Original article

Virus detection and cytokine profile in relation to age among acute exacerbations of childhood asthma

Masahiko Kato a, b, *, Kazuo Suzuki a, Yoshiyuki Yamada b, Kenichi Maruyama c, Yasuhide Hayashi d, Hiroyuki Mochizuki a

a Department of Pediatrics, Tokai University School of Medicine, Kanagawa, Japan
b Department of Allergy and Immunology, Gunma Children's Medical Center, Gunma, Japan
c Department of Nephrology, Gunma Children's Medical Center, Gunma, Japan
d Gunma Red Cross Blood Center, Gunma, Japan

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A B S T R A C T

Background: Little information is available regarding eosinophil activation and cytokine profiles in relation to age in virus-induced bronchial asthma. We therefore explored the association between age, respiratory viruses, serum eosinophil cationic protein (ECP), and cytokines/chemokines in acute exacerbations of childhood asthma.

Methods: We investigated viruses in nasal secretions from 88 patients with acute exacerbation of childhood asthma by using antigen detection kits and/or RT-PCR, followed by direct DNA sequencing analysis. We also measured peripheral eosinophil counts, and the serum levels of ECP and 27 types of cytokines/chemokines in 71 virus-induced acute asthma cases and 13 controls.

Results: Viruses were detected in 71(80.7%) of the 88 samples. The three major viruses detected were rhinoviruses, RS viruses, and enteroviruses; enteroviruses were found to be dominant in patients aged <3 years. There was no change in the levels of rhinoviruses and RS viruses between the two age groups, defined as children aged <3 years and children aged ≥3 years. Serum concentrations of ECP, IL-5, and IP-10 were significantly elevated in virus-induced acute asthma cases compared with controls. Serum ECP values were significantly higher in patients with virus-induced asthma at age ≥3 years compared with those aged <3 years. Among the 27 cytokines/chemokines, serum IP-10 was significantly higher in virus-induced asthma in patients <3 years than in those ≥3 years. Serum ECP and IL-5 production correlated significantly with age, whereas serum IP-10 showed an inverse correlation with age.

Conclusions: Age-related differences in cytokine profiles and eosinophil activation may be related to virus-induced acute exacerbations of childhood asthma.

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Introduction

Viral infection induces both the development and exacerbation of bronchial asthma. Accumulating evidence suggests that rhinovirus infection is a major cause of acute exacerbations of asthma in both adults and children. Kotaniemi-Syrjänä et al. showed that the most significant risk factor for the development of preschool childhood wheezing is the occurrence of symptomatic rhinovirus illness during infancy. The COAST (Childhood Origins of Asthma) study group also reported that wheezing attacks during childhood (2–16 years of age) can be linked to rhinovirus infection with atopy or eosinophilic airway inflammation.

Respiratory syncytial (RS) virus is another leading cause of serious lower respiratory tract infection in infants. RS virus infection exacerbates recurrent wheezing attacks in patients with...
established asthma. A number of case–control studies have established at least a statistical correlation between RS virus infection in infancy and the development of recurrent wheezing and asthma in young children (9–14 years old). However, RS virus infection appears unlikely to be a cause of atopic asthma later in life.\(^{9,14}\)

Heymann et al. found that patients of age <3 years with wheezing and positive tests for viruses showed a higher presence of RS virus compared with other viruses. In contrast, rhinovirus was dominant in children aged 3–18 years, suggesting that viral respiratory tract pathogens might differ at different ages in asthmatic children with acute exacerbation.\(^{15}\)

The purpose of this study was to investigate changes among the viruses detected, peripheral eosinophil counts, serum levels of eosinophil cationic protein (ECP), and several cytokines and chemokines in relation to age in cases of virus-induced acute exacerbation of childhood asthma.

**Methods**

**Patients and study setting**

We enrolled 88 subjects attending as outpatients or hospitalized with acute respiratory symptoms (55 boys, 33 girls, mean/median age 3.6/2.8 years) at the Gunma Children’s Medical Center between January 1, 2008 and December 31, 2013. All patients had a history of three or more different episodes of recurrent wheezing and documented evidence of wheezing by auscultation. Subjects with asthma were diagnosed according to the criteria of the Japanese guidelines.\(^{16}\) Briefly, a diagnosis of asthma was confirmed on the basis of a history of recurrent wheezing and dyspnea on at least three independent occasions, and reversible bronchoconstriction.\(^{16}\) Patients were prescribed short-acting β agonists and/or long-term controller medications. We excluded children with obvious bacterial infections, congenital heart diseases, and chronic lung diseases as well as those who showed the presence of a foreign body, had signs of severe infection, or were immunosuppressed, as these complications can interfere with the assessment of asthma-related outcome measures. The control group included 13 healthy children (8 boys, 5 girls, mean/median age 3.7/4.2 years) with no symptoms of wheezing at the time of examination. Exclusion criteria for the controls included immunosuppression, the presence of other respiratory tract symptoms, or a history of previous wheezing and asthma. Controls and patient cases were age- and sex-matched. This study was approved by the Ethics Committee of Gunma Children’s Medical Center. Informed consent was obtained from parents of patients and assent was obtained from the children if they were considered old enough (generally >9-year-old).

**Virus detection**

Nasal aspirates were obtained from 88 patients during acute exacerbations of asthma as previously reported.\(^{17}\) Nasal samples were then analyzed using antigen detection kits for 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.\(^{17}\) Some samples were tested by multiplex PCR (Seeplex RV15 OneStep ACE Detection kit, Seegene, Inc., Seoul, 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), as reported previously.\(^{18}\) The amplified PCR products were analyzed by automatic electrophoresis (MCE-202 MultiNA; Shimadzu, Kyoto, Japan).\(^{19}\)

**Fig. 1.** Virus detection in acute exacerbations of childhood asthma. Among the 88 samples from asthma exacerbation cases, rhinovirus was detected in 30; RS virus in 18; enterovirus in 9; human coronavirus in 2, human bocavirus in 1, influenza virus in 1, human parainfluenza virus in 1, ≥2 viruses in 9; and no viruses were found in 17.

**Fig. 2.** Monthly (A) and age-dependent (B) changes of virus detection in acute exacerbations of asthma. (A) Two-thirds of the patients were hospitalized or treated for asthma attacks between September and December. RS viruses were frequently detected from May to June and from November to December. Enteroviruses were dominant in September. In contrast, rhinoviruses were detected almost all year round. (B) More than half the patients with acute exacerbations of asthma were <3-year-old. Among the three major detected viruses, rhinovirus, RS virus, and enterovirus, enteroviruses were dominant in patients aged ≥3 years. However, there was no change in the levels of rhinoviruses and RS viruses between the two age groups.
Serum cytokines/chemokines and ECP

We measured peripheral eosinophil counts, and the concentrations of serum ECP and 27 cytokines/chemokines including [interleukin (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, and IL-17, interferon (IFN)-γ, IFN-γ-induced protein (IP)-10, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, monocyte chemoattractant protein 1, macrophage inflammatory protein (MIP)-1α, MIP-1β, eotaxin, regulated on activation normal T expressed and secreted chemokine, platelet-derived growth factor bb, fibroblast growth factor basic, and vascular endothelial growth factor] in samples from 71 patients with asthma who were not using systemic corticosteroids at the time of the examinations and from 13 control subjects. ECP contents in serum were determined with a fluoroenzyme immunoassay kit (Pharmacia, Uppsala, Sweden). Serum cytokines/chemokines were measured by the multi-cytokine detection system, Bio Plex (Bio-Rad, Hercules, CA, USA), following the manufacturer’s instructions, measured using a Luminex System (Luminex Corporation, Austin, TX, USA), and calculated using Bio-Plex software (Bio-Rad), as reported previously.17

Statistical analyses

Patient characteristics were evaluated by the Pearson χ² test statistic and Fisher’s exact test for categorical variables. Unpaired data were analyzed using the Mann–Whitney U test. Differences between more than three groups were analyzed by
the Kruskal–Wallis test followed by the Dunn’s multiple comparison test. Correlation coefficients for the parameters were calculated with Spearman rank correlation coefficient analysis. A statistically significant result was indicated by a value of $P < 0.05$ (two-sided). All analyses were performed using statistical software package SPSS for Windows (version 19.0, SPSS Japan Inc., Tokyo, Japan), or GraphPad Prism for Windows (version 5.0, GraphPad Software, San Diego, CA, USA) for multiple group comparisons.

**Results**

**Virus detection**

Of the 88 samples from child patients with acute exacerbations of asthma, rhinovirus was detected in 30 (34.1%); RS virus in 18 (20.5%); enterovirus in 9 (10.2%); human coronavirus in 2 (2.3%), human bocavirus in 1 (1.1%), influenza B in 1 (1.1%), human parainfluenza virus type 3 in 1 (1.1%), ≥2 viruses in 9 (10.2%); additionally, no viruses were detected in 17 (19.3%) (Fig. 1). The monthly changes in acute exacerbations of asthma are shown in Fig. 2A. Two-thirds of the patients were hospitalized or treated for asthma attacks from September through December. RS viruses were frequently detected from May to June and from November to December. Enteroviruses were dominant in September. In contrast, rhinoviruses were detected in almost all months of each year.

Next, we investigated the detection of viruses in relation to age. Patient characteristics are shown in Table 1. No significant differences in age or sex were found between asthma patients and controls. There was a significant difference in atopic status between asthma patients of age <3 years and those of age ≥3 years (Table 1).

More than half the patients with acute exacerbations of asthma were <3-year-old (Fig. 2B). Among the three major viruses detected, namely, rhinovirus, RS virus, and enterovirus, enterovirus was the most dominant in patients ≥3 years of age. However, there was no change in the levels of rhinoviruses and RS viruses between the two age groups (Fig. 2B).

**Serum cytokines/chemokines and ECP**

Concentrations of ECP, IL-5, and IP-10 were significantly elevated in virus-induced asthma cases compared with controls (Fig. 3). In contrast, only IL-1β was significantly lower in asthma cases than in controls (Fig. 3). We summarized the results of peripheral eosinophil counts, serum ECP and 27 types of cytokines/chemokines in Table 2. Furthermore, we evaluated the difference of virus-specific values of each cytokine/chemokine. As a result, in case of IL-5, enterovirus-induced asthma was greater than RS virus-induced asthma, in case of IP-10, RS virus-induced asthma was greater than enterovirus-induced asthma. The virus-dependent values of IP-10 [pg/ml, median (range)] were 14518 (594.7–6455.7) at rhinovirus-induced acute asthma, 21758 (576.9–13882.2) at RS virus-induced acute asthma, 795.4 (423.5–1656.4) at enterovirus-induced acute asthma, 2115.8 (982.5–9437.7) at other viruses-induced acute asthma, respectively. No significant correlation between each virus was observed in other cytokines/chemokines.

We then compared these values between the patients of age <3 years and those of age ≥3 years; the mean and median age of virus-induced asthma are 3.4 or 2.6 years, respectively. Serum ECP values were significantly higher in virus-induced asthma cases of age ≥3 years compared with those of age <3 years (Fig. 4). Of the 27 cytokines/chemokines, serum IP-10 was significantly higher in the presence of virus-induced asthma in patients <3-year-old than in

| Table 2 | Eosinophils, ECP, and cytokines/chemokines in acute asthma compared with control. |
|--------|----------------------------------------------------------------------------------|
|         | Asthma, All (n = 71)                                                                 |
| Eosinophils | 174 (0–2646)                                                                 |
| ECP     | 22.9 (3.0–1070)                                                                  |
| IL-1β   | 5.6 (0.0–52.7)                                                                  |
| IL-1αa  | 393.0 (137.0–4565.2)                                                             |
| IL-2    | 6.8 (0.4–248.8)                                                                  |
| IL-4    | 13.8 (3.5–151.5)                                                                 |
| IL-5    | 5.3 (0.1–52.8)                                                                   |
| IL-6    | 79.8 (6.8–893.8)                                                                 |
| IL-7    | 14.8 (1.5–42.8)                                                                  |
| IL-8    | 13.3 (0.2–236.1)                                                                 |
| IL-9    | 55.8 (5.4–2036.2)                                                                |
| IL-10   | 15.7 (0.1–145.4)                                                                 |
| IL-12   | 9.5 (0.9–10.7)                                                                   |
| IL-13   | 3.4 (0.1–3.7)                                                                    |
| IL-15   | 10.1 (0.0–71.3)                                                                  |
| IL-17   | 11.4 (0.0–217.9)                                                                 |
| IFN-γ   | 132.6 (3.5–570.1)                                                                |
| IP-10   | 1592.6 (423.5–13882.2)                                                           |
| TNF-α   | 20.5 (0.1–682.8)                                                                 |
| GM-CSF  | 33.5 (0.2–962.0)                                                                 |
| G-CSF   | 45.9 (4.2–1586.7)                                                                |
| MCP-1   | 48.2 (13.0–223.6)                                                                |
| MIP-1α  | 10.1 (1.5–46.8)                                                                  |
| MIP-1β  | 80.7 (6.0–228.4)                                                                  |
| Eotaxin | 77.9 (8.2–440.8)                                                                  |
| RANTES  | 7256.1 (3198.8–114340.4)                                                         |
| PDGF-bb | 5012.9 (433.3–14909.3)                                                           |
| FGF-basic | 73.3 (15.4–471.6)                                                                |
| VEGF    | 124.8 (5.2–729.1)                                                                |

1 Median (range); Mann–Whitney U test, *p < 0.05; **p < 0.01; ***p < 0.001 versus control; Dunn’s multiple comparison test, *p < 0.05, **p < 0.01, ***p < 0.001 versus control; p < 0.05, *p < 0.01 versus Asthma, ≥3 year. Eosinophil/mm$^3$; ECP: ng/ml; cytokines/chemokines: pg/ml.
Discussion

In this study, we found that two major viruses, rhinovirus and RS virus, detected in acute exacerbations of asthma, were not associated with age. However, the production of both serum ECP and IL-5 were correlated with age, and IP-10 production showed an inverse correlation with age.

Previous reports suggest that RS virus in wheezing children <3-year-old is the dominant infection compared with children 3–18 years old. Rhinovirus was more frequently detected in wheezing children aged 3–18 years compared with other viruses. However, in our study the frequency of rhinovirus and RS virus detection in acute exacerbations of asthma was almost the same between patients <3-year-old and those ≥3 years. In contrast to these two viruses, enterovirus was dominant in children aged ≥3 years, although this result might be influenced by the seasonal prevalence of the virus. Although the exact reasons for this conflicting result are unknown, it is possible that our methods of virus detection, including RT-PCR and sequence analysis, might be more sensitive than the methods used in previous reports.

Peripheral eosinophil counts and serum ECP levels were significantly higher in acute asthma in patients of age ≥3 years than in those <3-year-old. Furthermore, serum ECP and IL-5 levels, but not eosinophil counts, were significantly correlated with age. Notably, among the 27 cytokines and chemokines, only IP-10 was higher in acute asthma in patients <3-year-old than in those ≥3-year-old. In addition, IP-10 values in acute asthma subjects, but not in controls, were inversely correlated with age. These results are the first to show age-dependent cytokine/chemokine production, specifically IP-10, in acute asthmatic children.

IP-10 (CXCL10), a chemokine secreted from cells stimulated with type I and II IFNs and lipopolysaccharides, is a chemoattractant for activated T cells. IP-10 interacts with CXCR3, a receptor that is highly expressed in activated CD4+, CD8+, and natural killer T cells and stimulates directional migration of these cells. T cells expressing CXCR3 are usually associated with sites of Th1...
T cells recruited during viral infections are predominantly Th1 and are characterized by the production of a pattern of cytokines that includes IFN-γ and IL-2. IP-10 chemokine IP-10 attracts Th1 cells through interaction with CXCR3 and plays a prognostic role in viral infection. Although our results showed that IP-10 production in <3-year-old was significantly greater than that ≥3-year-old, there was no significant difference of IFN-γ production between two age groups. However, previous report showed IP-10 has been shown to be induced in bronchial epithelial cells infected by rhinovirus-16, with a close link to rhinovirus replication and through a mechanism that is not dependent on prior induction by either IFN-γ or the type I IFNs such as IFN-α and IFN-β. Further, IP-10 release might be triggered via double-stranded viral RNA reacting with Toll-like receptor 3, leading to the translocation of nuclear factor κB to the nucleus. Wark et al. showed that serum IP-10 levels in virus-induced acute asthma subjects aged 16–74 years are increased compared with non-virus-induced acute asthma cases, specifically rhinovirus infection. Our recent study also suggests that serum IP-10 is a novel marker of rhinovirus- and RS virus-induced acute exacerbations. Another report showed that the levels of plasma IP-10 during acute RS virus infection in infants are elevated compared with the convalescence period and controls. Although the present study could not specify which factor such as asthma, attack, or viral infection is most important on the IP-10 production, previous reports found that serum IP-10 was elevated by viral-induced acute exacerbations of asthma compared with stable asthma as well as acute non-virus-induced asthma. Further, serum IP-10 increased at exacerbations of patients with COPD by rhinovirus infection. These observations collectively suggest that IP-10 is a biomarker of virus infection.

Evidences suggest that attacks of wheezing that are induced by viral infection are one of the most exacerbation factors of asthma and related to hospitalization in preschool children. However, at the present time, there is no special recommendation of management for virus-induced asthma in the Japanese guidelines as well as the Global strategy for asthma management and prevention for children 5 years and younger, Global Initiative for Asthma (GINA). On the other hand, the former GINA report described that because exacerbations of asthma induced by viral infection are prolonged, early oral corticosteroids or 4 times dose inhaled corticosteroids as well as short-acting beta2-agonists should be recommended. Indeed, a randomized, placebo-controlled trials suggest that in preschool children with moderate to severe virus-induced wheezing/asthma, preemptive use of high-dose fluticasone reduced the use of rescue oral corticosteroids. Conversely, in preschool children with mild to moderate wheezing/asthma associated with a viral infection, oral prednisolone was not superior to placebo. Thus, the role of corticosteroids for virus-induced wheezing/asthma in younger children remains controversial. However, the diagnosis of virus-induced asthma due to high levels of IP-10 might have a benefit to how to treat and prevent the exacerbations of virus-induced childhood asthma. Previous reports showed age-related changes in cytokine responses in atopic children. Kawamoto et al. showed that in allergic diseases, such as atopic dermatitis and asthma, the Th1-dominant pattern changes to Th2 dominance in childhood by detecting IFN-γ and IL-4 production. Another report found that the production of IFN-γ as a Th1 cytokine and IL-4, IL-5, and IL-13 as Th2 cytokines were significantly related to age in both non-atopic and atopic children. Notably, non-atopic children <2 years of age were shown to have reduced Th2 responses compared with older, non-atopic children. On the other hand, IFN-γ response in atopic children were decreased when compared with non-atopic children in early childhood although the decreased IFN-γ response seen in early childhood did not persist beyond age 10 years. These results suggest that age-related changes in cytokine profile might be involved in the natural history of atopic disease during early childhood.
Although the exact reasons for the dependency of IP-10 production on age remain elusive, this could, at least in part, be related to Th1-dominant immune responses, such as virus infections, but not to Th2 immune responses including eosinophil activation. Furthermore, the balance of Th1 and Th2 cytokine/chemokine production on aging might also be involved in virus-induced acute exacerbations of childhood asthma.

Further work is needed to explore the mechanisms by which acute exacerbations of asthma occur and relationship between age and IP-10 production. These studies might ultimately lead to or prevent and/or treat the significant burden of asthma exacerbations caused by virus infections.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors’ contributions

MK conducted the study design, collected samples, performed data analysis, wrote first draft, and finalized the manuscript. KS, YY, and KM collected samples and performed data analysis. YH and HM interpreted the results. All authors read and approved the final manuscript.

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