2.5. Eosinophil adhesion assay

The percentage of adherent eosinophils was determined by measuring the content of EDN in adherent cells by enzyme immunoassay, as previously reported [24,25]. Briefly, after preincubation with or without IFN-γ, eosinophils were incubated with GM-CSF, IL-5, or medium, as described in the degranulation assay. After 1 h of incubation, the supernatants were collected and the plate was rinsed gently with warm RPMI 1640 to remove nonadherent cells. Adherent cells were then lysed with 0.5% Nonidet P-40 detergent, and EDN content in the lysate was measured by enzyme immunoassay. All experiments were performed in duplicate. Percent adhesion was calculated as the ratio of EDN content in adherent eosinophils to total available EDN after incubation according to the following equation:

$$\text{percent adhesion} = \left[\frac{\text{EDN in lysates of adherent cells after incubation}}{(\text{total EDN in lysates of cells before incubation} - \text{EDN release into supernatants during incubation})}\right] \times 100.$$

2.6. GM-CSFR, IL-5R, and CD11b expression

GM-CSFR, IL-5R, and CD11b expression were determined by flow cytometry, as previously described [24]. After preincubation with or without IFN-γ, eosinophils (2.5×10^5 cells/sample) were resuspended in 50 μl of PBS for incubation with an anti-GM-CSFR, anti-IL-5R or anti-CD11b mAb or isotype-matched immunoglobulin for 30 min at 4 °C. Cells were resuspended in PBS, and expression of GM-CSFR, IL-5R, or CD11b was determined by a flow cytometer (Epics XLII, Beckman Coulter, Tokyo, Japan), and reported as mean fluorescence intensity (MFI). Cell viability always exceeded 98%, as determined by propidium iodide (1 μg/ml) staining.

2.7. Analysis of phosphoproteins

Phosphorylation of signaling molecules induced by GM-CSF or IL-5 was assessed by Bio-Plex Suspension Array System (Bio-Rad Laboratories). For the examination of target phosphoproteins, we used the Bio-Plex 12-plex Phosphoprotein Assay Kit and the Phosphoprotein Testing Reagent Kit (Bio-Rad Laboratories).

After preincubation with or without IFN-γ, eosinophils were stimulated by GM-CSF or IL-5 in a HSA-coated, flat-bottom 96-well tissue culture plate. After 30 min of incubation, the plate was centrifuged at 1200 × g for 7 min. The supernatant was then removed and cells were lysed using the Phosphoprotein Testing Reagent Kit. Cell lysates were placed in a 96-well filter plate, coated with multiplex beads of antibodies against the anti-phosphoprotein, following by overnight incubation (from 15 to 18 h) on a rotary shaker at 300 rpm at room temperature. After three washes to remove unbound proteins, Bio-Plex Phospho 12-Plex Detection Antibodies solution was added to the reaction. Streptavidin–phycoerythrin solution was then added to bind to the Detection Antibodies on the beads. Twelve types of phosphoproteins (ATF-2, ERK1/2, IκB-α, JNK, p38 MAPK, STAT2/3, AKT, GSK-3α/β, p70s6K, p90RSK, and TrkA) were determined by the Bio-Plex Suspension Array System.

2.8. Statistical analysis

All data were expressed as mean ± standard errors of the means (S.E.M.). Variations between two groups were tested using the Student's *t*-test. Variations between more than two groups were tested using ANOVA, followed by Dunnett's test. A value of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Effects of IFN-γ on GM-CSF- or IL-5-induced superoxide anion generation

Superoxide anion generation is an important effector function of eosinophils. GM-CSF and IL-5 are cytokines involved in allergic inflammation and have been shown to induce generation of superoxide anion in eosinophils. We examined the effect of IFN-γ on GM-CSF- or IL-5-induced superoxide anion generation. To determine the optimal concentration for use, we performed experiments using 100, 500 and 1000 U/ml of IFN-γ. Both concentrations elicited a significant increase in GM-CSF or IL-5 stimulated superoxide generation dose-dependently, although 1000 U/ml showed less of an increase than 500 U/ml, as shown in Table 1.

Table 1
Effect of IFN-γ pretreatment on GM-CSF- or IL-5-induced superoxide anion generation from eosinophils

Stimulants	IFN-γ (U/ml)			
	0 (control)	100	500	1000
Medium	0.7 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
GM-CSF (ng/ml)				
0.1	11.3 ± 1.1	13.0 ± 1.1††	14.4 ± 0.9††	12.6 ± 1.3
1	12.4 ± 1.1	13.9 ± 0.9††	16.0 ± 1.2††	14.1 ± 1.5†
10	12.6 ± 1.2	14.1 ± 1.0††	16.1 ± 1.1††	14.0 ± 1.6
IL-5 (ng/ml)				
0.1	8.5 ± 1.5	9.1 ± 1.9	11.1 ± 1.3††	9.2 ± 1.7
1	12.2 ± 0.9	13.8 ± 1.4†	15.1 ± 1.3††	13.0 ± 1.7
10	12.9 ± 1.0	14.4 ± 1.4††	15.6 ± 1.5††	14.1 ± 1.6

Values are presented as mean ± S.E.M. of nmol/10^5 cells from four independent experiments. Variations compared with control were tested using ANOVA, followed by Dunnett's test, †$p < 0.05$, ††$p < 0.01$.

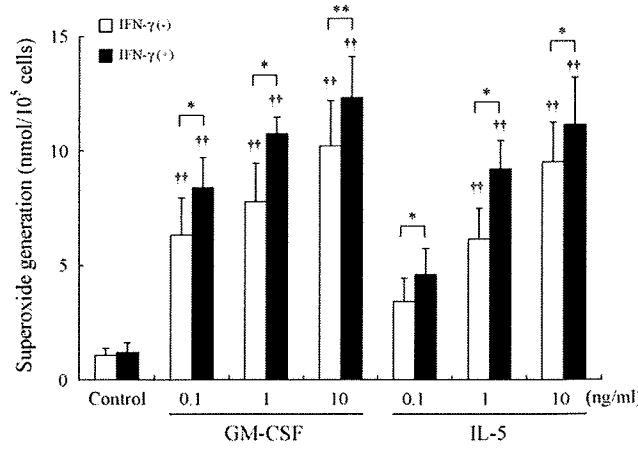

Fig. 1. Effect of IFN-γ on GM-CSF- or IL-5-induced superoxide anion generation of human eosinophils. Eosinophils (5×10^4 cells/well) were stimulated with GM-CSF or IL-5 after preincubation with or without IFN-γ (500 U/ml). Superoxide anion generation after 120 min of stimulation determined by cytochrome *c* reduction method and described as nmol/10^5 cells. Data are expressed as mean ± standard error of the mean (S.E.M.) of eight independent experiments. Variations between IFN-γ +/− groups were tested using Student's *t*-test, *$p < 0.05$; **$p < 0.01$. Variations between more than two groups were tested using ANOVA, followed by Dunnett's test, †$p < 0.05$; ††$p < 0.01$.

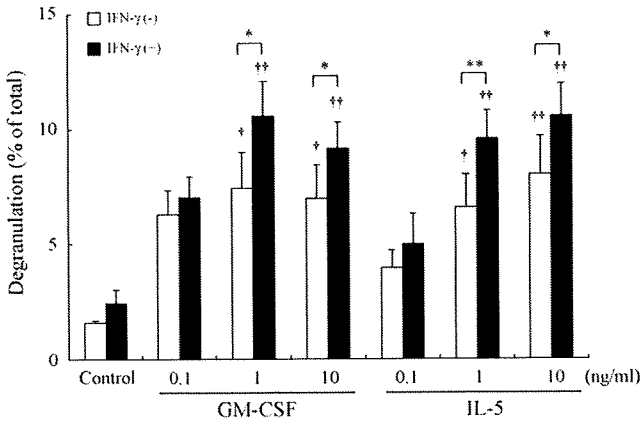

Fig. 2. Effect of IFN-γ on GM-CSF- or IL-5-induced degranulation of human eosinophils. Eosinophils (5×10^4 cell/well) were stimulated with GM-CSF or IL-5 after preincubation with or without IFN-γ (500 U/ml). Concentrations of EDN after 3 h of stimulation in the supernatant were determined and described as % of total. Total cellular EDN content was 2505 ± 405 ng/10^6 cells. Data are expressed as mean ± standard error of the mean (S.E.M.) of four independent experiments. Variations between IFN-γ +/− groups were tested using Student's *t*-test, $^{*}p < 0.05$; $^{**}p < 0.01$. Variations between more than two groups were tested using ANOVA, followed by Dunnett's test, $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$.

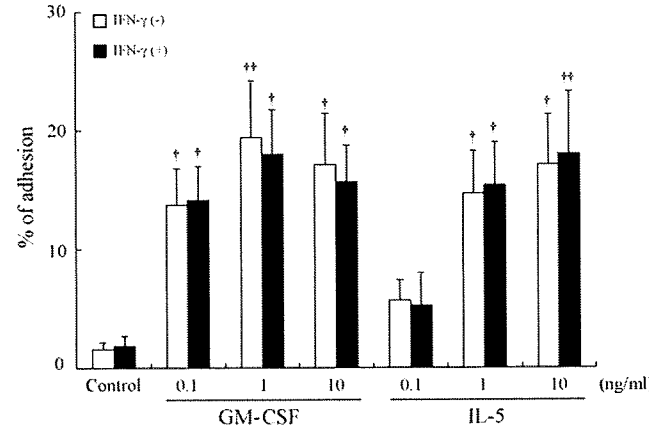

Fig. 3. Effect of IFN-γ on GM-CSF- or IL-5-induced cell adhesion. Eosinophils were stimulated with GM-CSF or IL-5 after preincubation with or without IFN-γ (500 U/ml). After 1 h, the percentage of adhesion was calculated as the ratio of EDN content in adherent eosinophils to total available EDN. Total cellular EDN content was 2943 ± 714 ng/10^6 cells. Data are expressed as mean ± S.E.M. of six independent experiments. Variations between IFN-γ +/− groups were tested using Student's *t*-test. Variations between more than two groups were tested using ANOVA, followed by Dunnett's test, $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$.

On the basis of these experiments, 500 U/ml was shown to be an optimal concentration and we therefore selected it for use in subsequent experiments. Thus, eosinophils were preincubated with or without IFN-γ (500 U/ml), and followed by GM-CSF or IL-5 stimulation. IFN-γ itself did not induce superoxide anion generation in human eosinophils. However, GM-CSF- or IL-5-induced superoxide anion generation from IFN-γ pretreated eosinophils was elicited at doses ranging from 0.1 to 10 ng/ml (Fig. 1).

3.2. Effects of IFN-γ on GM-CSF- or IL-5-induced degranulation

Degranulation is thought to be a major eosinophil effector function, in addition to superoxide anion generation. We next examined whether IFN-γ affected degranulation induced by GM-CSF or IL-5 stimulation. Preincubation with IFN-γ significantly enhanced degranulation induced by GM-CSF or IL-5, while IFN-γ itself did not induce degranulation of eosinophils (Fig. 2). Enhancement was not significant at low-dose (0.1 μM) stimulation. This result suggests that, in contrast to superoxide anion generation, high-dose stimulation is necessary to enhance degranulation.

3.3. Effects of IFN-γ on GM-CSF- or IL-5-induced cellular adhesion

β2 integrin-dependent cellular adhesion, particularly αMβ2 (CD11b/CD18), is critical for the effector functions of eosinophils during eosinophil activation. We examined the effect of IFN-γ on cellular adhesion of eosinophils to HSA-coated plates using an adhesion assay 1 h after stimulation. GM-CSF- or IL-5-induced eosinophil adhesion was unaffected by IFN-γ (Fig. 3).

3.4. Effects of IFN-γ on GM-CSFR, IL-5R or CD11b expression on eosinophils

To determine the effects of IFN-γ on the expression of GM-CSFR, IL-5R or an αMβ2 integrin (CD11b/CD18) on eosinophils, we examined whether GM-CSFR, IL-5R or CD11b expression was modulated by IFN-γ. As shown in Fig. 4, IFN-γ had no effect on GM-CSFR, IL-5R, or CD11b expression on the surface of eosinophils.

3.5. Effects of IFN-γ on phosphorylation of signal molecules

Next, we examined the phosphorylation of twelve signaling molecules including MAPK family in eosinophils stimulated by

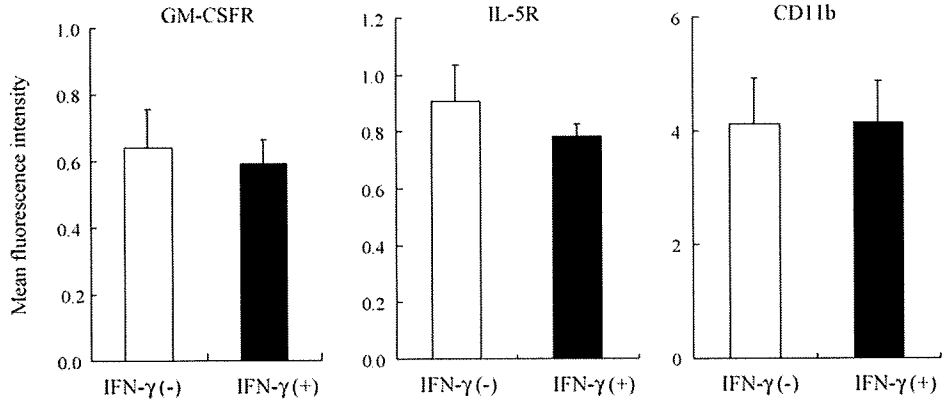

Fig. 4. Effect of IFN-γ on GM-CSFR, IL-5R, or CD11b expression. Eosinophils (2.5×10^5 cells/sample) were incubated with or without IFN-γ (500 U/ml). GM-CSFR, IL-5R, and CD11b expression after 15 min of stimulation were determined by means of flow cytometric analysis and described as mean fluorescence intensity (MFI). Data are expressed as mean ± S.E.M. of four independent experiments. Variations between IFN-γ +/− groups were tested using Student's *t*-test.

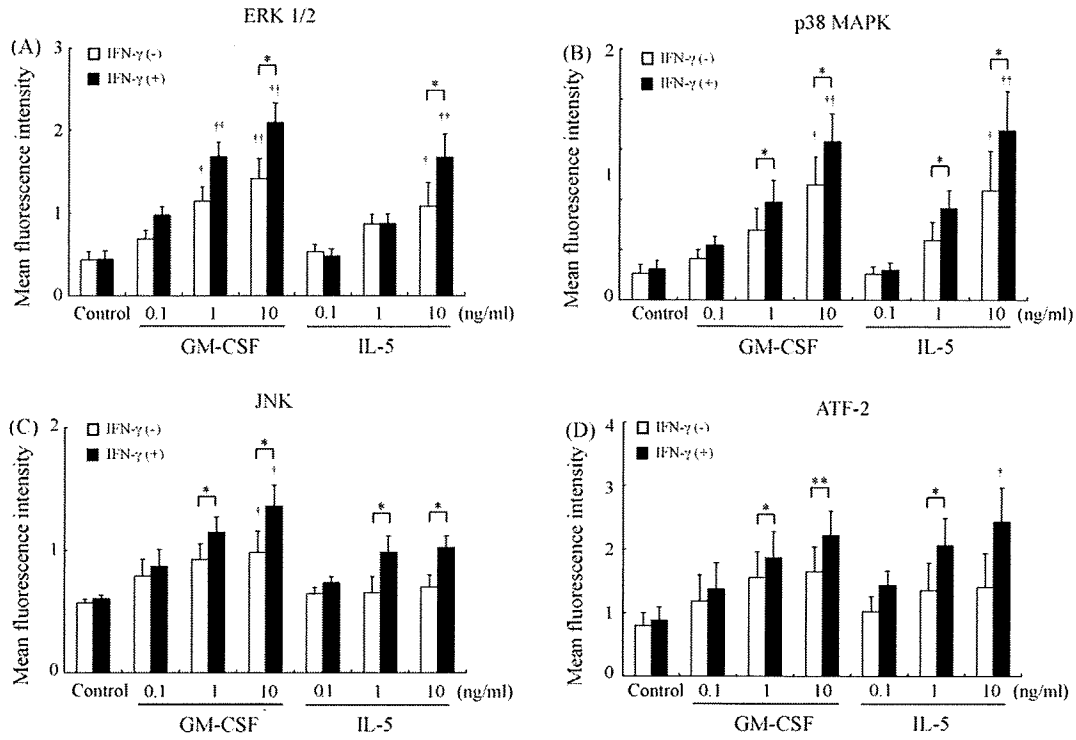

Fig. 5. Effect of IFN-γ on GM-CSF- or IL-5-induced ERK 1/2, p38 MAPK, JNK, and ATF-2 phosphorylation of human eosinophils. Eosinophils (0.7–1.4 × 10^5 cells/well) were stimulated with GM-CSF or IL-5 after preincubation with or without IFN-γ (500 U/ml). Phosphorylation of ERK1/2 (A), p38 MAPK (B), JNK (C), or ATF-2 (D) after 30 min of stimulation was determined by the Bio-Plex phosphoprotein assay and described as mean fluorescence intensity (MFI). Data are expressed as mean ± S.E.M. of five to ten independent experiments. Variations between IFN-γ +/− groups were tested using Student's *t*-test, $^{*}p < 0.05$; $^{**}p < 0.01$. Variations between more than two groups were tested using ANOVA, followed by Dunnett's test, $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$.

GM-CSF or IL-5. As shown in Fig. 5, GM-CSF- or IL-5-induced phosphorylation of ERK1/2 (A), p38 MAPK (B), JNK (C), and ATF-2 (D) as a downstream substrate of p38 MAPK or JNK dose-dependently. Accordingly, IFN-γ tended to increase phosphorylation of ERK1/2, p38 MAPK, JNK, and ATF-2 of eosinophils induced by GM-CSF or IL-5. Further, other signal molecules (IκB-α, STAT2/3, AKT, GSK-3α/β, p70s6K, p90RSK, and TrkA) were not affected by IFN-γ (data not shown).

3.6. Effects of MAPK inhibitors on GM-CSF- or IL-5-induced superoxide anion generation

Finally, we confirmed the role of ERK1/2, p38 MAPK, and JNK on superoxide anion generation in IFN-γ pretreated eosinophils induced by GM-CSF or IL-5. We used ERK inhibitor peptide II, SB 202190, and JNK inhibitor I to inhibit ERK1/2, p38 MAPK, and JNK activation, respectively. Concentration of all inhibitors above 0.3 μM blocked enhancement of superoxide anion generation from IFN-γ preincubated eosinophils (Fig. 6). SB 202190 and JNK inhibitor I caused concentration-dependent inhibition of superoxide anion generation induced by GM-CSF or IL-5, although ERK inhibitor peptide II caused no significant decrease. DMSO vehicle (0.1%) did not affect spontaneous or stimulus-induced superoxide anion generation (data not shown).

4. Discussion

In this study, we found that IFN-γ significantly enhanced GM-CSF- or IL-5- induced superoxide anion generation or degranulation of human eosinophils, whereas IFN-γ did not directly affect eosinophil functions. Stimulus-induced eosinophil adhesion was unaffected by IFN-γ and furthermore, IFN-γ did not influence the expression of GM-CSFR, IL-5R, or CD11b. Consequently, we assessed whether IFN-γ modulates phosphorylation of GM-CSF- or IL-5-stimulated signaling molecules, including ERK1/2, p38 MAPK, JNK, and ATF-2. Our results showed that IFN-γ enhanced phosphorylation of these molecules. Finally, we confirmed that MAPK inhibitors blocked enhancement of stimulus-induced superoxide anion generation. This is the first report to demonstrate that IFN-γ upregulated both effector functions and phosphorylation of MAPK family proteins induced by GM-CSF or IL-5 in human eosinophils.

Evidence suggests that the allergic diseases may be closely related to Th2 cytokine immune responses, and that Th1 cytokine responses may be protective against Th2-related diseases. Park et al. showed that IFN-γ pretreatment results in inhibition of the IL-5-induced human eosinophil shape change and mobility [26]. Fujisawa et al. demonstrated that IFN-γ pretreatment suppresses secretory IgA-induced EDN release [27]. However, there is increasing evidence that the roles of Th1 and Th2 cytokines are not entirely competitive. Indeed Kuwano et al. have demonstrated that IFN-γ pretreatment activates transcription of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 gene, and upregulates 4-phorbol 12-myristate 13-acetate (PMA) stimulated production of superoxide anion in human large intestinal epithelial cells [28]. In addition, Hamid et al. showed that human eosinophils pretreated with IFN-γ increases IL-6 release [29], and Lacy et al. showed that incubation with IFN-γ induces piecemeal degranulation and release of RANTES [30]. Moreover, incubation with IFN-γ induced CD69 expression on eosinophils [31], and preincubation with IFN-γ enhanced GM-CSF-induced eosinophil cytotoxicity [32]. Additionally, IFN-γ pretreatment has been shown to enhance, or prime, the production of superoxide anion from polymorphonuclear leukocytes (PMN) in combination with a secondary stimulus.

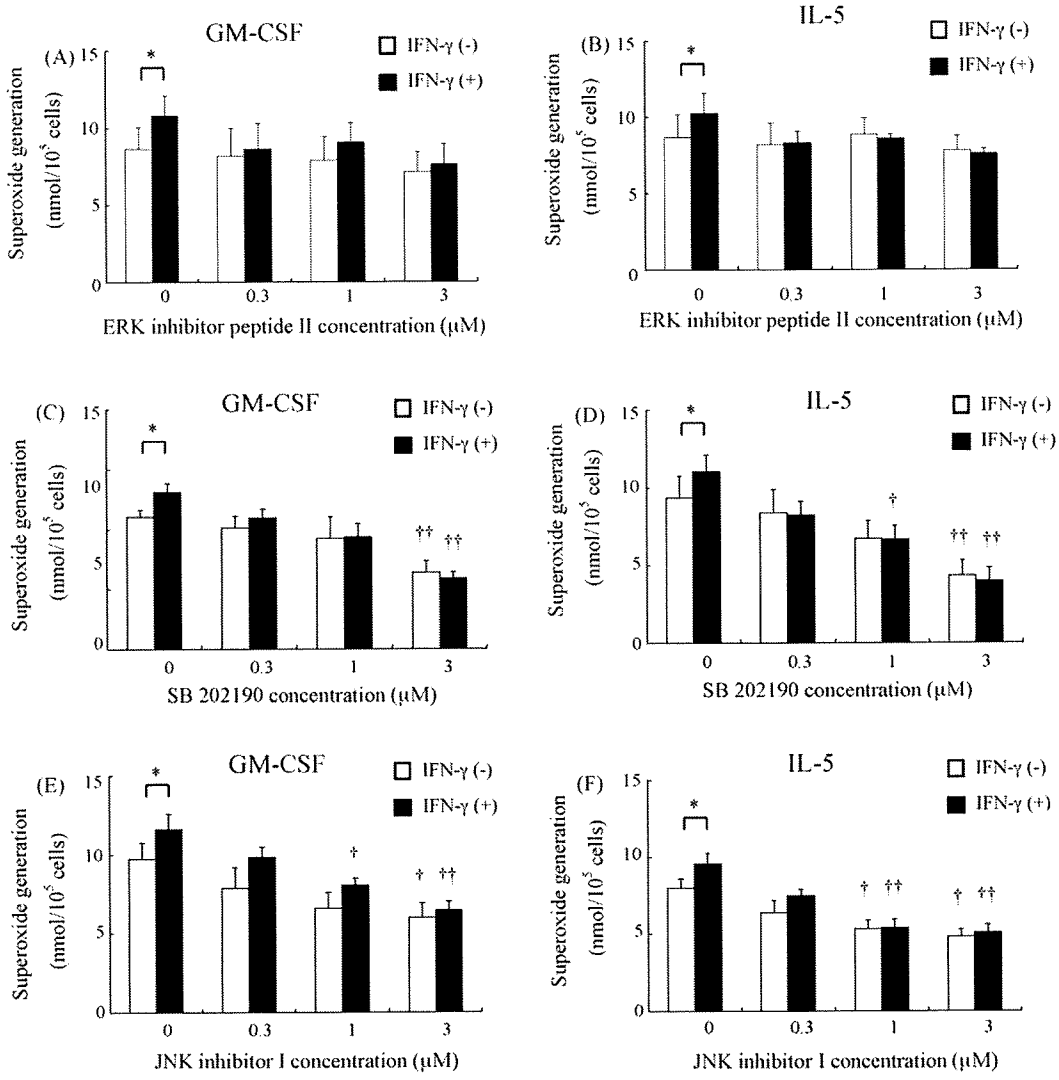

Fig. 6. Effect of MAPK inhibitors on GM-CSF- or IL-5-induced superoxide anion generation of IFN-γ preincubated eosinophils. Eosinophils (0.4–0.8 × 10^5 cells/well) preincubated with or without IFN-γ (500 U/ml) were treated with ERK inhibitor peptide II (A and B), SB 202190 (C and D), and JNK inhibitor I (E and F) for 30 min at 37 °C. After the incubation, the cells were stimulated by 1 ng/ml of GM-CSF (A, C and E) or IL-5 (B, D and F) for 120 min. Superoxide anion generation was determined by cytochrome c reduction method and described as nmol/10^5 cells. Data are expressed as mean ± S.E.M. of four to six independent experiments. Variations between IFN-γ +/− groups were tested using Student's *t*-test, $^{*}p < 0.05$; $^{**}p < 0.01$. Variations between more than two groups were tested using ANOVA, followed by Dunnett's test, $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$.

This priming response is observed in a number of species, including humans [33], rats [34], and cows [35]. In the present study, we found that the preincubation with IFN-γ significantly enhanced GM-CSF- or IL-5-induced superoxide anion generation or degranulation of human eosinophils. This result is comparable to that of Berton et al., who demonstrated the priming effect of IFN-γ on the PMN oxidative burst. When pretreated with IFN-γ, PMN stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) or concanavalin A show increased superoxide anion generation [33]. These results show that IFN-γ might enhance Th2 immune responses.

Cellular adhesion is one of the most critical steps during eosinophil activation [22]. We previously demonstrated that treatment with an antibody against β2 integrin can almost completely inhibit both eosinophil adhesion and degranulation induced by a lipid mediator (e.g., platelet-activating factor (PAF)), a cytokine (e.g., IL-5), or an immunoglobulin (e.g., IgG) [21,36]. These findings suggest that β2 integrin-dependent cellular adhesion, particularly αMβ2 (CD11b/CD18) induced by stimulation through the membrane receptors, is critical for eosinophil effector functions. Thus, we examined whether IFN-γ modulates cellular adhesion induced by GM-CSF or IL-5. As a result, stimulus-induced eosinophil adhesion was unaffected by IFN-γ. Further, IFN-γ did not influence the expression of GM-CSFR, IL-5R, or CD11b. These results demonstrate that IFN-γ enhanced GM-CSF- or IL-5-induced superoxide anion generation and degranulation from eosinophils, but did not affect CD11b/CD18-mediated cellular adhesion.

Our results raise the question of how IFN-γ enhances the GM-CSF or IL-5-induced eosinophils functions. Thus, we investigated the intracellular signaling pathway of GM-CSF or IL-5 in eosinophils. While the functional role of GM-CSF/IL-5 signaling is not well known, these cytokines activate the MAPK family, consisting of ERK and p38 MAPK, signaling cascade in eosinophils [37] and neutrophils [38,39]. Welham et al. demonstrated that GM-CSF and IL-5 induces tyrosine phosphorylation of p44 (ERK1) or p42 (ERK2) in hemopoietic cells [40]. Adachi et al. showed that ERK1/2 is important for eosinophil degranulation and cytokine production, and p38 MAPK is critical for eosinophil differentiation, degranulation, and cytokine production induced by IL-5 or eotaxin [41,42]. Bates et al. showed that ERK1/2 activation is essential for eosinophil

chemotaxis and activation by IL-5 [43], and Miike et al. demonstrated that ERK1/2 is involved in the degranulation but not in the survival of eosinophils stimulated by GM-CSF [44]. In addition, PD 098059, a MAPK kinase inhibitor, has no effect on CD11b/CD18-dependent eosinophil adhesion or NADPH oxidase activation [45]. In contrast, SB 203580, an inhibitor of p38 MAPK, does not influence eosinophil adhesion but inhibits superoxide anion generation in a concentration-dependent manner [46]. These data are in agreement with the present study, since IFN-γ activated p38 MAPK, while GM-CSF- or IL-5-induced eosinophil adhesion was unaffected. Although GM-CSF and IL-5 are known to also activate ERK and p38 MAPK, the point of this study is that IFN-γ activates ERK, JNK or p38/ATF-2 induced by GM-CSF or IL-5, leading to enhanced cytokine-induced eosinophil superoxide generation and degranulation. However, from our data, the role of ERK phosphorylation in IFN-γ enhanced superoxide anion generation might be limited compared with other MAP kinase family members.

Based on the evidence presented, we suggest a hypothesis for the possible intracellular signaling mechanism and the role of IFN-γ in eosinophil effector functions stimulated by GM-CSF or IL-5. Receptor-mediated stimulation by GM-CSF or IL-5 induces β2 integrin expression on eosinophil surfaces and a conformational change in the integrin molecule, so-called "inside-out" signaling [36]. The clustering of αMβ2 is followed by its focal adhesion to appropriate ligands. The αMβ2-ligand interaction triggers the "outside-in" signaling, for instance, phosphorylation of tyrosine kinase (PTK), followed by activation of phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K), which can contribute to the activation of protein kinase C (PKC) [46]. PKC is able to activate the MAPK cascade via Raf [47]. Various eosinophil effector functions such as superoxide anion generation and degranulation may also be induced by MAPK family proteins. IFN-γ may directly activate MAPK family proteins, probably independently of cellular adhesion and outside-in signaling. This may lead to enhanced GM-CSF- or IL-5-induced eosinophil functions largely through "outside-in" signaling.

In conclusion, this study demonstrated that IFN-γ upregulated GM-CSF- or IL-5-induced ERK 1/2, p38 MAPK, or JNK/ATF-2 activation and led to enhanced cytokine-induced eosinophil effector functions. This suggests that, in some instances, Th1-induced cytokines stimulate rather than suppress ongoing Th2-type responses related to allergic inflammation. Further studies are required to clarify the exact effects of IFN-γ on human eosinophils.

Acknowledgments

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Original Article

Genetic Changes of Coxsackievirus A16 and Enterovirus 71 Isolated from Hand, Foot, and Mouth Disease Patients in Toyama, Japan between 1981 and 2007

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SUMMARY: We characterized the genetic diversity of the complete VP1 region of coxsackievirus A16 (CA16) and enterovirus 71 (EV71) isolated from patients with hand, foot, and mouth disease in Toyama from 1981 to 2007 to evaluate the relationship between epidemics and genetic changes. The predominant genogroups of CA16 changed from B to C in 1995 - 1998, and genogroup C further changed from subgenogroup C1 to C2 around 2002, and to C3 in 2005 - 2007. The subgenogroups of the EV71 isolates were classified into B1, B4, C1, and C3 in 1983 - 1994, and into C4 in 1997 - 2006. However, changes of the amino acid sequences of the VP1 regions of CA16 were restored, and those of the EV71 isolates were not observed among the same subgenogroups during this survey period, indicating that the prevalence that occurred at intervals of several years seemed to depend on an accumulating number of immunologically naive children, not viral antigenic changes.

INTRODUCTION

Hand, foot, and mouth disease (HFMD) is a common febrile illness of children characterized by lesions on the skin and oral mucosa. HFMD occurs mainly in the summer in Japan, as is usually seen with enterovirus-related diseases (1,2). Coxsackievirus A16 (CA16) and enterovirus 71 (EV71) are the major causative agents of HFMD. EV71 infection is occasionally associated with severe neurological diseases such as aseptic meningitis, encephalitis, and acute flaccid paralysis (3-5). Several fetal cases have also been reported (6-9). In contrast, CA16-associated HFMD has a milder outcome (1,2,10).

EV71 has been classified into three genogroups (A, B, and C), and further divided into many subgenogroups, genotypes or lineages (B1-5, C1-5) based on the complete VP1 gene sequences (8,11-20). The VP1 region is considered to be variable and to play an important role in characterizing the antigenicity (21). Although few studies on genetic analysis have been reported on CA16 compared to EV71, CA16 has been classified into three genogroups (A, B, and C) according to the phylogenetic analysis of the VP4 or VP1 gene (22-24). While CA16 and EV71 usually co-circulate, EV71 has been predominant every 3 years since 1994 in Japan (1,2). Whereas genogroups or subgenogroups of CA16 and EV71 have been used to understand the origins of viral isolates and outbreaks (11,16), the influence of genetic variations in the VP1 region or prevalence has not been precisely investigated.

In this study, we characterized the genetic diversity of the complete VP1 region of CA16 and EV71 isolates from 1981 to 2007 to evaluate the relationship between epidemics and genetic changes.

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MATERIALS AND METHODS

Patients with HFMD: The weekly numbers of patients with HFMD were reported from 21 and 29 pediatric clinics in Toyama Prefecture during 1981 - 1998 and 1999 - 2007, respectively, by sentinel surveillance. The clinical specimens of 203 patients from pediatric clinics were used for isolation of viruses. They included 185 nasopharyngeal swabs, 195 stool samples, and 99 rash swabs. Fourteen cerebrospinal fluid samples were also collected from patients with aseptic meningitis or encephalitis complication.

Viral isolation and identification: The specimens were inoculated on appropriate tissue culture cells (Vero, MA-104, LLC-MK_2, RD-18S) for isolation of viruses. Virus isolates were identified by a neutralization test using anti-CA10, CA16, EV71, and pool enteroviruses polyclonal antibodies provided from the National Institute of Infectious Diseases in Japan. One hundred seventy-seven CA16 and 143 EV71 strains were respectively isolated from 79 and 92 patients. Randomly selected isolates (40 of 177 and 26 of 143, CA16 and EV71 strains, respectively) were used for further genetic analyses.

Phylogenetic analysis of CA16 and EV71: Viral genomic RNA was extracted from 140 μL of the culture fluid of cells that appeared cytopathic using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized for 1 h at 42°C by SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif., USA) with a random hexamer according to the manufacturer's procedures. Polymerase chain reaction

Table 1. Representative strains used for determination of genogroup or subgenogroup

Virus	Genogroup or subgenogroup	Strain	Accession no.	Reference
CA16	A	G-10	U05876	23, 26
	B	shzh99-83	AY895116	23
	B	shzh00-2	AY895127	23
	C	shzh02-14	AY895110	23
	C	shzh03-13	AY895114	23
	C	shzh04-J31	AY821796	23
EV71	A	BrCr	U22521	11-13, 16-20
	B1	2232/NY/77	AF135871	11
	B1	2258/CA/79	AF135880	11, 13
	B2	7423/MS/87	U22522	11
	B3	4F/AUS/4/99	AF376105	13
	B4	18/SIN/97	AF251359	13
	B5	2972/Yamagata/03	AB213650	16
	C1	0756/MAA/97	AF135935	11, 13
	C2	NCKU9822	AF136379	29
	C3	KOR-EV71-01	AY125966	18, 23
	C4	shzh02-40	AY895130	23
	C5	933V/VNM/05	AM490161	20

(PCR) was performed using an Ex Taq (TaKaRa, Otsu, Japan) to amplify the VP2/VP1/2A region of the viral genome (11,23,25). For CA16, we used the primers CA16F-2030 (sense 5′-AGG TAC TAC ACC CAG TGG TCA G-3′) and CA16R-3400 (antisense 5′-GCA AGG TGC CGA TTC ACT ACC CT-3′), which amplify 1371 bp corresponding to nucleotides (nt) 2030 to 3400 of G-10/South Africa/51, GenBank accession no. U05876 (23). For EV71, we used the primers 159 (sense 5′-ACY ATG AAA YTG TGC AAG G-3′, 2385-2403), 162 (antisense 5′-CCR GTA GGK GTR CAC GCR AC-3′, 2869-2850), 189 (sense 5′-CAR GCI GCI GAR ACI GGN GC-3′, 2612-2631), and 011 (antisense 5′-GCI CCI GAY TGI TGI CCR AA-3′, 3408-3389), which amplify a total of 1024 bp (nt 2385 to 3408 of BrCr/USA/70, U22521) (11,25).

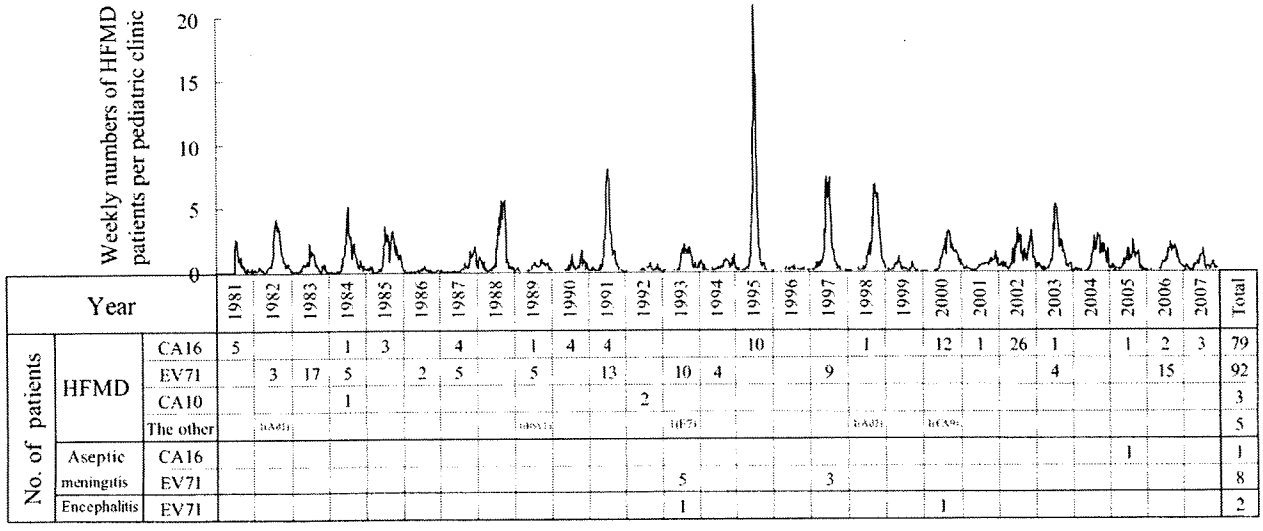
To determine the sequences of these viruses, the PCR products were directly applied for sequence analysis using an ABI Prism BigDye Terminators v3.1 cycle sequencing kit and an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, Calif., USA). The genogroups and subgenogroups were determined by comparing sequences with those of reference strains in GenBank (Table 1). The genetic relationship between the strains isolated in this study (Toyama strains) and the reference strains (8,11-13,15-21,23,24,26-29) were analyzed by MEGA 3.1 software (30), using the genomic sequences of the complete VP1 region of 891 bp (nt 2446 to 3336 for CA16, G-10/South Africa/51; 2439 to 3329 for EV71, BrCr/USA/70). A phylogenetic tree was constructed by the neighbor-joining method after estimation of genetic distance using the Kimura two-parameter method (31). A bootstrapping test was performed 1,000 times.

Nucleotide sequence accession numbers: Nucleotide sequences determined in this study were deposited in GenBank under accession no. AB465366 to AB465431.

RESULTS

Causative viruses of patients with HFMD: To determine the annual prevalence of HFMD, the numbers of patients were counted weekly at pediatric clinics from 1981-2007 (Fig. 1). As for monthly prevalence, HFMD occurred in either June to August, as typically observed in 1991 and 1997, or in August to November, as seen in 1988 and 1998. Remarkable prevalence was not observed in 1986, 1987, 1989, 1990, 1992, 1994, 1996, and 1999. The endemic was the largest in 1995, and comparatively large in 1984, 1988, 1991, 1997, 1998, and 2003, indicating that large outbreaks of HFMD occurred at an interval of several years.

The numbers of patients with HFMD, encephalitis, and aseptic meningitis from whom viruses were isolated are summarized in the lower part of Fig. 1. The numbers of patients with HFMD from whom CA16 and EV71 were detected dominated at 44.1% (79/177) and 51.4% (92/177), respectively. While CA16 was mainly isolated in 1995, 2000, and 2002, EV71 was dominantly isolated in 1983, 1991, 1993, 1997, 2003, and 2006. Therefore, the size of the annual preva-



			1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	Total
Year																														
No. of patients	HFMD	CA16	5			1	3		4		1	4	4				10			1		12	1	26	1		1	2	3	79
		EV71		3	17	5		2	5		5		13		10	4			9						4			15		92
		CA10				1								2																3
		The other		(Ad1)							(HSV1)				(E7)					(Ad1)		(CA9)								5
	Aseptic meningitis	CA16																									1			1
		EV71													5				3											8
	Encephalitis	EV71													1							1								2

Fig. 1. The numbers of hand, foot, and mouth disease (HFMD) patients and viruses detected from 1981 to 2007 in Toyama Prefecture, Japan. Upper chart: Weekly numbers of HFMD patients per pediatric clinic. Lower chart: The number of patients with HFMD detected CA16, EV71, CA10, and the other viruses, and the number of patients with encephalitis and aseptic meningitis detected CA16 and EV71. Ad1, adenovirus 1; HSV1, herpes simplex virus 1; E7, echovirus 7; CA9, coxsackievirus A9.

lence did not seem to correlate with the viral serotypes.

Phylogenetic analysis of CA16 and EV71 isolates: To examine the genogroups of CA16 and EV71, sequences of the complete VP1 region of the CA16 and EV71 isolates were determined, and phylogenetic analysis was conducted.

Genogroups of CA16 detected in 1981-1995 and 1998-2007 were classified into two genogroups, B and C (23), respectively (Fig. 2). The isolates of genogroup C were further divided into three lineages, tentatively named subgenogroups C1, C2, and C3 in this paper. The representative strains of each cluster were 260/Toyama/2002, 261/Toyama/2002, and 459/Toyama/2007, respectively. Differences of nucleotide sequences (total 891 nucleotides) among C1, C2, and C3 ranged from 3.7 to 8.0%. Remarkable differences in sampling areas, symptoms and ages of the subjects between C1 and C2 in 2002 were not observed, except the time-detected isolates when the former were in June to the beginning of July, and the latter at the end of July. Recent isolates in 2005-2007 were classified into subgenogroup C3, including 355/Toyama/2005, which was detected from a patient with aseptic

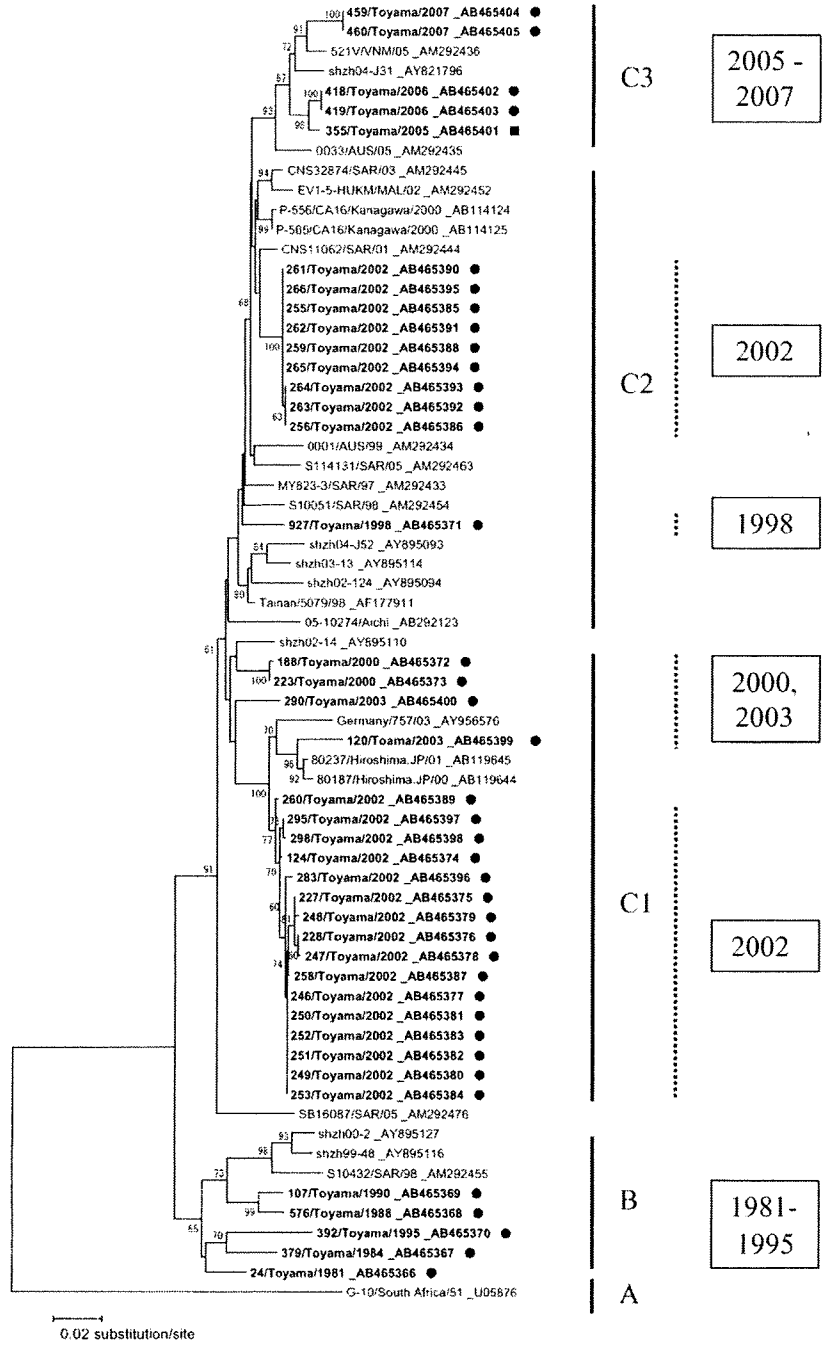


Fig. 2. Phylogenetic tree for CA16 using 891 nt, the complete VP1 region by the neighbor-joining method with isolates in Toyama and reference strain. Filled circles (●) and squares (■) indicate isolates in Toyama derived from patients with HFMD and aseptic meningitis, respectively. Isolates and reference strains were represented as strain's name _accession number. The scale bar denotes the genetic distance (nucleotide substitution per site).

meningitis complications.

The EV71 isolates were classified into five subgenogroups of B1, B4, C1, C3, and C4 (Fig. 3) (8,11,13,16,17,23). The isolates of EV71 in 1983, 1989, and 1994 were classified into B1, C1, and C3, respectively. 185/Toyama/2000 detected from patients with encephalitis belonged to subgenogroup B4. The isolates from patients with HFMD in 1997, 2003, and 2006 including 763/Toyama/1997 and 818/Toyama/1997 detected from patients with aseptic meningitis were classified into subgenogroup C4.

It thus appeared that the CA16 isolated in Toyama had predominantly changed from genogroup B to C in 1995-1998, and the genogroup C changed from C1 to C2 around 2002, and to C3 in 2005-2007. The subgenogroups of the EV71 isolates were classified into B1, B4, C1, C3 in 1983-1994, and C4 in 1997-2006.

Sequence diversities of CA16 and EV71 isolates: We compared nucleotide and amino acid sequences of the VP1 region to investigate diversities among Toyama strains. The differences in nucleotide sequences (total 891 nucleotides) of CA16 between genogroups B and C ranged from 5.8 to 11.0%, and those in the same subgenogroups of C1, C2, and

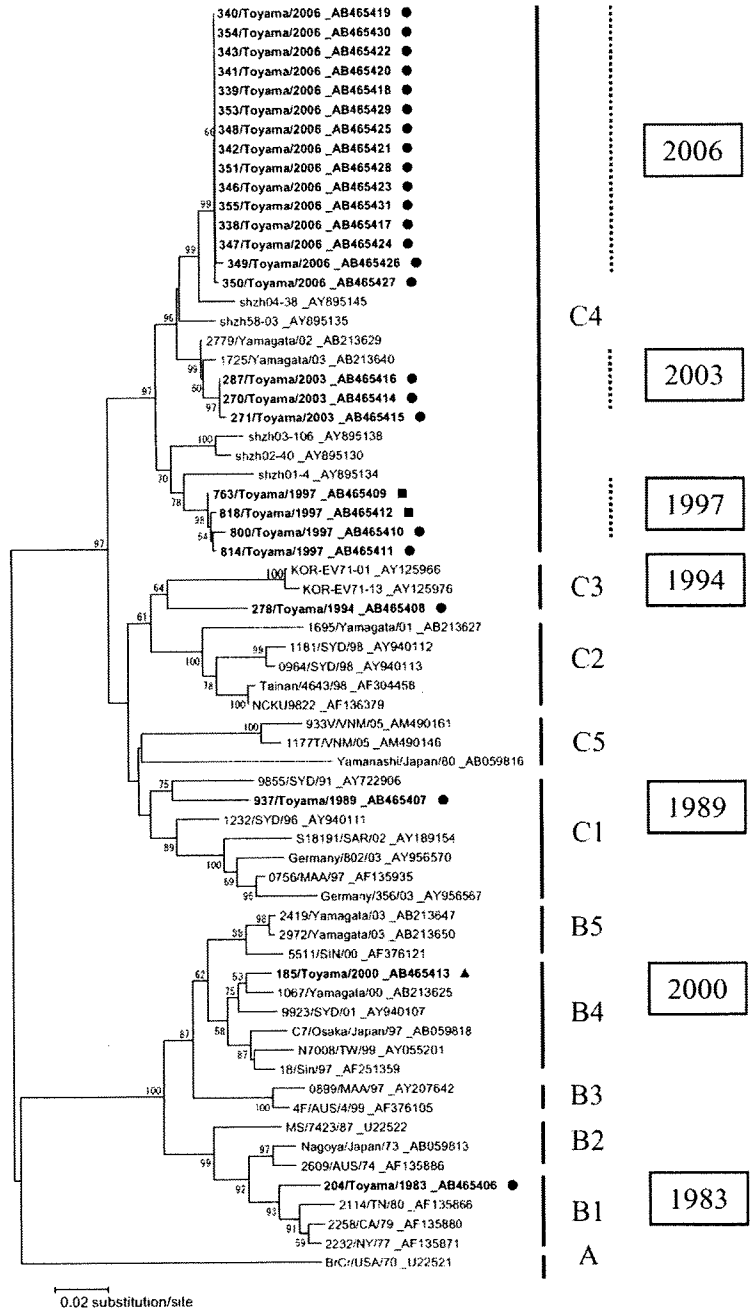
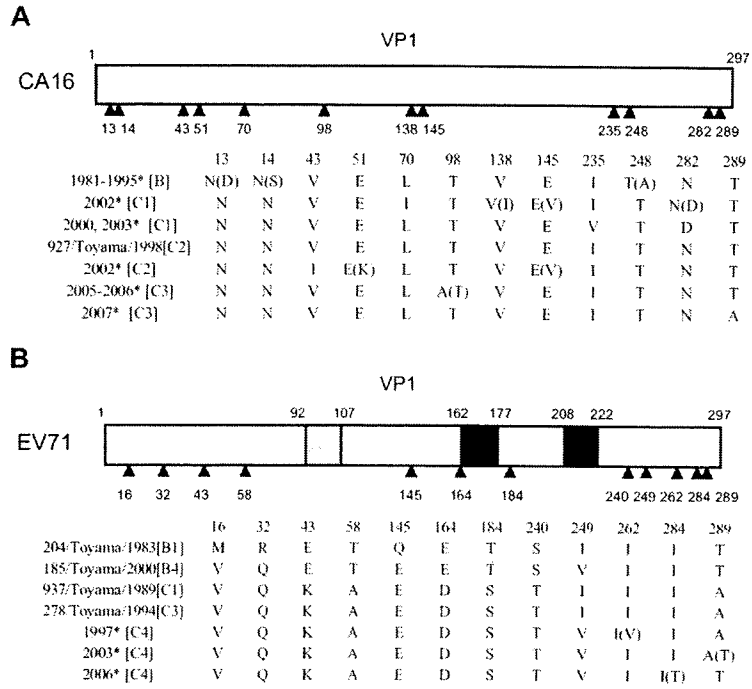


Fig. 3. Phylogenetic tree for EV71 using 891 nt, the complete VP1 region by the neighbor-joining method with isolates in Toyama and reference strain. Filled circles (●), triangle (▲), and squares (■) indicate isolates in Toyama derived from patients with HFMD, encephalitis, and aseptic meningitis, respectively. Isolates and reference strains were represented as strain's name _accession number. The scale bar denotes the genetic distance (nucleotide substitution per site).



	13	14	43	51	70	98	138	145	235	248	282	289
1981-1995* [B]	N(D)	N(S)	V	E	L	T	V	E	I	T(A)	N	T
2002* [C1]	N	N	V	E	I	T	V(I)	E(V)	I	T	N(D)	T
2000, 2003* [C1]	N	N	V	E	L	T	V	E	V	T	D	T
927/Toyama/1998[C2]	N	N	V	E	L	T	V	E	I	T	N	T
2002* [C2]	N	N	I	E(K)	L	T	V	E(V)	I	T	N	T
2005-2006* [C3]	N	N	V	E	L	A(T)	V	E	I	T	N	T
2007* [C3]	N	N	V	E	L	T	V	E	I	T	N	A

	16	32	43	58	145	164	184	240	249	262	284	289
204/Toyama/1983[B1]	M	R	E	T	Q	E	T	S	I	I	I	T
185/Toyama/2000[B4]	V	Q	E	T	E	E	T	S	V	I	I	T
937/Toyama/1989[C1]	V	Q	K	A	E	D	S	T	I	I	I	A
278/Toyama/1994[C3]	V	Q	K	A	E	D	S	T	I	I	I	A
1997* [C4]	V	Q	K	A	E	D	S	T	V	I(V)	I	A
2003* [C4]	V	Q	K	A	E	D	S	T	V	I	I	A(T)
2006* [C4]	V	Q	K	A	E	D	S	T	V	I	I(T)	T

Fig. 4. Variation of amino acid sequences in the VP1 region among isolates in Toyama. A: CA16. B: EV71. Filled triangles (▲) indicate amino acid residues that have variation. *: 1981-1995*, 2002*, 2000, 2003*, 2005-2006*, 2007*, 1997*, 2003*, and 2006* indicate the clusters, including the Toyama strains, isolated in each years. The abbreviation of amino acid in the parentheses shows variation of amino acids among the isolates that belonged to the same clusters.

C3 ranged from 0 to 4.6%, 0 to 3.5%, and 0 to 4.0%, respectively. The differences in nucleotide sequences of EV71 between different genogroups or subgenogroups ranged from 7.0 to 16.8%, and those in the same subgenogroups ranged from 0 to 4.8% (data not shown).

When the variations in amino acid sequences (total 297 amino acids) in the VP1 region among the CA16 or EV71 isolates were plotted, they were scattered in the entire region (Fig. 4). Most of the changes observed in CA16 were not accumulated, since the changes were temporary. The maximum number of different amino acids between strains of CA16 was only six, as observed between either genogroup B in 1981-1995 and subgenogroup C1 in 2002, or C1 and C2 in 2002 (Fig. 4A). On the other hand, 11 residues maximally differed between subgenogroup B1 in 1983 and C4 in 1997 of EV71 (Fig. 4B), since changes of amino acids were accumulated across the genogroups and subgenogroups. However, the amino acids of EV71 hardly changed among the same subgenogroup, for example, the C4 strains in Toyama, from 1997 to 2006.

DISCUSSION

In this study, we summarized the prevalence of HFMD in Toyama from 1981 to 2007. Large outbreaks of HFMD occurred at an interval of several years, which is consistent with the report by National Epidemiological Surveillance of Infectious Diseases in Japan showing that CA16 and EV71 are detected every year, and EV71 has been predominant every 3 years since 1994 (1,2). Since there are some patients with HFMD in winter, CA16 and EV71 seem to exist year round in the community in Toyama.

As for recent CA16 isolates in Asia, genogroup B was detected in 1999-2000, 1998-2000, and 1987-1998 and genogroup C was isolated in 1999-2004, 1997-2005, and 1995-2003 in China (23), Malaysia (24), and Fukushima (22), respectively. Moreover, the strains detected in Hiroshima from 2000-2001 and the strain of Aichi in 2005 were respectively classified as subgenogroups C1 and C2. As for EV71, while recent isolates in Toyama were classified as C4, C4 was also found in Yamagata in 2003 (16), China in 1998-2004 (23), and Vietnam in 2005 (20). It thus appeared that recent genogroups of CA16 and EV71 in Toyama were similar to those detected in these countries.

There are several factors influencing the large prevalence of HFMD occurring at intervals of several years. HFMD is prevalent mainly in young children aged 0 to 6 years old. A seroepidemiological study by Hagiwara et al. has shown that whether EV71 causes an endemic or an epidemic depends on the seropositive rate among children (32,33). They reported that the age distribution possessing the antibody against EV71 gradually shifted to a higher age until the next prevalence occurred. Therefore, once children are immunized by viruses, it may take several years to have another sensitive generation.

We found that there were only small amino acid variations of the VP1 region that correlate to the serotype of enteroviruses and play an important role in characterizing antigenicity (21) among the Toyama strains, since the variations were restored in CA16, and were not observed among the same subgenogroups of EV71. Only one variation of E or D at 164 between genogroups B and C of EV71 was detected in neutralization epitopes of the VP1 region (amino acid residues 162-177 and 208-222) (34), and no variation in the extended BC loop (residues 92-107), which is predicted to be immunogenic (35). These facts suggest that the VP1 region has not easily altered its antigenicity to escape immune pressure. Consistently, antibodies against EV71 have reportedly

shown a broad spectrum of neutralizing activity over a wide range of subgenogroups, even though they showed higher activity on the same subgenogroups than on different ones (14,36). Therefore, the increased numbers of HFMD cases every few years may correspond to the accumulating numbers of immunologically naive children between outbreaks. However, major antigenic epitopes of CA16 have not been determined, and other antigenic determinants in the VP2 or VP3 region instead of the VP1 region may exist in CA16 and EV71 (35,37). Further analyses are required to clarify these concepts.

In conclusion, our study showed that prevalent genogroups of CA16 and EV71 isolates in Toyama had shifted from B to C, but amino acid variations in the VP1 region of those isolates were small. Changes of the nucleotide sequences in the VP1 region of the Toyama strains might not be related to the viral antigenic changes.

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Continuous Presence of Noroviruses and Sapoviruses in Raw Sewage Reflects Infections among Inhabitants of Toyama, Japan (2006 to 2008)[▽]

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Various genotypes of norovirus (NoV) (genogroup I genotype 1 [GI.1], -2, -4, -5, -8, -11, -12, and -14; GII.3, -4, -6, -7, -10, -13, -14, and -15), and sapovirus (SaV) (GI.1 and GI.2, GII.1, and GIV.1) were detected from raw sewage from April 2006 to March 2008, while limited numbers of genotypes of NoV (GI.8, GII.4, GII.6, and GII.13) and SaV (GII.3 and GIV.1) and of NoV (GII.4, GII.7, and GII.13) were detected from clinical cases and healthy children, respectively. During the winter 2006 to 2008, a large number of sporadic gastroenteritis outbreaks and many outbreaks caused by NoV GII.4 occurred among inhabitants in Toyama, Japan. The copy number of genomes of NoV GII detected from raw sewage changed in relation to the number of outbreaks. NoV strains of the same genotypes observed in both raw sewage and human specimens belonged to the same cluster by phylogenetic analysis and had almost identical nucleotide sequences among each genotype. These data suggest that NoVs and SaVs detected from raw sewage reflect the viruses circulating in the community, irrespective of symptoms, and that subclinical infections of NoV are common in Japan. Combined surveys of raw sewage with those of clinical cases help us to understand the relationship between infection of these viruses and gastroenteritis.

Norovirus (NoV) and sapovirus (SaV), members of the *Caliciviridae* family, are considered to be a major cause of acute gastroenteritis in humans. Both NoV and SaV infect humans via the fecal-oral route and cause family or community-wide outbreaks, mainly in the winter season. NoVs are shed in feces at a level of 10^5 to 10^9 virus particles per gram during the symptomatic phase (32, 37), and viruses are continuously shed from patients after cessation of the symptoms (28, 37, 40). In addition, recent reports showed relatively high levels of shedding of the viruses from asymptomatic individuals (7, 8, 32, 37).

NoVs and SaVs show high diversity in their genomes (5, 9). According to such a genetic diversity, they are classified into several genogroups (genogroup I [GI], GII, and GIV for human NoV and GI, GII, GIV, GV for human SaV) and further divided into many genotypes (NoV GI genotypes 1 to 14 [GI.1-14] and GII.1-17 and SaV GI.1-5, GII.1-6, GIV.1, and GV.1) (10, 17, 18). In 2006 to 2007, NoV GII.4 caused a large number of outbreaks of acute gastroenteritis worldwide (1, 11, 35, 43, 45). However, the other genotypes of NoV and SaV may infect humans asymptomatically and persist in the environment.

Raw sewage could contain enteric viruses shed from affected people, and therefore, detectable viruses in raw sewage would reflect the actual state of the circulating viruses in the area. We previously reported that polioviruses in raw sewage and river water were isolated at the same time as oral vaccination in babies, and these isolates were derived from vaccine strains (13, 30). We also showed that the nucleotide sequences of echovirus type 13 isolated from river water were closely related to those from patients with aseptic meningitis during the outbreak in 2002 (14). For NoVs and SaVs, many epidemiological surveys have been conducted to determine the prevalence and virological properties of these viruses (42). Previous reports have shown that the nucleotide sequences of NoV strains from stools of outbreaks in nursing homes and from sewage were identical for an individual outbreak (26), and NoVs detected from gastroenteritis patients, domestic sewage, river water, and cultivated oysters in the area were related to each other (44). However, less is known about infection of the viruses with minor genotypes that are silently circulating in the population.

In this study, we investigated NoVs and SaVs in raw sewage from 2006 to 2008 in Japan and compared the results with the viruses detected from clinical cases as well as healthy individuals to show the comprehensive prevalence of these viruses in the community.

MATERIALS AND METHODS

Samples and preparation of viral suspension. (i) Raw sewage. Raw sewage was collected monthly from April 2006 to March 2008 at the threshold point of a waste tank in the sewage disposal plant located in Toyama Prefecture, Japan. This facility covers an area with about 300,000 inhabitants, which is the largest group served by 29 sewage disposal plants for a population of 1,100,000 in Toyama Prefecture. The raw sewage from each household in the area reaches the facility within 4 to 8 h. The temperature of raw sewage ranged from 13.8°C to 25.0°C during the year. The average inflows of raw sewage per day in the fiscal year 2007 were 46,063 m^3 (70.9%), 2,535 m^3 (3.9%), and 16,323 m^3 (25.2%) derived from household sewage, industrial wastewater, and unidentified wastewater, respectively.

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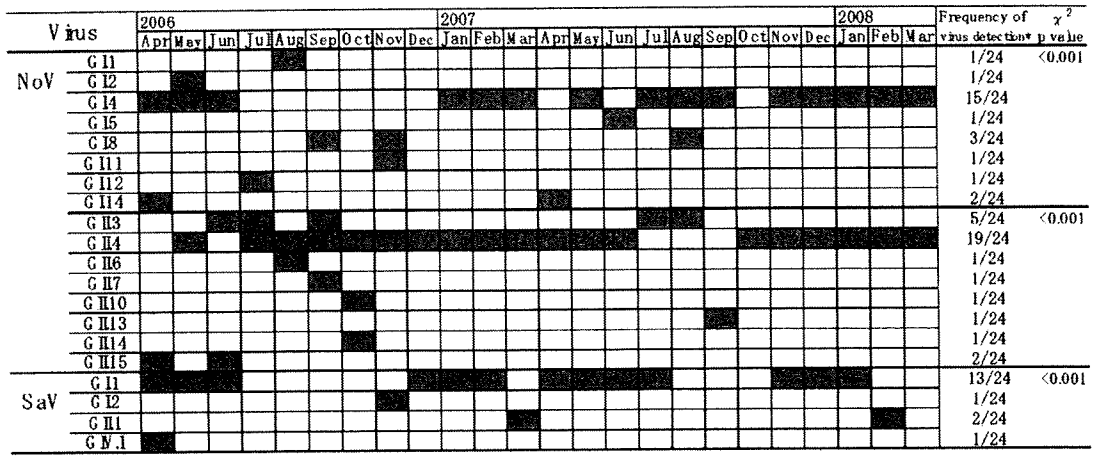

FIG. 1. Genotypes of NoV and SaV detected from raw sewage (2006 to 2008). Shading indicates times of virus detection. Frequency of virus detection (*) is defined as the ratio of the number of months when viruses were detected to total number of months in the investigation period.

Two liters of raw sewage was centrifuged at 3,000 rpm for 30 min (4°C), and the supernatants were applied for subsequent concentration of viruses using the filter adsorption and elution method and the polyethylene glycol (PEG) precipitation method, as described previously (24, 29).

For the filter adsorption and elution method, $MgCl_2$ (final concentration, 0.05 M) was added to 1 liter of the supernatant of the raw sewage, and the pH was adjusted to 3.5 with HCl. The supernatant was filtered through a mixed cellulose ester-type membrane to adsorb viruses. Then, the membranes were soaked in 10 ml of 3% beef extract solution, and the viruses were eluted by sonication.

For the PEG precipitation method, 80 g of PEG 6000 (WAKO) was added to 1 liter of the supernatant of raw sewage, and the supernatant was stirred for 2 h at 4°C to suspend PEG 6000. After the suspension was centrifuged at 10,000 rpm for 30 min (4°C), the pellet was collected and dissolved in 4 ml of 0.15 M Na_2HPO_4 (pH 9.0). The solution was recentrifuged at 10,000 rpm for 30 min (4°C) to recover the supernatant. The eluted solution or the recovered supernatant was used for viral RNA extraction.

(ii) Stool specimens. A total of 805 fecal specimens were collected from hospital patients with gastroenteritis or from outbreaks from April 2006 to March 2008. An outbreak was defined as occurring when at least three patients from the same area came down with similar clinical symptoms at roughly the same time. A total of 780 of the 805 samples could be assigned to 59 outbreaks according to the above definition. The remaining 27 samples were considered cases of sporadic gastroenteritis diagnosed at pediatric clinics.

With permission from guardians, 134 fecal specimens were collected from healthy children from 2006 to 2008. The ages of the healthy children ranged from <1 to 6 years old. The specimens were originally collected for a survey of poliovirus in September and January in 2006 to 2008, which is at least 2 months after the vaccination for poliovirus. Personal information of donors was disconnected and deidentified from the samples.

A 10% (wt/vol) suspension of stool was prepared by mixing with phosphate-buffered saline, followed by centrifugation at 13,000 rpm for 30 min (4°C). The supernatant fluids were used for viral RNA extraction.

Reverse transcription-PCR and genotyping. Viral RNA was extracted from 140 μl of concentrated raw sewage or supernatant of stool suspension, using a QIAamp viral minikit (Qiagen) according to the manufacturer's procedure. Extracted RNA was treated with 5 U of DNase I (TaKaRa), and cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen) with a random hexamer, according to the manufacturer's instructions. The cDNA was used for PCR and real-time PCR.

For NoV PCR, we used the primers SK1F (sense; 5'-CTG CCC GAA TTY GTA AAT GA-3'), SK1R (antisense; 5'-CCA ACC CAR CCA TTR TAC A-3'), SK2F (sense; 5'-CNT GGG AGG GCG ATC GCA A-3'), and SK2R (antisense; 5'-CCR CCN GCA TRH CCR TTR TAC AT-3'), which amplify 330 bp corresponding to nucleotides (nt) 5342 to 5671 of Norwalk/68/US (GenBank accession number M87661) for GI or 344 bp (nt 5046 to 5389) of Lordsdale/93/UK (accession no. X86557) for GII, encompassing the 3' end of ORF1 to the beginning of the capsid region, as described by Kojima et al. (21). For SaV PCR, we used the primers SV-F11 (sense; 5'-GCY TGG TTY ATA GGT GGT AC-3') and SV-R1 (antisense; 5'-CWG GTG AMA CCA TTK TCC AT-3'), which amplify about 780 bp (nt 5098 to 5878 of Manchester virus; accession number X86560) of the N-terminal of VP1 region, as described by Okada et al. (36). To determine the genotypes of these viruses, the PCR products were directly applied for sequence analysis using an ABI Prism BigDye Terminator, version 3.1, cycle sequencing kit and an ABI Prism 3100 DNA sequencer (Applied Biosystems). TA cloning (TOPO TA cloning kit; Invitrogen) of the NoV GII PCR products derived from raw sewage from July to October in 2006 was also performed to detect mixed populations of NoV GII.4 because the predominant genotype of NoV GII.4 had changed from 2006a and Chiba-4e to 2006b during that time, as described in the Results section. We sequenced 25 amplicons per month. The genotypes were determined by comparing sequences with those of reference strains in the GenBank (6, 10, 17, 18, 25, 35, 41, 42). The genetic relationship between the strains in this study (Toyama strains) and reference strains was analyzed by MEGA, version 3.1, software (23), using the genomic regions described above. The phylogenetic tree was constructed by the neighbor-joining method after estimation of genetic distance using the Kimura two-parameter method. A bootstrapping test was performed 1,000 times.

Real-time PCR. The cDNA samples of NoV were used for the TaqMan-based real-time PCR as previously described by Kageyama et al. (16). NoV probes labeled with the TaqMan dye VIC were synthesized at Applied Biosystems Japan, Ltd.

Counting of coliforms. The amounts of coliforms in raw sewage were measured using the pour plate method with desoxycholate agar (39). Briefly, each 1 ml of a 10-fold dilution of raw sewage was spread into the 90-mm-diameter dishes and then mixed with 20 ml of desoxycholate agar medium. The dishes were incubated at 35°C overnight. The number of colonies of coliforms was counted and defined as the number of CFU.

Statistical number of pediatric patients with gastroenteritis. The number of patients with gastroenteritis was counted according to the reports from 29 out of 141 pediatric clinics (20.6%) in Toyama Prefecture, from April 2006 to March 2008.

Nucleotide sequence accession numbers. Nucleotide sequences determined in this study were deposited in the GenBank database under accession numbers AB437141 to AB437235.

RESULTS

NoVs and SaVs in raw sewage. NoVs and SaVs detected from raw sewage from April 2006 to March 2008 are summarized in Fig. 1. Both the GI and GII genogroups of NoVs were observed almost every month during the survey period. They included eight genotypes of GI (GI.1, -2, -4, -5, -8, -11, -12, and -14) and eight genotypes of GII (GII.3, -4, -6, -7, -10, -13, -14, and -15). NoV GII.4 was the most frequently detected among the genotypes of NoVs, especially in winter and spring. NoV GI.4 was detected with the second highest frequency. Four

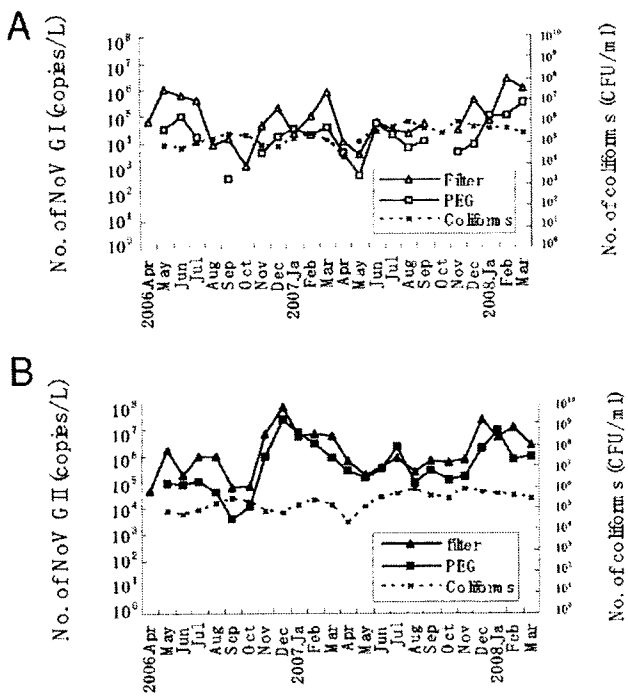

FIG. 2. Quantification of NoV in raw sewage. (A) Values indicate the number of NoV GI genome copies per liter of raw sewage concentrated by the filter absorption and elution method (filter) and the PEG precipitation method. (B) Values are the number of NoV GII genome copies per liter of raw sewage concentrated by each of the indicated methods. The dotted lines indicate the amounts of coliforms per milliliter of raw sewage.

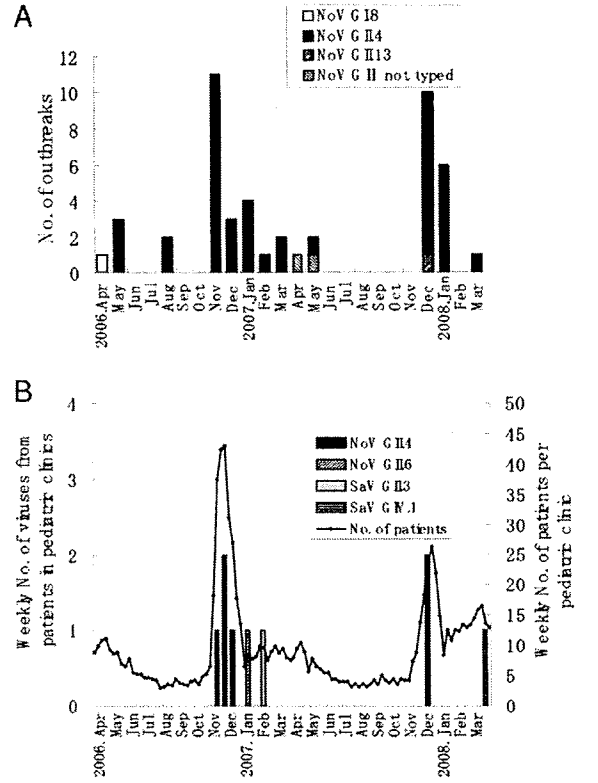

FIG. 3. (A) Numbers of outbreaks caused by NoVs in Toyama Prefecture (2006 to 2008). Values are the instances caused by NoV GI.8, GII.4, and GII.13 and instances caused by infection with NoV II agents whose genotypes were not determined, as indicated on the figure. (B) Weekly number of viruses detected from children with gastroenteritis in pediatric clinics and weekly number of patients with gastroenteritis per pediatric clinic in Toyama Prefecture, Japan (2006 to 2008). Data represent patients with NoV GII.4, NoV GII.6, SaV GII.3, and SaV GIV.1, as indicated. The dots indicate the weekly mean number of patients with gastroenteritis from 29 pediatric clinics from April 2006 to March 2008.

genotypes of SaVs, GI.1, GI.2, GII.1, and GIV.1, were observed, and GI.1 was the most frequently detected among four genotypes. The frequencies of detection of NoVs GI.4, GII.4, and SaV GI.1 were significantly high between each genotype of NoV GI, NoV GII, and SaV, respectively (χ^2 test, $P < 0.001$).

Thus, various genotypes of NoV and SaV were detected from raw sewage, while NoV GII.4 was predominantly detected in winter and spring.

Quantification of NoVs in raw sewage. We quantified the NoVs in raw sewage using real time PCR to examine the seasonal changes. To concentrate viruses, both the filter adsorption and elution method and the PEG precipitation method were employed since efficiency or preference of virus detection is believed to be different between these two methods. Although the filter adsorption-elution method was found to be more sensitive than the PEG precipitation method, both methods showed similar profiles of seasonal change (Fig. 2).

The raw sewage contained not only human excrement but also drainage, such as from a factory; therefore, the degree of dilution of raw sewage might differ every day. Therefore, we measured the number of coliforms as an index for the dilution level of raw sewage. The number of coliforms in raw sewage ranged from 2.0×10^4 to 8.1×10^5 CFU/ml, and the geometric mean titer was 2.0×10^5 CFU/ml (Fig. 2). The number of coliforms showed only a small dispersion (coefficient of variation [CV], 0.076) during the survey period. It indicates that the input of raw sewage into the sewage disposal plant is almost uniform. Furthermore, there was no correlation between levels of coliforms and either NoV GI or NoV GII ($r = -0.0751$ or $r = -0.0331$, respectively; $P < 0.05$).

The copy number of NoV GI in raw sewage ranged from 4.6×10^2 to 2.3×10^6 copies/liter (CV, 0.17) during the survey period, except for October 2006, when NoV GI was not detected. Although smaller amounts of NoV GI in raw sewage were observed than NoV GII, as described later, sewage tended to contain larger amounts of NoV GI in the period from winter to spring than from summer to fall (Fig. 2A).

The copy number of NoV GII ranged from 3.8×10^3 to 7.1×10^7 copies/liter (CV, 0.14). The amount of NoV GII was 10^4 to 10^6 copies/liter in July to November, and 10^6 to 10^8 copies/liter in December to June, indicating a clear correlation between the amount of NoV GII and the number of outbreaks (Fig. 3). Raw sewage contained a higher amount of NoV GII genome than NoV GI during the survey period.

NoVs and SaVs detected from clinical cases. Fifty-nine outbreaks of gastroenteritis occurred, mainly in November to January, during the survey period. Most outbreaks were caused by NoV GII.4, except for two instances caused by GI.8 in April

TABLE 1. Viruses detected from feces of healthy children in Toyama, Japan, 2006 to 2008

Date of sample	Age of subject (yr)	No. of stool samples	No. of samples (%) positive for[a]:		
			NoV GII.4	NoV GII.7	NoV GII.13
September 2006	<1	12	1		
January 2007	<1	2	1		
	1	10	1	5	2
	2	2			
	3	21			
	4	0			
	5	5			
	6	21		1	6
September 2007	<1	10			
January 2008	1	15			
	2	3			
	3	9			
	4	8			
	5	3			
	6	13			
Total		134	3 (2.2)	6 (4.5)	8 (6.0)

[a] No samples were positive for either NoV GI or SaV.

2006 and GII.13 in December 2007 (Fig. 3A). Genotypes were not determined in two instances. No outbreak caused by SaV occurred.

In 2006 and 2007, the total number of patients with gastroenteritis reported from pediatric clinics sharply increased in early November and then decreased in January (Fig. 3B). Gastroenteritis occurred in the winter season of 2006 to 2007 more frequently than in the season of 2007 to 2008. NoV GII.4 was detected from six patients diagnosed as sporadic gastroenteritis (Fig. 3B). NoV GII.6 and SaV GII.3 and GIV.1 were also observed from one patient each (Fig. 3B). For the other viruses, rotavirus group A (seven children), astrovirus (one child), adenovirus (two children), and parechovirus type 1 (one child) were also detected is samples from children with gastroenteritis (data not shown).

Thus, while NoV GII.4 was the main cause of outbreaks, NoV GII.4 and rotavirus group A were predominant among children with gastroenteritis in Toyama Prefecture from 2006 to 2008.

NoVs and SaVs detected from healthy children. Since the viruses detected from raw sewage were supposed to be of human origin, we investigated whether they existed in healthy individuals. For this purpose, we examined 134 available stool samples from healthy children and found that NoVs were detected in 17 stools (12.7%) (Table 1). Whereas NoV GII.4 was observed in three samples (2.2%), NoV GII.7 and GII.13 were detected in six (4.5%) and eight (6.0%) samples, respectively. Most NoVs derived from healthy children were observed in January 2007 when NoV GII.4 was prevalent in gastroenteritis cases (Fig. 3). Further investigation throughout the year will be needed to verify the presence of these viruses among overall healthy inhabitants.

These results indicate that there were certain healthy children shedding at least NoV GII.4, GII.7, and GII.13 in the winter of 2006 to 2007.

Phylogenetic analysis of NoVs detected from raw sewage and human specimens. The genetic variations of NoV GI.8 and GII.4, -7, and -13 strains detected from raw sewage were compared with those from human clinical cases by phylogenetic analysis (Fig. 4). NoV GII.4 strains were divided into three clusters: the types of 2006a, 2006b, and Chiba-4e (Fig. 4A) (6, 35, 41, 42). While a strain detected from raw sewage in May 2006 and the strains in July and August 2006 belonged to GII.4 strain 2006a (GII.4/2006a) and GII.4/Chiba-4e, respectively, most of the GII.4 strains from raw sewage belonged to the 2006b cluster. All GII.4 strains derived from patients with gastroenteritis and from healthy children also belonged to the 2006b cluster, except for two GII.4/2006a strains from outbreaks in May 2006 and one GII.4/Chiba-4e strain from a healthy child in September 2006 (Fig. 4B). In addition, the NoV GII.7, GII.13, and GI.8 strains detected from raw sewage formed a cluster with strains detected from healthy children or clinical cases. The identities of nucleotide sequences in 302 bases of the partial capsid regions among these strains were 96.4 to 97.4%, 98.0 to 99.3%, and 99.3 to 100%, respectively (Fig. 4A). Thus, the genotypes of NoVs detected from raw sewage showed a close relationship with those from human cases.

DISCUSSION

In this study, we compared NoVs and SaVs detected from raw sewage with those from human specimens. From 2006 to 2008, especially in winter, a large number of sporadic gastroenteritis cases and many outbreaks caused by NoV GII.4 occurred in Toyama, Japan (Fig. 3). NoV GII.4 was also predominantly detected from raw sewage in winter. In addition, the copy number of NoV GII in raw sewage of the winter season of 2006 to 2007 was higher than that of 2007 to 2008 (Fig. 2B), a result that correlates well with the prevalence of gastroenteritis and the number of outbreaks caused by NoV GII.4 infection. Clinical outbreaks preceded the high counts of NoV GII in raw sewage. Phylogenetic analysis showed that the nucleotide sequences of these NoV GII.4 strains were closely related to each other. Therefore, NoVs GII in raw sewage are thought to reflect mainly NoV GII.4 derived from clinical cases.

At least three clusters of NoV GII.4, 2006a, 2006b, and Chiba-4e, appeared to exist in Toyama Prefecture from 2006 to 2008. In Japan including Toyama, NoV GII.4/2006b has been dominantly prevalent since 2006, whereas a few NoV GII.4/2006a strains were detected from patients with gastroenteritis (20, 33). It is uncertain whether NoV GII.4/2006a and GII.4/Chiba-4e were locally extinct or persisted at low levels in Toyama Prefecture. Because NoV GII.4/2006a and GII.4/2006b epidemics had occurred in European countries beginning in December 2005 (22), these three clusters of NoV GII.4 might have migrated from Europe although migration routes have not been clarified.

NoV GI.8 and GII.13 were less frequently observed in outbreaks and were also detected from raw sewage, indicating that raw sewage contained minor genotypes of NoVs in the environment. Because GI.8 was still detected in sewage more than 1 year after the outbreak, GI.8 seemed to circulate over a long period of time in the community.

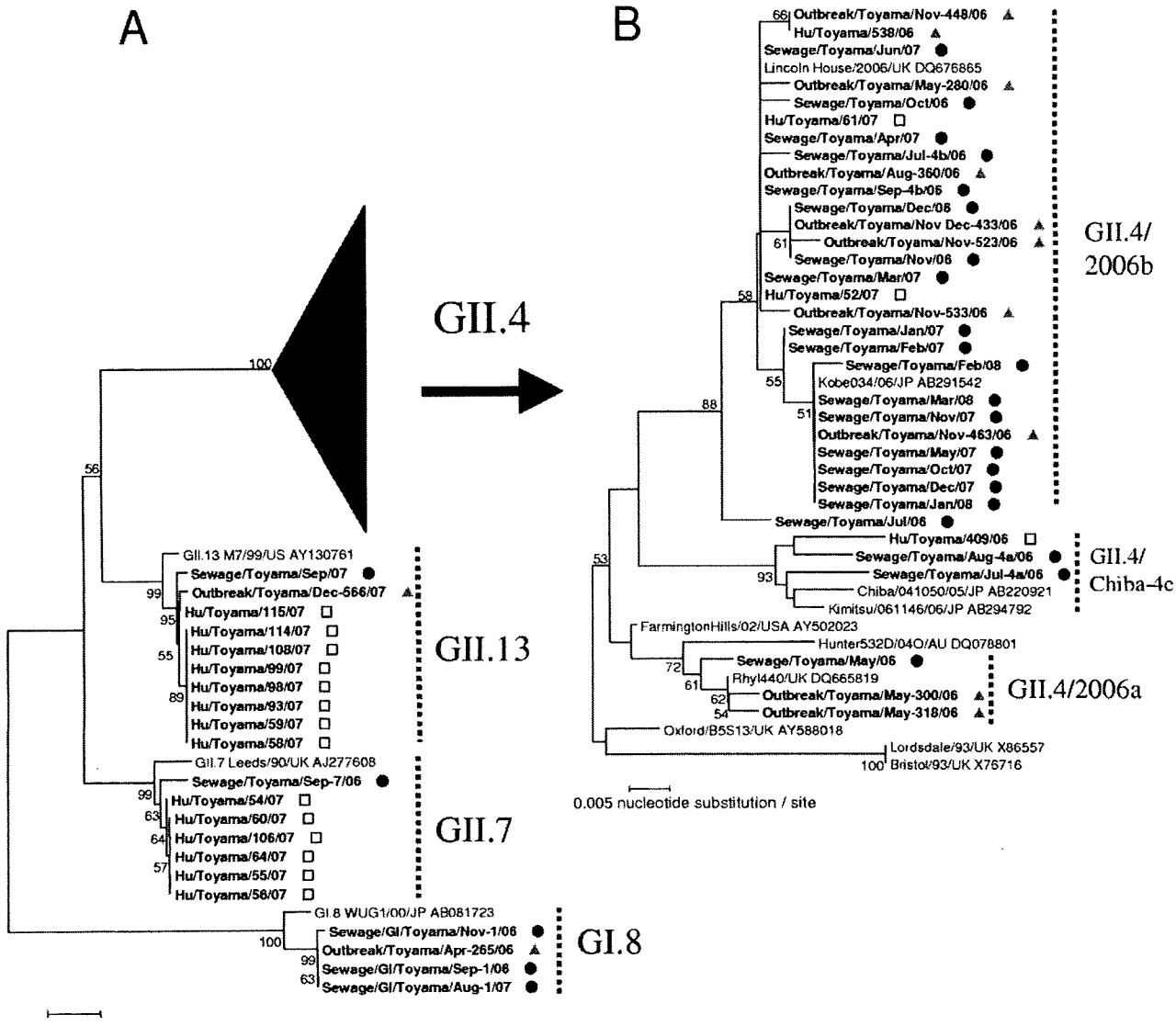

FIG. 4. Phylogenetic tree for NoV (GII.4, GII.7, GII.13, and GI.8) using about 300 nt from the 3′ end of ORF1 to the beginning of the capsid region; the tree was generated by the neighbor-joining method with Toyama strains derived from raw sewage and reference strains. (A) Phylogenetic tree of the strains of NoV GII.4, GII.7, GII.13, and GI.8. (B) Part of the phylogenetic tree for the strains of NoV GII.4 was magnified with a modification of branch indexes. Filled circles (●) and open squares (□) indicate the Toyama strains derived from raw sewage and asymptomatic healthy children, respectively. Filled triangles (▲) indicate the strains detected from the patients with gastroenteritis in hospitals or outbreaks from April 2006 to March 2008.

Various genotypes of NoV (GI.1, -2, -4, -5, -8, -11, -12, and -14; GII.3, -4, -6, -7, -10, -13, -14, and -15) and SaV (GI.1, GI.2, GII.1, and GIV.1) found in raw sewage are predicted to have originated from infected subjects. Among them, NoV GII.7, which was not detected in clinical cases, was found in raw sewage in September 2006 and in six stool specimens of healthy children in January 2007. Moreover, GII.7 and GII.13 were found more frequently than GII.4 in healthy children. These findings suggest that certain NoVs are shed from healthy children and that the population retains these viruses.

Although some other genotypes of NoV and SaV in raw sewage did not correlate with those from clinical cases, our findings suggest that they are also circulating in the environment throughout the year. NoV GI.4 was consistently detected in raw sewage but was not detected from fecal specimens of patients with gastroenteritis or from healthy children. The origin of NoV GI.4 remains to be clarified. Recent work by Okabayashi et al. showed that NoV GII.2, GII.3, GII.8, and GII.12 were detected from asymptomatic food handlers in 2005 and 2006 but not NoV GII.4, despite many outbreaks (34). Healthy adults may be infected with various genotypes of viruses that differ from the prevalent ones causing gastroenteritis. However, these viruses have the potential to be a source of an endemic or epidemic.

In a Mexican study by García et al. (7), nine different genotypes of NoV (GI.1, -3, -5, -7, and -14; GII.1, -2, -7, and -17) were detected in 48 out of 161 stool specimens (29.8%) from asymptomatic children under 2 years of age in June to August 1998. In an Indian study by Monica et al. (31), SaVs (GI.1, -2, and -3; GII.1 and -2) and NoVs (GI.3; GII.2, -3, and -4) were positive in 6 (3.5%) and 7 (4.0%) out of 173 asymptomatic children, respectively, under 3 years old living in an urban slum community in 2001 to 2004. On the other hand, a study in Australia by Marshall et al. (27) showed that NoV was not detected from 399 asymptomatic individuals aged between 5 months and 52 years in July to August 1997. Variation in the detection rate may depend on the differences in sanitation, such as the distribution of the sewage system, age groups of examinees, and methods of viral detection. Generally, improvement of waterworks or sewage facilities is necessary to prevent the transmission of enteric pathogens that infect humans by the fecal-oral route. However, even though the sewage facilities are widely maintained in the Toyama area (86.2% of the population was provided sewage facilities, and most of the rest treated wastewater individually, according to the Toyama Prefectural government in March 2006), the influence of the wastewater that bypasses the treatment system on viral prevalence could not be eliminated. Furthermore, Ueki et al. (44) reported that a few NoV genomes were also detected from treated wastewater of a sewage facility because of the difficulty to inactivate NoV thoroughly by present sewage treatments. The leakage of the NoVs from the sewage treatment system might be an additional cause of the viral prevalence. Continuous existence of NoVs is also probably due to their genetic and antigenic diversity that result from the high mutation rate (9, 25, 41). Another reason seems to be the physical stability of virions in the environment (3, 4, 19) and refractoriness against serum antibodies (2, 15, 38). Moreover, small numbers of NoV virions are reportedly able to establish infection in humans (3, 12), resulting in the easy expansion of viruses in the community. Thus, some genotypes of NoVs may infect healthy children, such as Mexican and Indian children, and outbreaks of gastroenteritis occur every year (7, 31). This study suggests that certain NoVs continuously exist in the community though certain NoVs can become locally extinct. Therefore, surveillance of circulating viruses in the inhabitants is necessary to control and prevent infection by NoVs and SaVs. The above concept correlates with our previous reports showing environmental surveillance of polioviruses and echoviruses that are mostly asymptomatic (14, 30). It is important to inform public health officials about the continuous existence of NoVs and SaVs in the community to prevent outbreaks among inhabitants.

In conclusion, NoVs and SaVs detected from raw sewage reflect their prevalence and circulation in the inhabitants, regardless of symptoms. A combination of the surveys of raw sewage with those of clinical cases helps us to understand the relationship between infection with these viruses and gastroenteritis.

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Cellular kinase inhibitors that suppress enterovirus replication have a conserved target in viral protein 3A similar to that of enviroxime

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Previously, we identified a cellular kinase inhibitor, GW5074, that inhibits poliovirus (PV) and enterovirus 71 replication strongly, although its target has remained unknown. To identify the target of GW5074, we searched for cellular kinase inhibitors that have anti-enterovirus activity similar or related to that of GW5074. With this aim, we performed screenings to identify cellular kinase inhibitors that could inhibit PV replication cooperatively with GW5074 or synthetically in the absence of GW5074. We identified MEK1/2 inhibitors (SL327 and U0126), an EGFR inhibitor (AG1478) and a phosphatidylinositol 3-kinase inhibitor (wortmannin) as compounds with a cooperative inhibitory effect with GW5074, and an Akt1/2 inhibitor (Akt inhibitor VIII) as a compound with a synthetic inhibitory effect with MEK1/2 inhibitors and AG1478. Individual treatment with the identified kinase inhibitors did not affect PV replication significantly, but combined treatment with MEK1/2 inhibitor, AG1478 and Akt1/2 inhibitor suppressed the replication synthetically. The effect of AG1478 in this synthetic inhibition was compensated by other receptor tyrosine kinase inhibitors (IGF-1R inhibitor II and Flt3 inhibitor II). We isolated mutants resistant to Flt3 inhibitor II and GW5074 and found that these mutants had cross-resistance to each treatment. These mutants had a common mutation in viral protein 3A that results in an amino acid change at position 70 (Ala to Thr), a mutation that was previously identified in mutants resistant to a potent anti-enterovirus compound, enviroxime. These results suggest that cellular kinase inhibitors and enviroxime have a conserved target in viral protein 3A to suppress enterovirus replication.

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INTRODUCTION

The genus *Enterovirus* consists of at least ten species of non-enveloped viruses with a single-stranded, positive-sense genomic RNA that belong to the family *Picornaviridae*. Enterovirus infection is mostly asymptomatic, but sometimes causes severe neurological symptoms exemplified by poliomyelitis. In infection by poliovirus (PV), which is the causative agent of poliomyelitis and belongs to the species *Human enterovirus C*, motor neurons are the major target for neurovirulence (Bodian, 1949). Enterovirus 71 (EV71), another neurotropic enterovirus belonging to the species *Human enterovirus A*, is a causative agent of hand, foot and mouth disease and herpangina, but sometimes causes severe neurological diseases, such as brainstem encephalitis and poliomyelitis-like paralysis (Chumakov *et al.*, 1979; McMinn, 2002; Wang *et al.*, 2003). EV71 causes fatal pulmonary oedema and pulmonary haemorrhage in young children by destruction of the vasomotor and respiratory centres in the brainstem (Chang *et al.*, 1999; Ho *et al.*, 1999; Huang *et al.*, 1999; Komatsu *et al.*, 1999; Lum *et al.*, 1998; Wang *et al.*, 1999). For PV, live-attenuated and inactivated vaccines have been established and are currently being used for global eradication of poliomyelitis (Sabin, 1965; Salk *et al.*, 1954). However, there is no effective therapeutic means for vaccine-associated paralytic poliomyelitis caused by virulent revertants of vaccine strains, which occurred at a rate of one case per 520 000 vaccinations with the first dose of the live-attenuated vaccine (Nkowane *et al.*, 1987). For EV71, various vaccine candidates and therapeutic means are being developed, but no vaccine has been established (Arita *et al.*, 2005a, 2007; Chen *et al.*, 2006; Chiu *et al.*, 2006; Liu *et al.*, 2005, 2007; Shih *et al.*, 2004; Tan & Cardosa, 2007; Tung *et al.*, 2007; Wu *et al.*, 2007; Yu *et al.*, 2000).

To date, several anti-enterovirus compounds that target cellular factors and inhibit various stages of virus replication have been identified. Brefeldin A blocks membrane traffic between the *cis*- and *trans*-Golgi compartments by targeting a cellular guanine nucleotide-

A supplementary figure showing viability of cells treated with kinase inhibitors and a supplementary table listing details of the kinase inhibitor library used are available with the online version of this paper.