstrated that rhinovirus and RS virus are almost equally prevalent in children under 3 years old with asthma [14]. This discrepancy might be because in the study by Heyman *et al.*, the children hospitalized for wheezing were not divided into groups of transient wheezing and asthma. Furthermore, the ages of the children in the first wheeze and asthma groups in this study were essential-

Table 2 Eosinophils/neutrophils, IgE, and cytokines/chemokines in acute asthma and first wheeze

	Asthma	Wheeze	Control
Eosinophils	111.0 (0.0–1510.0)[1]	134.0 (0.0–936.0)	148.0 (0.0–936.0)
Neutrohpils	5130.0 (256.0–15781.0)	4680.0 (1539.0–12240.0)	3234.0 (2418.0–6512.0)
IgE (Total)	119.0 (1.9–3150.0)	61.0 (4.0–693.0)	45.5 (4.0–76.6)
IL-1β	6.7 (1.5–19.0)	4.2 (1.7–23.1)	6.9 (2.2–31.0)
IL-1ra	406.3 (137.0–2946.0)	258.6 (142.9–1956.0)	177.2 (100.6–410.1)
IL-2	5.9 (1.7–57.5)	19.0 (6.3–51.1)	7.2 (3.6–9.6)
IL-4	13.2 (3.5–33.7)	7.3 (4.0–8.9)e	13.3 (2.0–22.7)
IL-5	6.5 (1.5–52.8)[b]	16.3 (3.3–26.8)[c]	2.3 (0.0–4.6)
IL-6	67.5 (6.8–893.8)	22.6 (5.9–71.9)[d]	50.1 (14.1–238.5)
IL-7	16.8 (2.6–38.1)	20.7 (11.4–25.5)	16.1 (0.8–27.1)
IL-8	19.6 (4.5–236.1)[c]	55.6 (23.1–73.2)[c,d]	8.2 (0.4–16.6)
IL-9	46.1 (8.8–2036.0)	16.6 (8.1–29.2)[a,e]	33.8 (29.5–72.9)
IL-10	29.5 (7.3–145.4)	29.5 (5.5–94.1)	13.8 (0.4–64.3)
IL-12	15.1 (0.9–110.7)	49.4 (24.8–105.3)[a,d]	6.9 (0.3–52.3)
IL-13	3.4 (0.1–3.7)	6.9 (0.1–23.3)	3.7 (1.7–4.0)
IL-15	10.6 (1.2–71.3)	30.3 (10.2–30.3)	15.4 (5.9–90.1)
IL-17	11.7 (0.1–217.9)	205.5 (1.5–240.4)	0.1 (0.1–0.1)
IFN-γ	164.7 (41.4–570.1)	161.7 (80.8–716.6)	132.9 (44.8–1061.0)
IP-10	1786.0 (423.5–13882.0)[b]	3573.0 (1135.0–6335.0)[c]	442.3 (330.9–776.3)
TNF-α	58.7 (1.5–124.8)	89.5 (4.9–151.6)	36.1 (0.3–392.1)
GM-CSF	30.1 (0.6–114.6)	35.2 (11.0–69.4)	24.3 (0.6–72.0)
G-CSF	58.6 (15.4–1587.0)	66.5 (44.3–101.8)	35.4 (19.4–129.6)
MCP-1	48.0 (13.0–223.6)	65.6 (1.4–89.4)	50.6 (30.2–80.6)
MIP-1α	9.3 (2.6–46.8)	6.7 (2.0–10.3)	12.5 (10.6–14.5)
MIP-1β	81.0 (14.4–186.5)	133.1 (84.6–169.1)	110.3 (15.3–168.0)
Eotaxin	81.0 (8.2–354.9)	62.8 (16.5–476.4)	104.4 (69.8–577.4)
RANTES	7256.0 (3199.0–114340.0)	8798.0 (8798.0–8798.0)	5831.7 (4632.5–7030.9)
PDGF-bb	5100.0 (433.3–14891.0)	2638.0 (1016.0–4004.0)	5777.0 (2623.0–12494.0)
FGF-basic	75.4 (15.4–471.6)	76.7 (50.7–137.7)	72.3 (35.2–156.8)
VEGF	135.3 (5.2–729.1)	94.1 (73.5–181.2)	70.3 (25.6–171.0)

[1]Median (range), Dunn's multiple comparison test, [a]p<0.05; [b]p<0.01; [c]p<0.001 versus Control: [d]p<0.05, [e]p<0.01 versus Asthma.
Eosinophils/neutrophils: /μl; IgE: IU/ml; cytokines/chemokines: pg/ml.

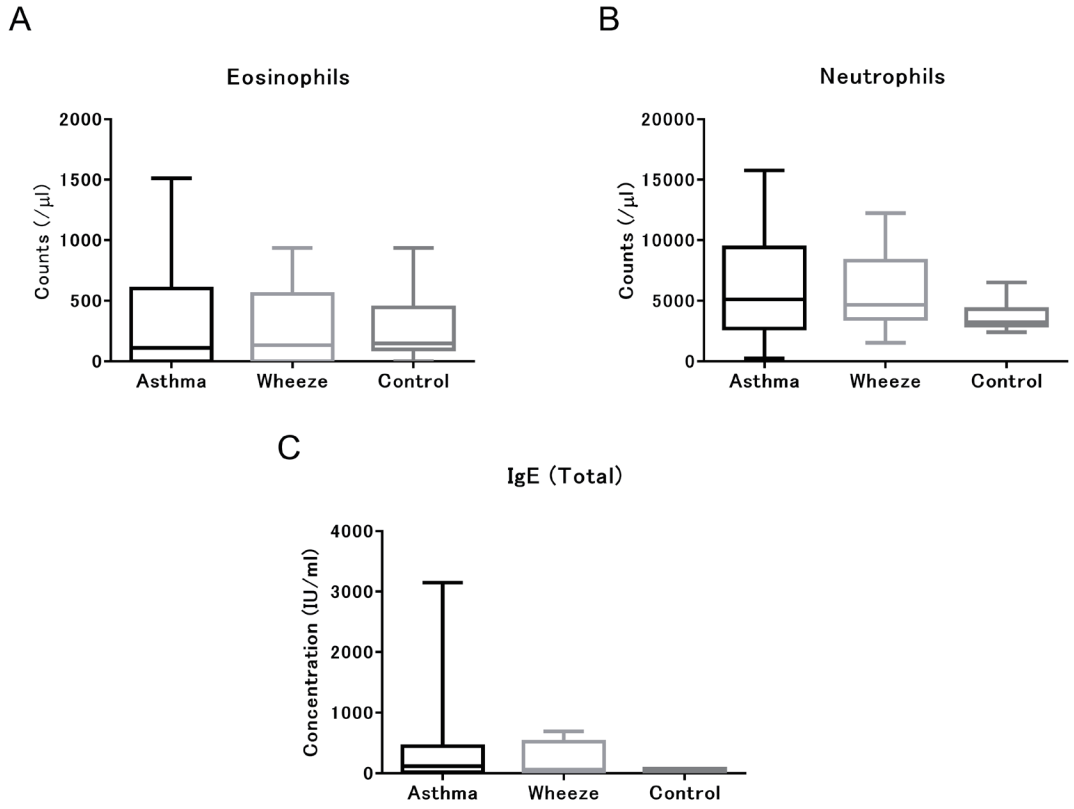

Fig. 2 Peripheral eosinophil and neutrophil counts, and serum IgE levels in cases of virus-induced acute exacerbations of asthma, first wheeze, and controls. Horizontal bar represents the median. Data were analyzed using the Dunn's multiple comparison test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

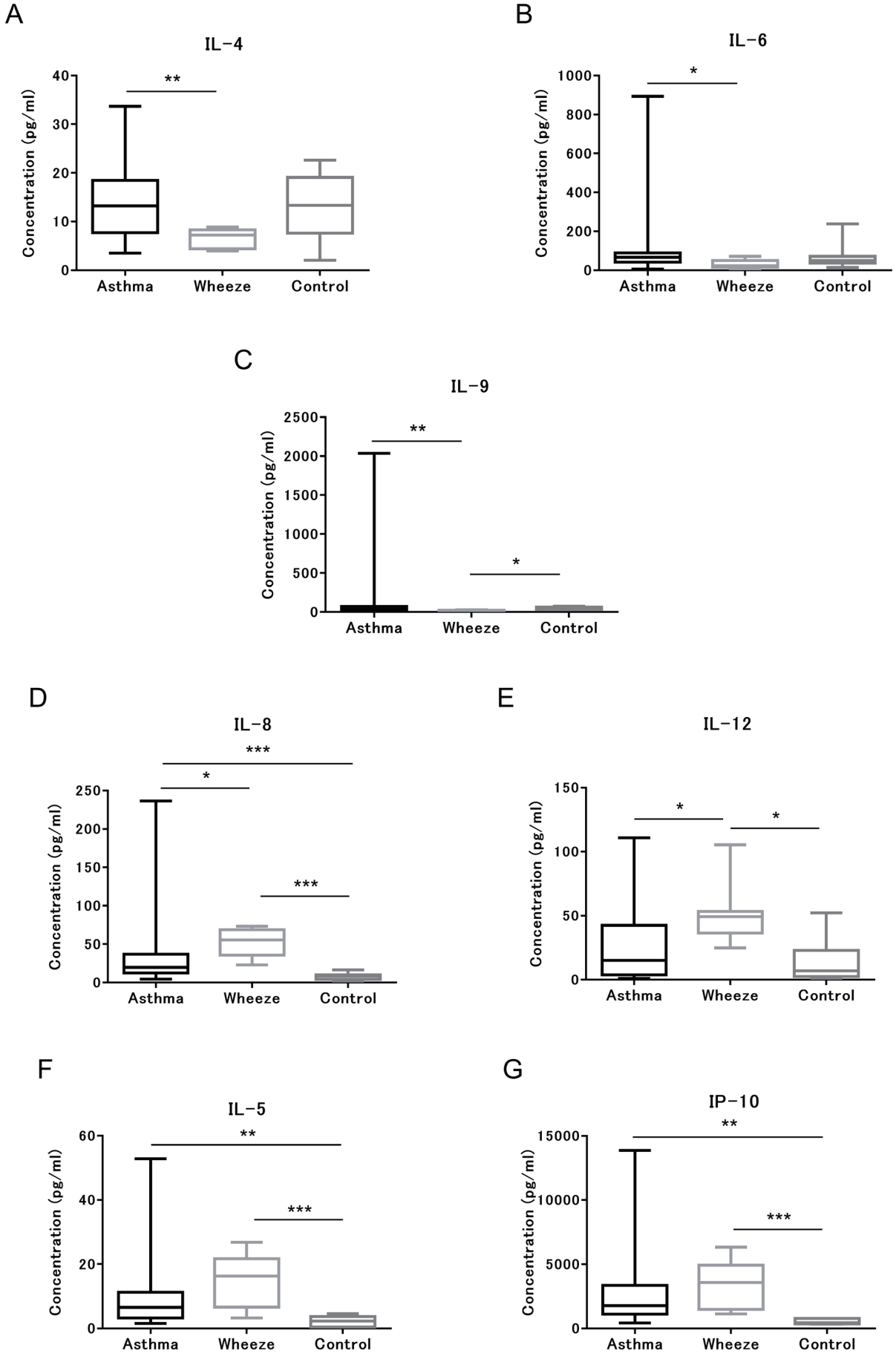

Fig. 3 Serum cytokines/chemokines in cases of virus-induced acute exacerbations of asthma, first wheeze, and controls. Horizontal bar represents the median. Data were analyzed using the Dunn's multiple comparison test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

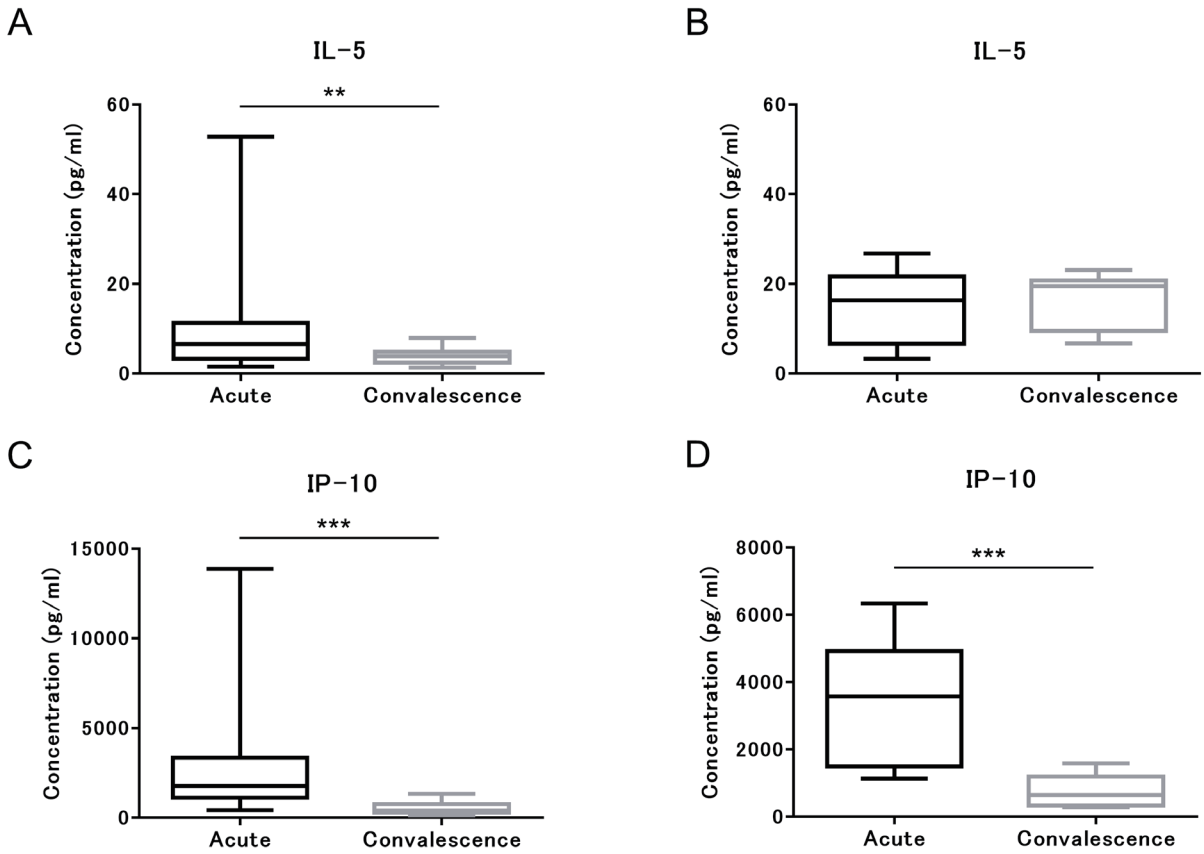

Fig. 4 Comparison of serum levels of IL-5 (A, B) and IFN-γ-induced protein (IP)-10 (C, D) between cases of virus-induced acute exacerbations of asthma (Acute A, C), first wheeze (Acute B, D), convalescence stage of asthma (Convalescence; A, C) or convalescence stage of first wheeze (Convalescence; B, D). Data were analyzed using the Mann-Whitney U test. $^{**}P < 0.01$; $^{***}P < 0.001$.

ly the same, which suggests that the viruses affecting the two groups were different. Specifically, rhinovirus was isolated much more frequently in cases of asthma than first wheeze. This susceptibility could be caused, at least in part, by defective immune responses to rhinovirus infection in children with asthma [17, 18]. Nevertheless, a European birth cohort study (COPSAC) demonstrated that the total number of respiratory infections contracted in early childhood, but not a specific viral trigger, is associated with the subsequent development of asthma [19]. Another cohort study (COAST) suggested that the presence of asthma in adolescence is strongly associated with outpatient wheezing, rhinovirus infection, and aeroallergen sensitization [20]. These observations suggest that the triggers leading to susceptibility and exaggerated responses to lower respiratory tract infections require further investigation.

This is the first report to show different patterns of serum cytokine and chemokine production between first wheeze and asthma patients. Several studies have demonstrated a predominance of T helper 2 (Th2)-type cytokines, chiefly IL-4, IL-5, and IL-13 in bronchial biopsy specimens from patients with asthma [21–24]. The Th2 cytokine IL-9 is also involved in allergic responses in asthma, which suggests that it might be a suitable marker of atopic asthma [25–27]. These results agree with the findings of our study, which showed serum IL-4 and IL-9 were higher in acute asthma compared with first wheeze, and that IL-12 levels were lower. Furthermore, Rothers *et al.* demonstrated that Th2 cytokines such as IL-4, IL-5, and IL-13 adjust the production of IFN-γ from PBMCs in early life, which predicts the subsequent development of asthma [28]. In addition, epithelial and immune cells produce IL-8, a potent neutrophil chemoattractant and activator, in response to RS virus infection and replication [29, 30], and higher levels in the upper airways are associated with markers of severity of acute bronchiolitis and recruitment of IL-8 dependent neutrophils to the lungs [31]. In this study, peripheral neutrophil counts did not differ between cases of first wheeze and asthma; however, the mechanisms leading to elevated levels of IL-8 in cases of first wheeze might be similar to those in bronchiolitis caused by RS virus. A recent report showed that production of plasma chemokines such as IP-10, thymus- and activation-regulated chemokine (TRAC), and macrophage-derived chemokine (MDC) in early wheeze is associated with the development of atopic asthma [32]. In contrast, our results showed that compared with controls, serum IP-10 was elevated in both asthma and first wheeze groups, but there was no difference between the two groups. This result might have been caused by the different samples obtained, such as those from cases of convalescence stage of early-life wheezing. Indeed, our study also showed that there were no significant changes in IP-10 values between cases of convalescence stage of first wheeze and convalescence stage of asthma (data not shown). Collectively, these results indicate that IP-10 is closely related to viral infection [33–36]. Previously, we have shown that serum eosinophil cationic protein and IL-5 were higher in rhinovirus-induced acute asthma and

recurrent wheeze than in convalescence stage of asthma and recurrent wheeze [12]. These results suggest that the increased levels of IL-5 in acute exacerbations of asthma might be, at least in part, related to eosinophil activation although exact reasons for which IL-5 levels were increased both in first wheeze and in convalescence stage of first wheeze are unclear. The present study was limited in that it was an observational, retrospective study. Prospective studies are required to identify potential cytokine/chemokine markers for the development of asthma following first wheeze.

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DISCLOSURE STATEMENT

The authors declare that no financial or other conflicts of interest exist in relation to the contents of this article.

AUTHOR CONTRIBUTIONS

MK conducted the study design, collected samples, performed data analysis, wrote the first draft, and finalized the manuscript. SM collected samples, performed data analysis, and wrote the first draft. TK, KS, MN, ME, HT, KH, and YY collected samples and performed data analysis. HM interpreted the results. All authors read and approved the final manuscript.

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