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Laboratory and Epidemiology Communications

Detection of Various Respiratory Viruses in Patients with Influenza-Like Illness before and after Emergence of the 2009 Pandemic H1N1 Influenza Virus in Okinawa

Masaji Nakamura[1,2*], Katsuya Taira[1], Hiroyuki Tsukagoshi[3], Kiyomasa Itokazu[1], Minoru Nidaira[1], Shou Okano[1], Jun Kudaka[1], Masahiro Noda[4], Makoto Takeda[4], and Hirokazu Kimura[5]

[1]Okinawa Prefectural Institute of Health and Environment, Okinawa 901-1202;
[2]Okinawa Prefectural Chuo Meat Inspection Center, Okinawa 901-1202;
[3]Gunma Prefectural Institute of Public Health and Environmental Sciences, Gunma 371-0052; and
[4]Department of Virology III and [5]Infectious Disease Surveillance Center,
National Institute of Infectious Diseases, Tokyo 208-0011, Japan

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The 2009 pandemic H1N1 influenza virus (AH1 pdm) emerged suddenly in Mexico and spread rapidly throughout the world, including Japan (1). Seasonal influenza was prevalent from November to March in Okinawa before the emergence of AH1 pdm, whereas large numbers of patients with influenza-like illness (ILI) were reported from May to December in 2009 (Fig. 1). We therefore decided to analyze the trends of the various respiratory viruses before and after the emergence of an unexpected, pandemic respiratory virus such as AH1 pdm. Herein we report on the variation of the respiratory viruses detected in patients with ILI before and after the emergence of AH1 pdm.

*Corresponding author: Mailing address: Department of Biological Sciences, Okinawa Prefectural Institute of Health and Environment, 2085 Ozato, Nanjo-shi, Okinawa 901-1202, Japan. Tel: +81-98-945-0785, Fax: +81-98-945-9366, E-mail: nakamuma@pref.okinawa.lg.jp

Nasopharyngeal swabs were obtained from 1,089 patients with ILI from January to December 2009. All patients were residents of Okinawa Prefecture, Japan. Viral RNA was purified using a commercial kit (QIAamp Viral RNA Mini kit; Qiagen, Valencia, Calif., USA), then suspended in DNase/RNase-free water. Initially we attempted to detect AH1pdm and seasonal influenza viruses (subtypes AH1, AH3, and B) using real-time RT-PCR (2) or conventional RT-PCR (3), as described previously. When the ILI sample was negative for influenza virus, we attempted to detect other viruses using RT-PCR methods for respiratory syncytial virus (RSV) (4), human parainfluenza virus (HPIV) (5), human metapneumovirus (HMPV) (6), enteroviruses (EV) (7,8), and human rhinovirus (HRV) (7,8). All amplicons were confirmed by agarose gel electrophoresis. Adenoviruses were confirmed by cell culture and immun-chromatographic methods.

The number of ILI patients in Okinawa peaked in January, August, and December 2009 (Fig. 1). The age

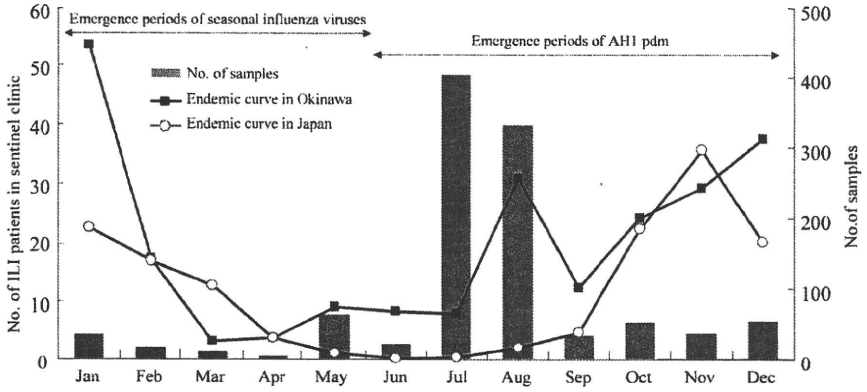

Fig. 1. The endemic curve of influenza-like illness (ILI) and monthly sample numbers in the present study.

Table 1. Monthly detection of the various viruses in the present study

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total (%)
AH1	15	2											17 (1.6)
AH3	4				42	10	31						87 (8.0)
B	1	1			9	4							15 (1.4)
AH1pdm						1	292	293	27	43	36	45	737 (67.7)
HRV					1		3	3		3			10 (0.9)
RSV		1	1				5	2					9 (0.8)
HMPV			2				2	1					5 (0.5)
HPIV		2	1		1		1						5 (0.5)
AdV	8	7	4	1				1		1			22 (2.0)
EV	1	1					4						6 (0.6)
Total no. of InfV	20	3			51	15	323	293	27	43	36	45	856 (78.6)
Total no. of non-InfV	9	11	8	1	2		15	7		4			57 (5.2)
Not detected	9	5	5	5	13	8	67	33	9	9	3	10	176 (16.2)
Total	38	19	13	6	66	23	405	333	36	56	39	55	1,089

AH1, influenza virus subtype AH1; AH3, influenza virus subtype AH3; B, influenza virus subtype B; InfV, influenza virus; AH1pdm, 2009 pandemic H1N1 influenza virus; HRV, human rhinovirus; RSV, repiratory syncytial virus; HMPV, human metapneumovirus; HPIV, human parainfluenza virus; AdV, adenovirus; EV, enterovirus.

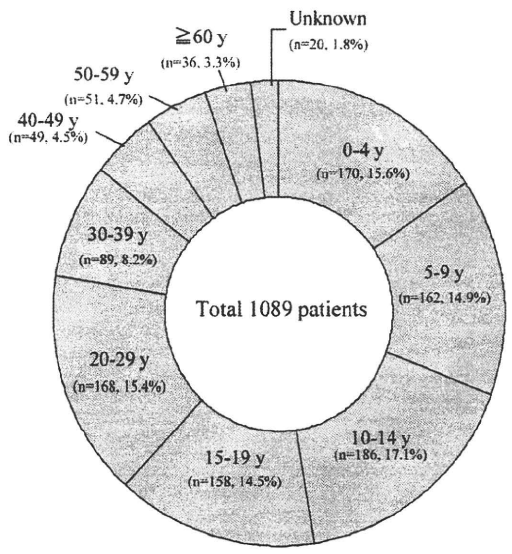

Fig. 2. The age groups of the patients who provided the samples collected in the study.

groups of the patients from whom samples were obtained, over 50% of whom were under 20 years of age, are shown in Fig. 2. The monthly detection rates of the various viruses are shown in Table 1. AH1 pdm and seasonal influenza viruses were detected in about 80% of patients, with AH1 pdm (68%) being the most prevalent virus detected. Non-influenza respiratory viruses were detected in 5% of patients, although none of the viruses tested were detected in about 16% of patients. Seasonal influenza viruses (subtype AH1, AH3, and B) were the most prevalent between January and June. AH1 pdm, together with AH3 and other viruses, was first detected in July, after which AH1 pdm was predominant. Non-influenza viruses were detected from January to October. Our previous report suggested that RSV circulated in Okinawa during the summer, whereas in other regions of Japan RSV is detected in autumn and winter (9). In addition, the endemic curve of ILI in Okinawa Prefecture in 2009 differed from the pattern observed in other regions of Japan (Fig. 1). A possible reason for this could be that Okinawa is located in a subtropical region, thus suggesting that the epidemiology of some respiratory viruses in Okinawa may be unique (10). Our findings suggest that the ILI outbreak and AH1 pdm occurred together with other non-influenza viruses, with non-influenza viruses being

detected throughout the year. The epidemiology of the various respiratory viruses in Okinawa is not currently known. However, these findings show the sustained impact of non-influenza viruses on ILI even during the outbreak of the newly emerged pandemic influenza virus, and provided useful information to allow clinicians to make an accurate diagnosis. Further epidemiological studies based on systematic virus surveillance may, however, still be needed.

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Conflict of interest None to declare.

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Laboratory and Epidemiology Communications

Detection of Human Metapneumovirus Genomes during an Outbreak of Bronchitis and Pneumonia in a Geriatric Care Home in Shimane, Japan, in Autumn 2009

Tamaki Omura, Setsuko Iizuka, Kenji Tabara, Hiroyuki Tsukagoshi[1], Katsumi Mizuta[2], Shunji Matsuda[3], Masahiro Noda[4], and Hirokazu Kimura[1,5]*

Shimane Prefectural Institute of Public Health and Environmental Science, Shimane 690-0122;
[1]Gunma Prefectural Institute of Public Health and Environmental Sciences, Gunma 371-0052;
[2]Yamagata Prefectural Institute of Public Health, Yamagata 990-0031;
[3]National Hospital Organization Ehime National Hospital, Ehime 791-0281; and
[4]Department of Virology III and [5]Infectious Disease Surveillance Center,
National Institute of Infectious Diseases, Tokyo 208-0011, Japan

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Human metapneumovirus (HMPV), which belongs to the family *Paramyxoviridae*, genus *Metapneumovirus*, is an important causative agent of acute respiratory infections (ARIs) in humans (1). Despite this, the molecular epidemiology of HMPV in Japan is not well understood. We described herein an outbreak of HPMV infection in a geriatric care home in Shimane, Japan in autumn 2009 and the results of genetic analyses of the HMPV detected in samples obtained from residents of this home. An epidemiological investigation in late September 2009 found that 2 of the 99 residents of this home exhibited symptoms such as high fever (>38°C), cough, and inflammation of the lower respiratory tract. Other residents were identified with similar symptoms up until late October 2009. The overall prevalence during this outbreak was around 30% (27/99 persons), although the infection route could not be determined. Nine throat swab samples were collected from these patients after obtaining verbal informed consent and attempts made to detect and/or isolate influenza virus subtype A, human rhinovirus, enteroviruses, respiratory syncytial virus, parainfluenza viruses, and/or adenoviruses using previously reported reverse transcriptase-polymerase chain reaction (RT-PCR) and cell culture methods (Vero E6, RD, MDCK, and HEp-2 cells) (2–5). Viral nucleic acid was extracted from the samples using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) and suspended in DNase/RNase-free water. After RNA extraction, RT-PCR was performed as described previously (6,7). Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Germantown, Md., USA) and the nucleotide sequences were determined by direct sequencing (6). Phylogenetic analysis based on the fusion (*F*) gene of HMPV strains was then performed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 4 (8). Evolutionary distances were estimated using Kimura's two-parameter method and a phylogenetic tree was constructed using the neighbor-joining method (9,10). The reliability of the tree was estimated on the basis of 1,000 bootstrap replications.

A summary of patient and viral data is shown in Table 1. HMPV was detected in samples from 7 patients; no other viruses were detected. In addition, serum IgG against HMPV was detected in 2 patients using an indirect immunofluorescence assay (11), with significantly higher levels being found in the convalescent phase. Nucleotide sequence analysis of different HMPV genes, with *F* gene being the most common, allows the virus to be divided into two major genetic groups (A and B) and four subgroups (A1, A2, B1, and B2) (12,13). The phylogenetic tree determined here showed that all strains detected in the patient samples were clustered in subgroup B2 (Fig. 1). The nucleotide identity among the present strains was 100%, with a nucleotide identity of 99.7% with respect to the Yamaguchi 09-15 strain detected in Yamaguchi Prefecture during the same season. A very recent study suggested that HMPV subgroups A2 and B2 are the major types circulating in Japan (14). Indeed, subgroups A2, B2, and B1 were found in 3, 4, and 2 strains, respectively, of the 9 HMPV strains detected by the sentinel surveillance system for viral diseases in Shimane Prefecture from March 2009 to January 2010. Furthermore, a high degree of nucleotide identity (98.7–100%) was seen between the subgroup B2 strains.

It is suggested that HMPV infection mainly occurs in children, although recent reports indicate that outbreaks of HMPV infection also occur in the elderly (15). Indeed, a similar outbreak to the present case occurred in another geriatric care home in Japan (16). However, despite these occurrences, the epidemiology of HMPV infection still remains unclear. A high prevalence (around 30%) of HMPV infection was seen in the present study, with some patients presenting with severe infections such as pneumonia. HMPV infection should therefore be considered in outbreaks among elderly peo-

*Corresponding author: Mailing address: Infectious Disease Surveillance Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan. Tel: +81-42-561-0771, Fax: +81-42-565-3315, E-mail: kimhiro@nih.go.jp

Table 1. Patient and human metapneumovirus data

Patient	Age (y)	Sex	Diagnosis	Onset date	Sampling date	Strain	Subgroup	GenBank accession no.
1	65	F	Pneumonia	14 Oct. 2009	23 Oct. 2009	Shimane 09-17	B2	AB594742
2	55	M	Pneumonia	16 Oct. 2009	23 Oct. 2009	Shimane 09-18	B2	AB594743
3	69	M	Fever, Cough	17 Oct. 2009	23 Oct. 2009	Shimane 09-16	B2	AB594741
4	56	F	Fever, Cough	20 Oct. 2009	23 Oct. 2009	Shimane 09-15	B2	AB594740
5	65	F	Pneumonia	21 Oct. 2009	23 Oct. 2009	Shimane 09-19	B2	AB594744
6	56	F	Fever, Cough	22 Oct. 2009	23 Oct. 2009	Shimane 09-14	B2	AB594739
7	61	M	Fever, Cough	29 Oct. 2009	30 Oct. 2009	Shimane 09-20	B2	AB594745

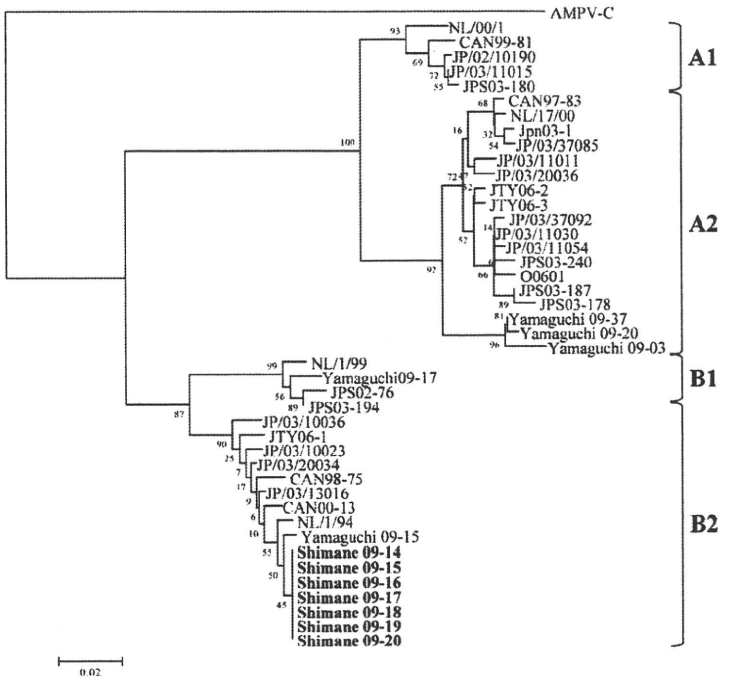

Fig. 1. Phylogenetic tree constructed on the basis of partial sequences of the human metapneumovirus *F* gene. Distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining method. Numbers above the branches are bootstrap probabilities (%). Reference strains were NL/00/1 (AF371337), CAN99-81 (AY145294), JP/02/10190 (AB113377), JP/03/11015 (AB113372), JPS03-180 (AY530092), CAN97-83 (AY145296), NL/17/00 (AY304360), Jpn03-1 (AB503857), JP/03/37085 (AB119485), JP/03/11011 (AB113371), JP/03/20036 (AB126612), JTY06-2 (EU127918), JTY06-3 (EU127919), JP/03/37092 (AB119486), JP/03/11030 (AB119489), JP/03/11054 (AB119491), JPS03-240 (AY530095), O0601 (EF589610), JPS03-187 (AY530093), JPS03-178 (AY530091), Yamaguchi 09-37 (AB533251), Yamaguchi 09-20 (AB533245), Yamaguchi 09-03 (AB533239), NL/1/99 (AY304361), Yamaguchi09-17 (AB533244), JPS02-76 (AY530089), JPS03-194 (AY530094), JP/03/10036 (AB126611), JTY06-1 (EU127917), JP/03/10023 (AB126608), JP/03/20034 (AB119493), CAN98-75 (AY297748), JP/03/13016 (AB126607), CAN00-13 (AY145298), NL/1/94 (AY304362), and Yamaguchi 09-15 (AB533243). Avian metapneumovirus type C (AMPV-C, AY579780) was also included as an outgroup.

ple with severe ARIs.

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Conflict of interest None to declare.

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Laboratory and Epidemiology Communications

Sequencing and Phylogenetic Analyses of Saffold Cardiovirus (SAFV) Genotype 3 Isolates from Children with Upper Respiratory Infection in Gunma, Japan

Hiroyuki Tsukagoshi*, Yutaka Masuda[1], Tetsuya Mizutani[2], Katsumi Mizuta[3], Mika Saitoh, Yukio Morita, Atsuyoshi Nishina[4], Kunihisa Kozawa, Masahiro Noda[5], Akihide Ryo[6], and Hirokazu Kimura[7]

Gunma Prefectural Institute of Public Health and Environmental Sciences, Gunma 371-0052;
[1]Masuda Pediatric Clinic, Gunma 370-0321; [2]Department of Virology I,
[5]Department of Virology III, and [7]Infectious Disease Surveillance Center,
National Institute of Infectious Diseases, Tokyo 208-0011;
[3]Yamagata Prefectural Institute of Public Health, Yamagata 990-0031;
[4]Yamagata Prefectural Yonezawa Women's Junior College, Yamagata 992-0025; and
[6]Department of Molecular Biodefence Research, Yokohama City University Graduate School of Medicine, Kanagawa 236-0004, Japan

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Saffold cardiovirus (SAFV) is a newly discovered virus that belongs to family *Picornaviridae* and genus *Cardiovirus* (1). Recent studies suggest that SAFV is relatively common in children with various diseases, with PCR-positive rates varying from 0.5–12% (2–6). For example, SAFV has been detected in stool samples from children with respiratory tract infections, gastroenteritis, and non-polio acute flaccid paralysis cases (2–6). Recently, we showed that SAFV genotype 2 was associated with typical exudative tonsillitis in Japanese children (7). However, as this virus is also detected in asymptomatic humans, the pathogenicity of SAFV is not yet clearly understood (5). In addition, Zoll et al. suggested that different SAFV strains may also exhibit different in vitro tropisms (6), thus suggesting that it may be relatively difficult to isolate SAFV using culture methods, which may explain why few studies have been conducted to date on its isolates (6). Herein we report the isolation of two strains of SAFV genotype 3 from children with acute respiratory infections, and the results of sequence and phylogenetic analyses (*VP1* coding region) of these strains.

We analyzed nasopharyngeal samples from two male patients, aged 5 and 6 years, who presented with fever (>38°C), canker sores, and upper respiratory inflammation. A pediatrician had diagnosed upper respiratory infection (URI) with sinusitis, pharyngitis, and laryngitis (8). The samples were obtained by the local health authority of Gunma Prefecture in 2008 for the surveillance of viral diseases in Japan. Informed consent was obtained from the parents of both subjects for donation of the nasopharyngeal samples used in this analysis.

The throat swab samples were centrifuged at 3,000 × g at 4°C for 30 min and the supernatants used for virus isolation. Cell lines (HEp-2, Vero, MDCK, A549, and RD18s cells) were inoculated and subjected to three freeze/thaw cycles.

The observation of enterovirus-like cytopathic effects (CPEs), such as collapsed balloons and small fragmented cells floating in the medium inoculated with HEp-2 cells (Fig. 1), led us to initially suspect another virus such as an enterovirus. However, we failed to amplify the *VP1* and *VP4* coding region of the hypothetical enterovirus in culture supernatants by RT-PCR with broad detectable primers (9,10). Next, we carried out a

*Corresponding author: Mailing address: Gunma Prefectural Institute of Public Health and Environmental Sciences, 378 Kamioki-machi, Maebashi-shi, Gunma 371-0052, Japan. Tel: +81-27-232-4881, Fax: +81-234-8438, E-mail: tsuka-hiro@pref.gunma.jp

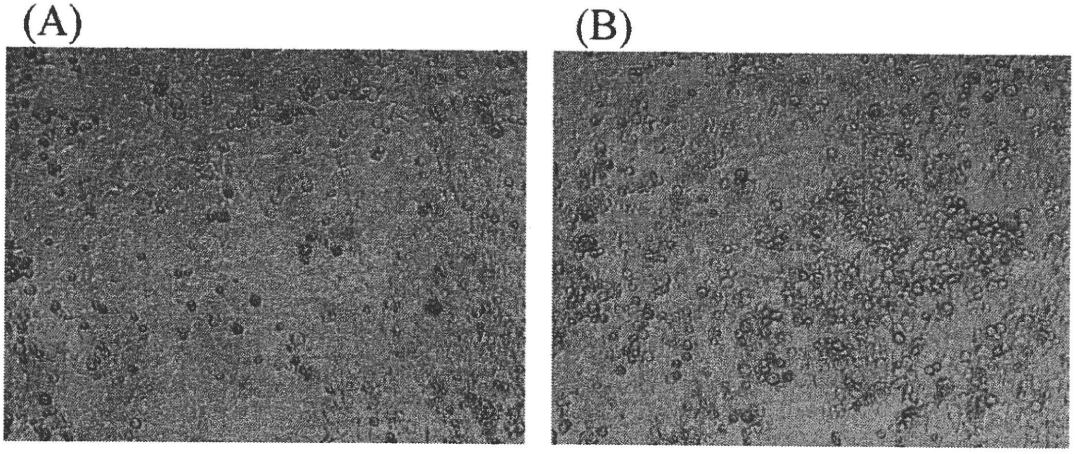

Fig. 1. Cytopathic effects of Saffold cardiovirus (SAFV) type 3 in HEp-2 cells. (A) No infection. (B) SAFV type 3 infected-HEp-2 cells. Images were taken following three passages after specimen (throat swab) inoculation.

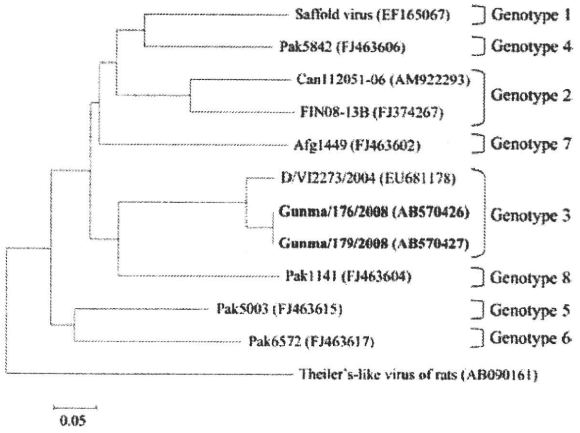

Fig. 2. A phylogenetic tree of SAFV type 2 based on the *VP1* coding region (327 nt). The phylogenetic tree was constructed using the *VP1* coding region. Numbers in parentheses are the GenBank accession numbers.

comprehensive sequencing (using the RDV method) for viral RNA determination, as described previously (11). The partial viral nucleotide sequences obtained upon analysis of the sequence data with BLAST (11) suggested SAFV. Finally, we analyzed the *VP1* coding region of SAFV, as described previously (7). Briefly, viral RNA was extracted from 140 μL of the supernatants using a QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., USA) and the RT-PCR procedure performed according to the manufacturer's instructions (One-step RT PCR kit; Qiagen). The primers for RT-PCR (for amplification of the *VP1* coding region) were as follows: 5′-HAA RCA RGR YTG GAR YTT YNT NAT GTT-3′ (primer 315F) and 5′-DGG BCK DGG RCA RWA VAC YCT CAT-3′ (primer 738R) (7). The following protocol was used: incubation for 30 min at 50°C, 15 min at 95°C, followed by 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, ending with elongation for an additional 5 min at 72°C. The DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen) and the nucleotide sequence determined using an automated DNA sequencer (7). Amplicons were sequenced and aligned (327 bp), then analyzed phylogenetically using the CLUSTAL W program on the website of DNA Data Bank of Japan (DDBJ; http://hypernig.nig.ac.jp/homology/clustalw-e.shtml) and TreeExplorer (version 2.12) (http://evolgen.biol.metro-u.ac.jp/TE/). Evolutionary distances were estimated using Kimura's two-parameter method and the phylogenetic trees were constructed using the neighbor-joining (N-J) method (12). The reliability of the tree was estimated using 1,000 bootstrap replications.

Phylogenetic analysis based on the *VP1* coding region revealed two strain clusters in the SAFV genotype 3 (Fig. 2), with an interstrain nucleotide identity of 100%. The high diversity of SAFV is highlighted by the identification of at least eight *VP1* coding regions (5). No pathogen other than SAFV was isolated using the cell-culture method.

Although a wide range of viruses is known to cause respiratory infections, the etiology of 20–30% of these currently remains unidentified (13). This may be due to the lack of sensitivity of some of the detection methods used, and it has also been suggested that unrecognized infectious agents, including other viruses, have yet to be discovered (14). Viral diagnosis is mostly based on clinical symptoms; thus, it is important to collect appropriate clinical samples early in the infection process, and the type of sample collected is somewhat dependent on the clinical symptoms. In this study, we succeeded in isolating SAFV from the throat swabs of two URI patients, thus we suppose that the fever and URI were associated with SAFV. Using the tissue culture method, we isolated 67 viruses from 124 samples taken during 2008 in Gunma Prefecture for the surveillance of viral diseases. Although 39 strains of influenza virus were isolated, only two of these were SAFV. This could suggest that SAFV is seldom the cause of URI, although much larger numbers of strains will be required to address its epidemiology more comprehensively.

In conclusion, SAFV might be a causative agent of acute respiratory infection. A previous report has suggested that SAFVs are ubiquitous human viruses with a global prevalence, therefore SAFV might be the cause in samples with enterovirus-like CPEs (6). The growth characteristics of our strains are similar to those seen

previously in other slow-growing SAFVs (5), and the HEp-2 cell line has been shown to be an effective cell line for further research into SAFV, including clarification of its epidemiology and etiology, although blind passages may be necessary to isolate the virus (15). A more detailed analysis, including serological surveys of SAFV, is needed to determine its exact role in human disease.

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Conflict of interest None to declare.

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Different cytokine profile and eosinophil activation are involved in rhinovirus- and RS virus-induced acute exacerbation of childhood wheezing

Kato M, Tsukagoshi H, Yoshizumi M, Saitoh M, Kozawa K, Yamada Y, Maruyama K, Hayashi Y, Kimura H. Different cytokine profile and eosinophil activation are involved in rhinovirus- and RS virus-induced acute exacerbation of childhood wheezing.
Pediatr Allergy Immunol 2010.

Because little information is available on eosinophil activation and cytokine response in virus-induced wheezing, we attempted to detect respiratory viruses and measure eosinophil cationic protein (ECP), and 27 types of cytokines/chemokines in both serum and nasal secretions from children with wheezing. This study was an observational, case–control investigation of 267 subjects, who were visited and/or hospitalized with acute respiratory symptoms (with wheezing: men, 115; women, 59; mean/median age, 3.6/3.0 years) or who were visited for regular physical examination and treatment (non-symptomatic wheezing: men, 48; women, 31; mean/median, 5.0/4.7 years), and 14 control subjects (controls: men, 9; women, 5; mean/median, 3.6/3.7 years). We detected viruses in nasal secretions from 174 patients with acute exacerbations of wheezing using antigen detection kits or reverse transcription-polymerase chain reaction, followed by direct DNA sequencing analysis. We measured peripheral eosinophil counts, and serum concentrations of ECP and 27 cytokines/chemokines using a multiplex bead-based assay in patients with wheezing or non-symptomatic wheezing. We also examined nasal ECP and 27 cytokines/chemokines in patients with wheezing. Of 174 samples from wheezing exacerbations, rhinovirus was detected in 59; respiratory syncytial (RS) virus in 44; enterovirus in 17; other viruses in 19; and no viruses in 35. Serum concentrations of ECP, IL-5, IL-6, IL-1ra, and IP-10 were significantly elevated in rhinovirus-induced wheezing compared with non-symptomatic wheezing. Similarly, serum ECP, IL-5, and IP-10 were significantly higher in rhinovirus-induced wheezing than in controls. On the other hand, IL-1ra and IP-10, but not ECP and IL-5 were significantly higher in RS virus-induced wheezing than in controls. Furthermore, only IL-5 was significantly elevated in the rhinovirus group compared with the RS virus group in both serum and nasal secretions. Different cytokine profile and eosinophil activation might be involved in rhinovirus- and RS virus-induced acute exacerbation of childhood wheezing.

Masahiko Kato[1,2], Hiroyuki Tsukagoshi[2], Masakazu Yoshizumi[2], Mika Saitoh[2], Kunihisa Kozawa[2], Yoshiyuki Yamada[1], Kenichi Maruyama[3], Yasuhide Hayashi[4], and Hirokazu Kimura[5]

[1]Departments of Allergy and Immunology, Gunma Children's Medical Center, Shibukawa, Gunma, Japan, [2]Gunma Prefectural Institute of Public Health and Environmental Sciences, Maebashi, Gunma, Japan, [3]Internal Medicine, Gunma Children's Medical Center, Shibukawa, Gunma, Japan, [4]Hematology and Oncology, Gunma Children's Medical Center, Shibukawa, Gunma, Japan, [5]National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan

Key words: eosinophils; rhinovirus; cytokines; wheezing; asthma

Masahiko Kato, Department of Allergy and Immunology, Gunma Children's Medical Center, 779 Shimohakoda, Hokkitsu-machi, Shibukawa, Gunma 377-8577, Japan
Tel.: +81 279 52 3551
Fax: +81 279 52 2045
E-mail:mkato@gcmc.pref.gunma.jp

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Abbreviations: ECP, eosinophil cationic protein; IL, interleukin; IFN, interferon; IP, interferon-γ-induced protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; RS, respiratory syncytial; PI, parainfluenza.

Respiratory infections can cause wheezing illnesses in children of all ages and can also influence the causation and disease activity of

asthma (1). For years, it has been recognized that respiratory syncytial (RS) virus infections often produce the first episode of wheezing in children who go on to develop chronic asthma (1). Furthermore, respiratory viral infections are detected in the majority of asthma exacerbations in both children (80–85%) (2) and adults (75–80%) (3), and of these, approximately 60% are rhinoviruses. However, at the present time, there is no specific treatment for virus-induced exacerbations of wheezing/asthma, and the precise mechanisms responsible are still unclarified.

Two studies compared responses to experimental rhinovirus infections in asthmatic or allergic rhinitic subjects and normal subjects, and both implicated an increased bronchial eosinophil infiltrate in the pathogenesis of virus-induced exacerbations of asthma (4, 5). Eosinophils are important effector cells in host defense against parasites in allergic diseases such as bronchial asthma (6). In allergic inflammation, mediators released from epithelial cells or inflammatory cells induce migration of eosinophils from the blood into affected tissues. After migration, eosinophils are activated by appropriate stimuli resulting in the release of inflammatory mediators, including arachidonic acid metabolites such as platelet-activating factor, or cystenyl leukotrienes, oxygen radicals, and toxic cationic granule proteins such as major basic protein, eosinophil peroxidase, eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (6). Eosinophils also play a critical role on airway remodeling (7).

The aims of this study were to assess the occurrence of viral infections in acute exacerbations of wheezing and to evaluate the association of these viral findings with cytokine/chemokine profiles and eosinophil activation in wheezing children.

Methods

Patients and study setting

This study was an observational, case–control investigation of 267 subjects, who were visited and/or hospitalized with acute respiratory symptoms (with wheezing: men, 115; women, 59; mean/median age, 3.6/3.0 years) or who were visited for regular physical examination and treatment (non-symptomatic wheezing: men, 48; women, 31; mean/median, 5.0/4.7 years), and 14 control subjects (controls: men, 9; women, 5; mean/median, 3.6/3.7 years) at the Gunma Children's Medical Center from November 1, 2003, through October 31, 2006.

All recruited patients had a history of three or more different episodes of recurrent wheezing and documented evidence of wheezing by auscultation. The subjects with asthma were diagnosed according to the criteria of the National Institutes of Health (8). In this study, we analyzed the patients as wheezing children including recurrent wheezing and asthma because the diagnosis of asthma in children < 5 years old presents particularly difficult problems (8). Also, studies of the natural history asthma have established that in almost 80% of cases, it begins during the first 6 years of life (9). We excluded children with congenital heart disease, cystic fibrosis, or other chronic lung disease, presence of foreign body or signs of severe infection or who were immunosuppressed that could interfere with the assessment of wheezing/asthma-related outcome measures. An acute exacerbation of wheezing/asthma was diagnosed by the emergency department physician based on the presence of wheezing with increased difficulty of breathing and had a history of a cold before the onset of the exacerbation. The criterion of 'non-symptomatic wheezing' patients was defined as physician-diagnosed wheezing/asthma and is currently stable at least 3 months before and at the timing of examinations. The patients were prescribed short-acting β2 agonists and/or long-term controller medications. The control group included 14 healthy children with no symptoms of wheezing at the time of investigation. Exclusion criteria for the controls included immunosuppression, the presence of other respiratory tract symptoms or a history of previous wheezing and asthma. This study was approved by the Ethics Committee of Gunma Children's Medical Center. Informed consent was obtained from parents, and assent was obtained from older children (usually over 9 years old).

Virus detection

Nasal aspirates were obtained from 174 patients during acute exacerbation of wheezing. However, we could not take these samples from patients with non-symptomatic wheezing patients and controls. Nasal secretions were aspirated into a mucus trap (attached to wall suction) by inserting the tip of a flexible 5F Argyle suction catheter (Nippon Sherwood, Tokyo, Japan) into the anterior nares. Nasal samples were analyzed by antigen detection kits (Becton Dickinson, Franklin Lakes, NJ, USA) for RS virus, influenza types A and B, and adenoviruses. The remaining secretions were frozen at −80°C until examination

by further reverse transcription-polymerase chain reaction (RT-PCR), and then by direct DNA sequencing analysis. Viral RNA was purified from nasopharyngeal samples diluted in virus transport medium. The samples were centrifuged at 3000 *g* at 4°C for 30 min. RNA extraction from samples was performed using a commercial RNA isolation procedure (QIAamp viral RNA purification kit; QIAGEN, Hilden, Germany). RNA solution was treated with DNase I (Takara, Tokyo, Japan), and the reverse transcription reaction mixture was incubated with random hexamers at 42°C for 90 min, followed by incubation at 99°C for 5 min, and then amplification by thermal cycling. The RT-PCR method for rhinoviruses and enteroviruses, developed by Blomqvist et al., was used for rhinovirus and enterovirus identification (10). The primers for RT-PCR were as follows: 5′-GAA ACA CGG ACA CCC AAA GTA-3′ (human rhinovirus primer 1; sense) and 5′-TCC TCC GGC CCC TGA ATG-3′ (human rhinovirus primer 2; antisense). The PCR protocol was as follows: incubation for 3 min at 94°C, followed by 40 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min, followed by an additional 7 min for elongation at 72°C after the last cycle. The RT-PCR method used for RS virus and parainfluenza (PI) virus types 1, 2, and 3 is as previously described with minor modifications (11). The primers for RT-PCR were as follows: 5′-GGG AGA GGT GGC TCC AGA ATA CAG GC-3′ (RS virus N3; sense), 5′-AGC ATC ACT TGC CCT GAA CCA TAG GC-3′ (RS virus N5; antisense), 5′-TCT GGC GGA GGA GCA ATT ATA CCT GG-3′ (PI virus type 1 PR3; sense), 5′-ATC TGC ATC ATC TGT CAC ACT CGG GC -3′ (PI virus type 1 PR5; antisense), 5′- AAC TAT GTC CAG AGG AGA GGT GCT GG -3′ (PI virus type 2 PR3; sense), 5′-CCA TGC CTG CAT AAG CAC ACT GTA GC -3′ (PI virus type 2 PR5; antisense), 5′-ACC AGG AAA CTA TGC TGC AGA ACG GC-3′ (PI virus type 3 PR3; sense), and 5′-GAT CCA CTG TGT CAC CGC TCA ATA CC-3′ (PI virus type 3 PR5; antisense). To avoid cross-contamination in RT-PCR, procedures for the extraction of viral RNA were carried out in a room physically separated from that used for performing RT-PCR. Furthermore, positive and negative controls were included in all PCR assays. The size of the amplified DNA fragment was determined by electrophoresis on 3% agarose gel. The DNA fragments were purified using a QIAquick PCR Purification kit (QIAGEN), and the nucleotide sequence was determined with an automated DNA sequencer ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems). For identification of the virus, newly determined sequences were compared with those available in the sequences using the GenBank DNA databases (http://www.ncbi.nlm.nih.gov) and the standard nucleotide–nucleotide BLAST algorithm. The identities of the sequences were determined on the basis of the highest percentage of total nucleotide match in GenBank.

Serum and nasal cytokines/chemokines and eosinophil cationic protein (ECP)

We measured peripheral eosinophil counts and the concentrations of serum ECP and 27 cytokines/chemokines [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, IL-17, interferon (IFN)-γ, IFN-γ-induced protein (IP)-10, tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, eotaxin, regulated upon activation, normal T expressed and secreted (RANTES), platelet-derived growth factor (PDGF)-bb, fibroblast growth factor (FGF) basic, and vascular endothelial growth factor (VEGF)] from 79 patients with wheezing and 79 non-symptomatic wheezing patients who had not used systemic corticosteroids at the timing of the examinations, and 14 control subjects. ECP contents in serum and nasal secretions were measured with a fluoroenzyme immunoassay kit (Pharmacia, Uppsala, Sweden). Serum or nasal cytokines/chemokines were determined by the multi-cytokine detection system, Bio-Plex (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions, measured using a Luminex System, Bio-Plex Pro Assay (Austin, TX, USA), and calculated using Bio-Plex software (Bio-Rad).

Statistical analyses

Patient characteristic data were evaluated by the Pearson X^2 test statistic and Fisher's exact test for categorical variables. Multivariate analyses were conducted by using multivariate linear regression or multivariate logistic regression analysis, allowing assessment simultaneously for each factor from patients with acute wheezing group, non-symptomatic wheezing group, or controls. The age, sex, and atopic status were

potential confounders, and adjustments were made for the multivariate analyses. 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 by using Spearman rank correlation coefficient analysis. A statistically significant result was defined by a value of $p < 0.05$ (two-sided). All analyses were performed using a statistical software package (SPSS for Windows, version 14.0; SPSS Japan Inc., Tokyo, Japan; or for multiple group comparison, GraphPad Prism for Macintosh, version 4.0, GraphPad Software, San Diego, CA, USA).

Results

Virus detection

Patient characteristics in viral detection are shown (Table 1). No significant differences for age, sex, or atopic status between each group were found. We detected 59 rhinoviruses, 44 RS viruses, 17 enteroviruses, and 19 other viruses, and no viruses were detected in 35 samples in a total of 174 patients with acute exacerbations of wheezing (Table 1). Of the 17 enteroviruses, 15 were enterovirus type 68 and 2 were coxsackievirus, or an echovirus. The other virus group consisted of 11 positive for both rhinovirus and RS virus, five for two other viruses, two for influenza A, and one for influenza B. The monthly changes in acute exacerbations of wheezing were shown (Fig. 1). Two-thirds of the patients were hospitalized or treated for wheezing/asthma attacks from September through December. RS viruses and enteroviruses were frequently detected from October through December, or October and November, respectively. In contrast, rhinoviruses were detected in almost all months of the year.

Serum and nasal cytokines/chemokines and eosinophil cationic protein (ECP)

Patient characteristics in relation to cytokine/chemokine measurements are shown (Table 2). No significant differences for age, sex, or atopic status between each group were found. We compared peripheral eosinophil counts, the concentrations of serum ECP and 27 cytokines/

Table 1. Characteristics of patients in virus detection

Virus	Number (% of total)	Mean/median age year (range)	Gender, % male	≥1 positive aeroallergen CAP-RAST, % positive
Rhinovirus	59 (33.9)	3.1/2.8 (0.3–9.2)	64.4	71.4
Respiratory syncytial virus	44 (25.3)	3.3/2.4 (0.8–11.2)	72.7	67.5
Enterovirus	17 (9.8)	5.5/5.4 (2.1–11.3)	52.9	88.2
Others	19 (10.9)	3.9/3.0 (1.3–9.8)	73.7	52.6
Not detected	35 (20.1)	3.8/3.1 (0.4–11.8)	68.6	65.6
Total	174 (100)	3.6/3.0 (0.3–11.8)	67.2	68.5

CAP-RAST, capsulated hydrophilic carrier polymer-radioallergosorbent test.

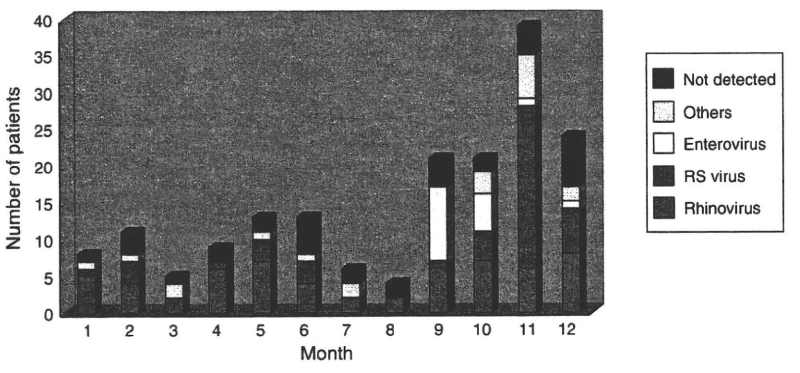

Fig. 1. Monthly changes of virus detection in acute exacerbation of wheezing. Two-thirds of the patients were hospitalized or treated for wheezing attacks from September through December. Respiratory syncytial viruses or enteroviruses were frequently detected from October through December, or October and November, respectively. In contrast, rhinoviruses were detected in almost every month of the year.

Table 2. Patient characteristics in cytokines/chemokines measurement

	Number	Mean/median age year (range)	Gender (% male)	≥1 positive aeroallergen CAP-RAST, % positive
Acute wheezing exacerbation group				
Rhinovirus-induced wheezing	28	2.9/2.7 (0.3–9.2)	60.7	70.4
Respiratory syncytial virus-induced wheezing	17	3.4/2.1 (0.8–11.2)	76.5	71.4
Enterovirus-induced wheezing	9	6.0/6.2 (2.8–11.3)	66.7	88.9
Other viruses-induced wheezing	10	3.5/1.9 (1.3–9.8)	60.0	40.0
Not detected viruses-induced wheezing	15	4.6/4.3 (0.4–11.8)	53.3	76.9
Non-symptomatic wheezing	79	5.0/4.7 (1.1–13.6)	60.8	78.2
Control	14	3.6/3.7 (1.3–6.4)	64.3	0.0

CAP-RAST, capsulated hydrophilic carrier polymer-radioallergosorbent test.

chemokines in each virus group against non-symptomatic wheezing. Table 3 showed the significantly elevated parameters among them. In comparison with each virus group, the concentrations of serum ECP, IL-5, IL-6, IL-1ra, and IP-10 were significantly elevated in rhinovirus-induced wheezing compared with non-symptomatic wheezing. On the other hand, peripheral eosinophil counts and any other cytokines/chemokines including IL-1β, IL-2, IL-4, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, TNF-α, GM-CSF, G-CSF, MCP-1, MIP-1α, MIP-1β, eotaxin, RANTES, PDGF-bb, FGF basic, and VEGF were not significantly elevated in the same comparison. By contrast, IL-6, IL-8, IL-1ra, and IP-10, but not ECP and IL-5 were significantly higher in RS virus-induced wheezing than in non-symptomatic wheezing. Notably, only IP-10 was significantly elevated in all wheezing groups compared with non-symptomatic wheezing. On the other hand, IL-5 was significantly higher in the rhinovirus, enterovirus, and other virus groups, but not in the RS virus group, than in non-symptomatic wheezing. Next, we compared each group against controls. Table 3 and Figure 2 showed the significantly elevated parameters among them. Serum ECP and IL-5 were significantly elevated in the rhinovirus, enterovirus, and other virus groups but not in the RS virus group, compared with controls. By contrast, serum IL-1ra was significantly higher in only RS virus group than in controls. Furthermore, we compared these parameters between non-symptomatic wheezing patients and controls. As a result, only ECP was significantly elevated in non-symptomatic wheezing patients than in controls ($p < 0.01$). Finally, we compared these parameters among three major viruses in acute exacerbations of wheezing, that is, rhino-, RS- and enterovirus groups, and only IL-5 was significantly elevated in the rhinovirus group compared with the RS virus group, in both serum and nasal secretions (Fig. 3). In contrast, only serum IL-1ra was significantly elevated in the RS virus group compared with the rhinovirus and enterovirus groups. Nasal IL-1ra was higher in some patients with RS virus group but not significantly elevated in the same comparison (Fig. 3).

Discussion

Johnston et al. (2) reported that in 9- to 11-year-old children with asthma, viral infections were associated with more than 80% of asthma exacerbations, and rhino/enteroviruses were responsible for 50% of these episodes, as detected by PCR or conventional methods including cell culture, immunofluorescence, and ELISA. Viruses such as corona, influenza, parainfluenza 1, 2, and 3, RS virus, and other viruses are responsible for 13.0, 7.2, 7.2, 4.1, and 1.0% of exacerbations, respectively. Brouard et al. (12) reported that in 118 children hospitalized for

Table 3. Elevated serum eosinophil cationic protein (ECP) and cytokines/chemokines in acute wheezing groups compared with non-symptomatic wheezing or control

	Compared with non-symptomatic wheezing (n = 79)	Compared with control (n = 14)
Rhinovirus-induced wheezing (n = 28)	ECP*, IL-5***, IL-6**, IL-1ra**, IP-10***	ECP†, IL-5††, IP-10††
Respiratory syncytial virus-induced wheezing (n = 17)	IL-6*, IL-8*, IL-1ra***, IP-10***	IL-1ra††, IP-10††
Enterovirus-induced wheezing (n = 9)	IL-5**, IL-8*, IP-10*	ECP††, IL-5†
Other viruses-induced wheezing (n = 10)	IL-5**, IL-6**, IL-8*, IL-10**, IL-1ra*, G-CSF**, IP-10***	ECP†, IL-5†, IL-10†, IP-10††
Not detected viruses-induced wheezing (n = 15)	IL-8**, IL-1ra*, IP-10**	ECP†, IP-10†

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with non-symptomatic wheezing: †$p < 0.05$; ††$p < 0.01$ compared with control.

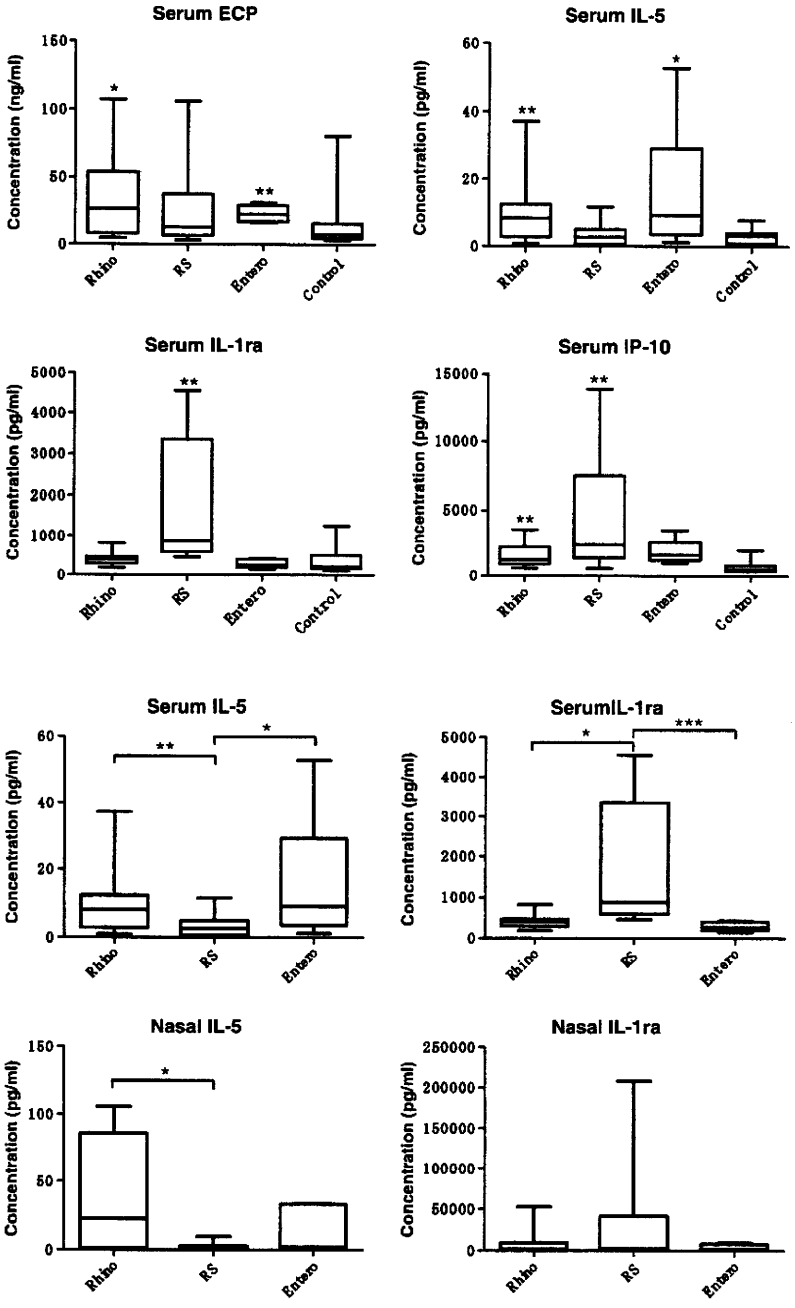

Fig. 2. Elevated serum eosinophil cationic protein and/or cytokines/chemokines in rhinovirus-, respiratory syncytial virus-, and enterovirus-induced acute exacerbation of wheezing compared with controls. The median is represented as horizontal bars. Data were analyzed using the Mann–Whitney *U* test, $*p < 0.05$; $**p < 0.01$.

Fig. 3. Elevated IL-5 and IL-1ra in serum and nasal secretions among rhinovirus-, respiratory syncytial (RS) virus-, and enterovirus-induced acute exacerbation of wheezing. Only IL-5 was significantly elevated in the rhinovirus group compared with the RS virus group in both serum and nasal secretions. In contrast, only serum IL-1ra was significantly elevated in the RS virus group compared with the rhinovirus or enterovirus groups. The median is represented as horizontal bars. Data were analyzed using the Kruskal–Wallis test, followed by Dunn's multiple comparison test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

acute asthma, rhinoviruses (45%) were prevalent, followed by RS viruses (28%) and enteroviruses (8.5%), as detected by conventional techniques, such as viral culture and immunofluorescence, and molecular techniques, such as PCR. They also noted that rhinoviruses and RS viruses have a similar prevalence (42% and 36%) before two years of age, when compared with 66% and 27%, respectively, in older children. Our methods, using a combination of an antigen detection kit and PCR followed by direct sequence analysis, revealed rhinoviruses (33.9%), RS viruses (25.4%), enteroviruses (9.8%), other viruses (10.9%), and no viruses detected (20.1%) in 174 acute exacerbations of patients with wheezing aged 3.6 years (mean). Comparing this study to the report by Brouard et al., the mean age in the rhinovirus and RS virus groups is almost the same, namely, 3.2 vs. 3.3 years, respectively. Indeed, Kotaniemi-Syrjänen et al. (13) reported that rhinoviruses were identified in 33% of wheezing children aged 1–23 months. They also

found that rhinoviruses were associated with the development of subsequent asthma, suggesting that rhinovirus, as well as the RS virus, are important inducers of wheezing even in infancy and play an important role in the development of asthma. In addition, our results showed much higher RS virus detection than the previous report. This could be attributable to improved detection by the high-sensitivity antigen detection kit. We also used DNA direct sequence analysis for more accurate and complete virus detection, and specifically, to distinguish between rhinovirus and enterovirus, compared with the previous report (2). The limitation of this study is the absence of the data from nasal samples in asymptomatic patients and control subjects. We thought that these children would be difficult to recruit to an intensive and invasive study of this type. Indeed, in Japan it is ethically difficult to do such procedure in these children.

Although previous reports have shown virus detection and eosinophil activation and/or cytokine profiles in virus-induced acute exacerbations of wheezing/asthma (14–19), this is the first report to show ECP and various cytokine/chemokine profiles in nasal and serum samples from patients with virus-induced, particularly rhinovirus-induced, acute exacerbations of wheezing. While there were some reports regarding serum or nasal ECP in asthma and RS virus infection (14, 15), Rakes et al. (16) showed that rhinovirus infection in wheezing children is associated with positive serum nasal eosinophilia, elevated nasal ECP, and positive radioallergosorbent testing, suggesting that wheezing attacks by rhinovirus infection are most likely in conjunction with evidence of eosinophilic airway inflammation or atopy. The same group also reported that experimental rhinovirus infection in mild adult asthma induced higher total blood eosinophil counts and increased nasal ECP (17). Furthermore, Johnston's group showed that nasal major basic protein was elevated in acute exacerbations of childhood asthma induced by virus infection, and most of the viruses were rhinoviruses (18). Previously, Oymar et al. (14) reported that serum IL-5 was elevated in childhood asthma and RS virus-induced bronchiolitis. In our study, ECP and IL-5 showed a significant association in both serum and nasal samples (data not shown).

These results collectively suggest that virus-induced wheezing/asthma, particularly by rhinovirus, might enhance eosinophil activation through IL-5 production. However, at the present time, we do not know the exact mechanism by which rhinoviruses might induce acute exacerbations of wheezing/asthma and enhance eosinophil activation. There are a number of articles relating to experimental rhinovirus infection and the induction of a variety of cellular responses. Human rhinoviruses induce an increase in airway reactivity and epithelial (4) or sputum (20) eosinophils in asthmatic patients. Rhinovirus infection in human respiratory submucosal glands up-regulates the expression of intracellular adhesion molecule-1 mRNA, the major rhinovirus receptor, and increased production of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF in supernatants (21). Similarly, rhinovirus infection up-regulates GM-CSF (22), eotaxin, and eotaxin-2 expression in bronchial epithelial BEAS-2B cells (23). In human airway submucosal glands, eosinophil chemotaxis was augmented during rhinovirus infection (24). A very recent article found that rhinovirus infection enhances airway epithelial remodeling through VEGF production (25). Collectively, evidence suggests that rhinoviruses could induce eosinophil activation, particularly through eosinophil-active cytokines/chemokines, such as IL-5, GM-CSF, and eotaxin, as well as an increase in the number of eosinophils.

In contrast to elevated ECP and IL-5 in rhinovirus group, our results showed that serum IL-1ra was elevated in only RS virus group. IL-1ra has anti-inflammatory properties and contributes to limit the inflammatory process in asthma (26). IL-1ra in bronchial lavage was increased in status asthmatics than in stable asthmatics and control patients (27). Another report found that nasal IL-1ra levels were significantly higher in the non-asthmatics than in asthmatics during experimental rhinovirus infection (28). These reports and our results suggest that IL-1ra might play an anti-inflammatory role in RS virus-induced acute exacerbations of childhood wheezing/asthma. IP-10 (or CXCL10) is a chemokine ligand for CXCR3 receptor that has been shown to be induced in human bronchial epithelial cells after infection with rhinovirus (29). A recent report showed that serum IP-10 levels were increased in acute virus-induced asthma compared with non-virus-induced acute asthma (30). Our results also suggest that serum IP-10 is a novel marker of both rhinovirus- and RS virus-induced acute wheezing/asthma exacerbations. In the differences of cytokine profiles between rhinovirus and RS virus infection, previously, Gern et al. showed that there were significant correlations between nasal IL-8 levels and symptom scores during infections with rhinovirus, but not RS virus, suggesting that the evidence of a close

relationship between the generation of IL-8 and symptoms during acute infections with rhinovirus (19).

Collectively, our observations and the previous report clearly indicate that different cytokine profile and eosinophil activation might be involved in rhino- and RS virus-induced wheezing/asthma exacerbations.

Further work is needed to better explore the mechanisms behind the association between wheezing/asthma exacerbations and rhinovirus or RS virus infections. These studies might ultimately lead to treatment modalities to prevent and/or treat the significant burden of wheezing/asthma exacerbations caused by virus infection.

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None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Relationships between cytokine profiles and signaling pathways (IκB kinase and p38 MAPK) in parainfluenza virus-infected lung fibroblasts

Masakazu Yoshizumi[1,2], Hirokazu Kimura[3]*, Yoshimichi Okayama[4]*, Atsuyoshi Nishina[5], Masahiro Noda[6], Hiroyuki Tsukagoshi[1], Kunihisa Kozawa[1] and Masahiko Kurabayashi[2]

[1] Gunma Prefectural Institute of Public Health and Environmental Sciences, Maebashi-shi, Gunma, Japan
[2] Department of Medicine and Biological Science, Gunma University Graduate School of Medicine, Maebashi-shi, Gunma, Japan
[3] Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Musashimurayama-shi, Tokyo, Japan
[4] Division of Molecular Cell Immunology and Allergology, Advanced Medical Research Center, Nihon University Graduate School of Medical Science, Tokyo, Japan
[5] Department of Health and Nutrition, Yamagata Prefectural Yonezawa Women's Junior College, Yonezawa-shi, Yamagata, Japan
[6] Department of Virology III, National Institute of Infectious Diseases, Musashimurayama-shi, Tokyo, Japan

Edited by:
Akihide Ryo, Yokohama City University, Japan

Reviewed by:
Isao Kosugi, Hamamatsu University School of Medicine, Japan
Masatoshi Nakazawa, Yokohama City University, Japan

***Correspondence:**
Hirokazu Kimura, National Institute of Infectious Diseases, Infectious Disease Surveillance Center, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan.
e-mail: kimhiro@nih.go.jp; Yoshimichi Okayama, Division of Molecular Cell Immunology and Allergology, Advanced Medical Research Center, Nihon University Graduate School of Medical Science, 30-1 Oyaguchi-Kamimachi, Itabashi-ku, Tokyo 173-8610, Japan.
e-mail: yokayama@med.nihon-u.ac.jp

Respiratory viruses such as parainfluenza virus (PIV) in individuals with certain genetic predispositions in early life are associated with the induction of wheezing, which can progress to the development of asthma. It has been suggested that aberrant production of various cytokines due to viral infection are associated with virus-induced asthma. However, the mechanisms of how respiratory viruses induce and exacerbate asthma have yet to be clarified. To examine cytokine responses to PIV infection, we assessed 27 cytokine levels released from PIV-infected human fetal lung fibroblasts. In addition, we examined relationships between these cytokine responses and signaling pathways (IκB kinase and p38 MAPK) in PIV-infected cells. At 24 h after infection, PIV-infected cells significantly released a number of cytokines, namely, proinflammatory cytokines [interleukins (IL)-1β, IL-6, and tumor necrosis factor-α], anti-inflammatory cytokine (IL-1ra), Th1 cytokines (interferon-γ, and IL-2), Th2 cytokines (IL-4, IL-5, and IL-10), granulopoiesis-inducing cytokines (granulocyte colony-stimulating factor and granulocyte–macrophage colony-stimulating factor), neutrophil recruitment-inducing cytokines (IL-8 and interferon-inducible protein-10), and eosinophil recruitment-inducing cytokines (eotaxin and regulated on activation normal T-cell expressed and secreted). PIV infection enhanced phosphorylation of both IκB and p38 MAPK, but not Akt, in the cells. Signaling pathway inhibitors, BMS-345541 (a specific IκB kinase inhibitor) and SB203580 (a specific p38 MAPK inhibitor), significantly suppressed release of these cytokines from PIV-infected cells. The results indicate that PIV infection induces aberrant production and release of various cytokines through IκB kinase and p38 MAPK pathways in human lung fibroblasts. Overproduction and imbalance of these cytokines may be partially associated with the pathophysiology of virus-induced asthma.

Keywords: cytokines/chemokines, parainfluenza virus, human fetal lung fibroblast, inflammation, signaling pathway

INTRODUCTION

Respiratory viruses are responsible for most acute respiratory illnesses in humans, including the common cold, bronchiolitis, and pneumonia (Johnston, 2004). These major pathogens include human parainfluenza viruses (PIV), rhinoviruses (RV), respiratory syncytial virus (RSV), and influenza viruses (Monto, 2004). Respiratory infections resulting from PIV, RV, and RSV have also been implicated in the induction and exacerbation of asthma (Folkerts et al., 1998; Stein et al., 1999; Azevedo et al., 2003; Kotaniemi-Syrjänen et al., 2003; Martinez, 2003; Matsuse et al., 2005; Hershenson and Johnston, 2006; Khetsuriani et al., 2007; Kusel et al., 2007). Most children are infected at least once with PIV early in life, but reinfections occur throughout life. Serologic surveys indicate that at least 60% of children have been infected with PIV3 by 2 years of age while approximately 80% have been infected by 4 years of age. PIV are associated with 0–12% of acute lower respiratory tract infections in adults. PIV infections may be associated with not only respiratory infections but also the induction and exacerbation of asthma (virus-induced asthma) (Azevedo et al., 2003; Matsuse et al., 2005). However, many children who wheeze with viral infections during infancy will not go on to develop asthma, suggesting a role of virus- and/or host-specific factors in the pathogenesis of the disease.

Most types of cytokines may be associated with the pathophysiology of virus-induced asthma or excessive virus-induced inflammation (Mallia and Johnston, 2006; Proud and Chow, 2006). In extreme cases, an overproduction of cytokines due to viral infection (e.g., influenza virus infection) may induce a cytokine storm resulting in systemic inflammatory response syndrome (SIRS) (Watanabe et al., 2003). Thus, viral infection-induced overproduction of cytokines *in vivo* might be closely associated with the pathophysiology of various diseases including asthma (Message and Johnston, 2004; Mallia and Johnston, 2006; Proud and Chow, 2006). However, cytokine profiles of viral infections including PIV infection remain largely unknown.