3. *TLC and HPLC conditions*

Analytical and preparative HPLC conditions: pump, PU-1580 (JASCO); detector, UV-1575 (JASCO); column, Mightysil RP-18 (4.6 mm i.d.×250 mm (for analysis), 20 mm i.d.×250 mm (for preparation), Kanto Chemical); column temp., 30℃; solvent, acetonitrile(CH_3CN)–water (H_2O)–acetic acid(AcOH)=90 : 10 : 0.3; injection volume, 10 μL (for analysis), 100 μL (for preparation); flow rate, 1.0 mL/min (for analysis), 8.0 mL/min (for preparation); detection, UV 210 nm.

TLC conditions: developing solvent, chloroform ($CHCl_3$)–methanol(MeOH)=75 : 1. After development to about 10 cm, the spots of constituents were visualized by spraying the plate with 50% sulfuric acid (H_2SO_4) followed by gentle heating.

4. *Isolation of compounds 1, 2 and 3*

Sandarac resin product (5.0 g) was dissolved in $CHCl_3$. The solution was added to a small amount of silica gel with stirring and then the solvent was evaporated *in vacuo*. The silica gel with absorbed sandarac resin was applied on an open silica gel column and fractionated by eluting with $CHCl_3$–MeOH (100:0→0:100 gradient), with monitoring by TLC. The eluates were concentrated *in vacuo*, affording seven fractions (Frs. 1–7). Fr. 2 (610 mg), including compounds 1–3, was fractionated by preparative HPLC (see preparative HPLC conditions) to give pure compounds 1 (146 mg), 2 (94 mg) and 3 (113 mg), respectively.

Compound 1: sandaracopimaric acid[7], colorless needles, mp 171–172℃ (from MeOH), $[\alpha]_D^{26}$ −17.7° (*c* 0.13, EtOH) (lit.[8]: mp 171–173℃, $[\alpha]_D^{26}$ −19.8° (*c* 0.2, EtOH)). HR-EI-MS: *m/z* 302.2220 M^+ (Calcd. for $C_{20}H_{30}O_2$ *m/z* 302.2246).

Compound 2: sandaracopimarinol[9], amorphous solid, $[\alpha]_D^{26}$ −9.5° (*c* 0.092, MeOH) (lit.[9]: $[\alpha]_D^{26}$ −20° (*c* 0.1, MeOH)). HR-EI-MS: *m/z* 288.2440 M^+ (Calcd. for $C_{20}H_{32}O$ *m/z* 288.2453).

Compound 3: 4-epidehydroabietic acid[10], colorless prisms, mp 149–150℃ (from aq. EtOH), $[\alpha]_D^{26}$ +128° (*c* 0.094, MeOH) (lit.[11]: mp 144–145℃, $[\alpha]_D^{26}$ +106.6° (*c* 0.8, MeOH)). HR-EI-MS: *m/z* 300.2094 M^+ (Calcd. for $C_{20}H_{28}O_2$ *m/z* 300.2089).

^{1}H- and ^{13}C-NMR data of compounds 1, 2 and 3 are shown in Table 1.

5. *Quantification of sandaracopimaric acid (1)*

Sandarac resin product (50 mg) was dissolved in MeOH (10 mL). The quantity of sandaracopimaric acid (1) was determined by using an absolute calibration

Table 1. NMR Data of Compounds Isolated from Sandarac Resin

Position	Sandaracopimaric acid (1)			Sandaracopimarinol (2)			4-Epidehydroabietic acid (3)		
	δ_C[a]	δ_H[b] (*J* in Hz)		δ_C[c]	δ_H[d] (*J* in Hz)		δ_C[c]	δ_H[d] (*J* in Hz)	
1	38.3	1.13	dt, 5.0, 12.8	39.0	1.00	dt, 5.1, 12.4	39.4	1.38	dt, 4.3, 13.2
		1.64	br. d, 12.8		1.71	br. d, 12.4		2.24	m overlapped
2	18.6	1.55	m overlapped	18.4	1.45–1.60	m overlapped	20.0	1.6	m
								1.98	m overlapped
3	37.0	1.31	br. d, 12.8	35.5	1.32–1.47	m overlapped	37.5	1.08	dt, 4.0, 13.2
		1.78	dt, 5.0, 12.8					2.24	m overlapped
4	47.3	—		37.9	—		43.9		
5	48.9	1.91	dd, 2.7, 12.8	47.9	1.34	m overlapped	52.9	1.57	dd, 1.5, 12.1
6	24.9	1.26	m overlapped	22.5	1.30	m overlapped	21.0	2.16	m
		1.45	m overlapped		1.47	m overlapped		2.01	m overlapped
7	35.5	2.11	br. dt, 5.5, 14.2	35.8	2.06	br. t, 11.8	32.1	2.86	br. dd, 4.6, 14.2
		2.21	ddd, 1.8, 4.6, 14.2		2.23	ddd, 1.8, 4.3, 11.8		2.78	dt, 4.6, 14.2
8	136.6	—		137.1	—		135.1	—	
9	50.6	1.80	br. t, 7.8	50.6	1.75	br. t, 7.5	145.5	—	
10	37.8	—		38.2	—		38.4	—	
11	18.2	1.50	m overlapped	18.9	1.44	m overlapped	125.5	7.17	d, 8.0
		1.60	m overlapped		1.58	m overlapped			
12	34.5	1.36	dt, 3.6, 12.3	34.6	1.32–1.47	m overlapped	124.1	6.99	dd, 1.7, 8.0
		1.45	m overlaped						
13	37.4	—		37.5	—		145.8	—	
14	129.2	5.21	br. s	128.8	5.20	br. s	126.9	6.88	d, 1.7
15	148.9	5.76	dd, 10.5, 17.3	149.2	5.77	dd, 10.6, 17.4	33.5	2.81	hep., 6.9
16	110.2	4.87	dd, 1.5, 10.5	110.1	4.87	dd, 1.4, 10.6	24.1	1.21	d, 6.9
		4.90	dd, 1.5, 17.3		4.90	dd, 1.4, 17.4			
17	26.1	1.03	s	26.0	1.03	s	24.1	1.21	d, 6.9
18	184.4	—		72.3	3.12	d, 10.9	183.7	—	
					3.39	d, 10.9			
19	16.8	1.20	s	18.0	0.80	s	28.8	1.32	s
20	15.2	0.83	s	15.7	0.83	s	23.3	1.11	s

All signals were assigned based on DQF-COSY, HMQC, and HMBC experiments.
a) Recorded at 200 MHz. b) Recorded at 800 MHz. c) Recorded at 125 MHz. d) Recorded at 500 MHz.

curve based on peak height at UV 210 nm of compound (1) isolated from sandarac resin product.

Results and Discussion

1. Identification of the main constituents in sandarac resin

The silica gel TLC profile of the sandarac resin product is illustrated in Fig. 1. Several spots were observed on the TLC plate along with the tailing spot after spraying the plate with H_2SO_4, followed by gentle heating. The spot at *Rf* 0.28 was the most intense one. The HPLC profile of the product is illustrated in Fig. 2. Six to seven peaks were observed, and the largest peak (peak B) at T_R 13.0 min corresponded to the most intense spot on the TLC plate. In order to identify the major constituents, the sandarac resin product was fractionated *via* silica gel and preparative HPLC, affording compounds **1** (peak B), **2** (peak C), and **3** (peak A). The structures were elucidated on the basis of the spectral data.

Compound **1** showed a molecular ion peak at *m/z* 302.2220 in HR-EI-MS. The ion peak indicated that the molecular formula could be represented as $C_{20}H_{30}O_2$. The ^{1}H-NMR spectrum showed three singlet methyl signals [δ 0.83 (3H, s), 1.03 (3H, s), 1.20 (3H, s)], vinyl groups [δ 5.76 (1H, dd, J=17.3, 10.5 Hz), 4.87 (1H, dd, J=10.5, 1.5 Hz), 4.90 (1H, dd, J=17.3, 1.5 Hz)], a detached olefinic group [δ 5.21 (1H, br. s)], and many methylene signals. The ^{13}C-NMR spectrum showed a carboxyl group (δ 184.4), two olefinic signals including an exo-methylene group (δ 110.2, 129.2, 136.6, 148.9), and 15 other signals due to methyl, methylene, and methine carbons. All the ^{1}H- and ^{13}C-NMR signals of compound **1** were assigned based on 2D-NMR results (DQF-COSY, HMQC, HMBC). Compound **1** was identified as sandaracopimaric acid[7,8] (Fig. 3). The reported ^{13}C-NMR assignments[5] at C-4, 5 and 18 of **1** should be revised according to our assignment.

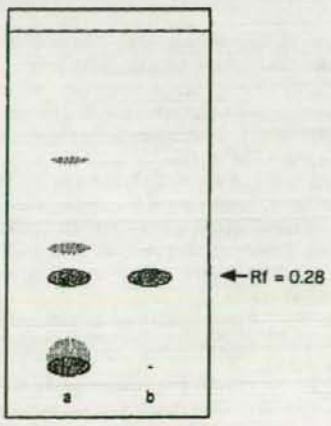


Fig. 1. Silica gel TLC profiles of sandarac resin product and sandaracopimaric acid (1)

a) Sandarac resin product. b) Sandaracopimaric acid (1). Solvent: $CHCl_3$: MeOH = 75 : 1. Spots were visualized with H_2SO_4/heat.

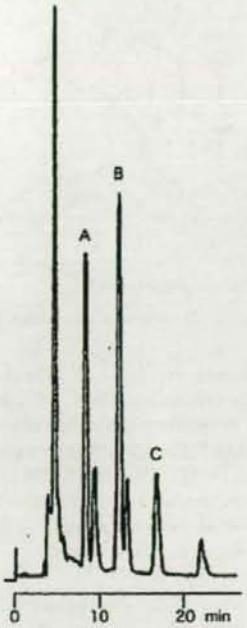


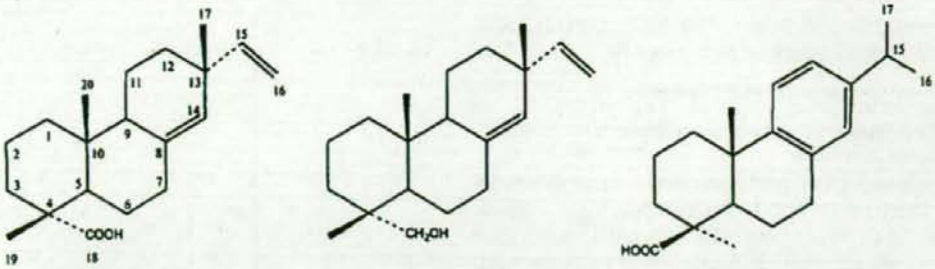
Fig. 2. HPLC profile of sandarac resin product

Peak A = 4-epidehydroabietic acid (3). Peak B = sandaracopimaric acid (1). Peak C = sandaracopimarinol (2).

Compound **2** showed a molecular ion peak at *m/z* 288.2440, indicating that its molecular formula is $C_{20}H_{32}O$. The ^{1}H- and ^{13}C-NMR spectra of compound **2** were very similar to those of sandaracopimaric acid (**1**). Comparison of compound **2** with sandaracopimaric acid (**1**) indicated that compound **2** is also an isopimarane derivative having one hydroxyl methyl group [^{1}H: δ 3.12, 3.39 (each ^{1}H, d, =10.9 Hz), ^{13}C: δ 72.3], instead of the carboxyl group at C-18 on sandaracopimaric acid (**1**). Since the ^{13}C-NMR data of compound **2** were the same as the literature data for sandaracopimarinol, compound **2** was identified as sandaracopimarinol[9] (Fig. 3).

The molecular formula of compound **3**, $C_{20}H_{28}O_2$, was deduced from the molecular ion peak at *m/z* 300.2094. The ^{1}H-NMR spectrum showed two singlet methyl signals [δ 1.11 (3H, s), 1.32 (3H, s)], a doublet methyl signal [δ 1.21 (6H, d, J=6.9 Hz)], three aromatic signals [δ 6.88 (1H, d, J=1.7 Hz), 6.99 (1H, dd, J=1.7, 8.0 Hz), 7.17 (1H, d, J=8.0 Hz)], and many methylene signals. The ^{13}C-NMR spectrum showed a carboxyl group [δ 183.7] and an aromatic group [δ 124.1, 125.5, 126.9, 135.1, 145.5, 145.8], and 13 other signals derived from methyl, methylene and methine carbons. NOE was observed for the methyl group [δ 1.32 (3H, s)] on C-4 with H-5α [δ 1.57 (1H, dd, J=1.5, 12.1 Hz)]. By comparison of the spectral and physical data with published data[11,12], compound **3** was identified as 4-epidehydroabietic acid[10,11] (Fig. 3).

Other peaks on HPLC were still mixtures of two or



sandaracopimaric acid (1) sandaracopimarinol (2) 4-epidehydroabietic acid (3)

Fig. 3. Structures of sandaracopimaric acid (1), sandaracopimarinol (2) and 4-epidehydroabietic acid (3)

more diterpenoids, and hence could not be identified at this time. The concentrations of the other diterpenoids appear to be lower than that of sandaracopimaric acid (1), because peak B due to sandaracopimaric acid (1) was the largest on HPLC. Therefore, the main constituent, sandaracopimaric acid (1), was selected as a characteristic constituent to develop a quality standard and/or verification test of sandarac resin.

2. Quantification of sandaracopimaric acid (1) in sandarac resin product

In order to quantify sandaracopimaric acid (1), a calibration curve based on peak height was prepared within the range of 0.25–2.0 mg/mL of sandaracopimaric acid (1). The concentration of sandaracopimaric acid (1) in the sandarac resin product was found to be 11.6% by HPLC. Since neither other sandarac resin products nor *T. articulata*, the origin of sandarac resin, are available in Japan, comparisons could not be made. However, we concluded that the sandarac resin product used in this research had been derived from *T. articulata*, since sandaracopimaric acid (1), a characteristic constituent in sandarac resin, was detected as the main constituent.

Conclusion

This report is the first investigation of the major constituents of commercial sandarac resin product used as a food additive. The major constituents were isolated from the product and identified as sandaracopimaric acid (1), sandaracopimarinol (2) and 4-epidehydroabietic acid (3) by 2D-NMR. Based on TLC and HPLC analyses, we confirmed that the main constituent was sandaracopimaric acid (1) and its content was found to be 11.6%. This result will be useful for setting official standards for sandarac resin as a natural food additive.

Acknowledgments

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Note

Standard Infrared Absorption Spectrum of Betaine and Optimal Conditions for its Measurement

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The infrared absorption (IR) spectrum is often used as a standard reference in identification tests of food additives in Japan. In the case of betaine, many different IR spectra have been reported and, therefore, it is necessary to establish an IR spectrum that is reproducible and reliable enough to be used as a standard for identification. In the present study, suitable conditions to obtain a standard IR spectrum were examined from various viewpoints, including pretreatment, selection of method, and measuring technique. The KBr disk method, which has generally been used to identify betaine, was found to be humidity-dependent, and there was also an interaction between betaine and KBr. A reproducible IR spectrum suitable as a standard could be obtained by drying betaine at 105℃ for 3 hours over phosphorus pentoxide, and then measuring the IR spectrum by the liquid paraffin (Nujol) paste method.

Key words: betaine; seasoning; infrared spectrum; reference spectrum; anhydride; hydrate; identification

Introduction

Betaine (Fig. 1) occurs widely in nature, especially in beets, and is used as a food additive for seasoning or as a flavor-improving agent in Japan. An identification-test for betaine is described in "Voluntary Specifications of Existing Food Additives: 3rd Ed"[1], which suggests identification of betaine on the basis of the wave numbers of the absorption bands in an infrared absorption (IR) spectrum measured by the KBr method, as well as retention time in HPLC, and the like. However, the criteria for identification employed in the test are not comprehensive; for example, the defined wave numbers do not include that of the absorption band of the COO^- group, which is one of the characteristic bands of betaine.

Apart from their use as food additives, betaine and related compounds have attracted much interest in terms of the relationship between IR spectra and structure, specifically, the influence of hydrogen bonding[2)-5)]. IR spectra of betaine are available from databases, for example, "The Sigma Library of FT-IR Spectra"[6], "Spectral Database for Organic Compounds, SDBS"*1 of the National Institute of Advanced Industrial Science and Technology, Japan (AIST), and "WebBook"*2 of the National Institute of Standards and Technology, USA (NIST). However, these IR spectra are very different from each other and hardly appear to represent the same compound.

In this study, we examined the influence of pretreatment, measuring method, and measurement conditions on the IR spectrum of betaine in order to establish a method to obtain a reliable and reproducible IR spectrum of betaine, to serve as a standard for an identification test.

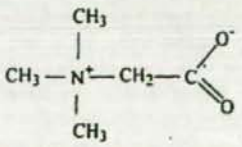

Fig. 1. Structure of betaine

Materials and Methods

1. *Samples and reagents*

Betaines (products of Nippon Beet Sugar Manufacturing Co., Ltd., and Danisco Japan Co., Ltd. Tokyo, Japan)

*1 http://www.aist.go.jp/RIODB/SDBS/cgi-bin/direct_frame_top.cgi

*2 http://webbook.nist.gov/cgi/cbook.cgi?ID=C590476&Units=SI&Mask=80

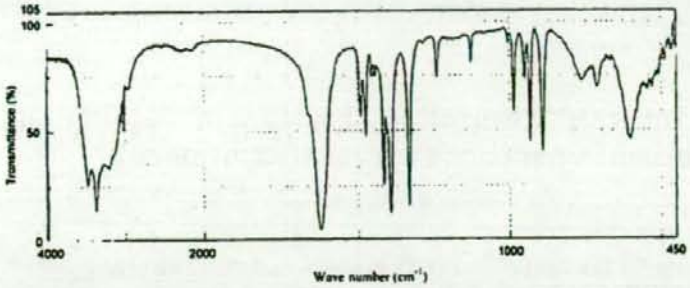

Fig. 2. IR spectrum of betaine dried over phosphorus pentoxide at 105℃ for 3 hours (the KBr method)

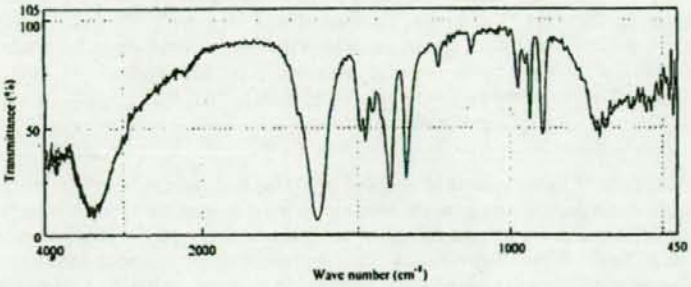

Fig. 3. IR spectrum of betaine dried over phosphorus pentoxide at 105℃ for 3 hours (the KCl method)

were provided by Japan Food Additives Association (Tokyo, Japan) and used as received. Both betaines were in the form of white crystalline powders.

Liquid paraffin (Nujol®) and potassium bromide (KBr) for IR spectroscopy were purchased from Merck AG. (Germany) and JASCO Co., Ltd. (Hachiouji, Japan), respectively. Potassium chloride (KCl) for IR spectroscopy was from JASCO Co., Ltd. (Japan).

2. Measurements of IR spectra

Measurements were carried out by the KBr method and the paste method using liquid paraffin as described in the 7th Ed. of Japan's Specifications and Standards for Food Additives[7] for measuring a solid sample. A KBr disk without any sample and a KBr optical plate were used as references in the KBr method and the paste method, respectively. The resolution was about 4 cm^{-1} (32 or 64 scans). The Fourier transform (FT)-IR device used was an Impact 400 FT-infrared spectrophotometer (Nicolet Co., Madison, Wis., USA) which could nominally measure up to 400 cm^{-1}, though the practical limit was about 450 cm^{-1}. The measurement was conducted in a room used exclusively for IR spectral measurement, where the humidity and temperature were controlled to 30–40% and 23℃, respectively. KBr disks and pastes were usually prepared in the controlled room, but sometimes in an ordinary laboratory. Measurements using the KCl disk or paste method were carried out under the same conditions.

3. Recommended procedure for measurement of a standard IR spectrum of betaine

Betaine as a sample was dried over phosphorus pentoxide at 105℃ for 3 hours. The IR spectrum was measured by using the paste method as described in the General Methods of the 7th Ed. of Japan's Specifications and Standards for Food Additives[7]. As a reference, a KBr optical plate was used.

Results and Discussion

1. Examination by the disk method

In the measurement by the KBr disk method, commercial betaine was used after having been dried at 105℃ for 3 hours according to the "Loss on Drying Test" in the Voluntary Specifications of Existing Food Additives: 3rd Ed.[1] The dried betaine was first subjected to measurement by the KBr method according to the General Methods of the 7th Ed. of Japan's Specifications and Standards for Food Additives[7]. The IR spectrum obtained is shown in Fig. 2. The IR spectrum agreed well with that described in SDBS, but differed from those described in The Sigma Library (the paste method), NIST (the paste method) and reference 2 cited above (the KBr method), suggesting the importance of moisture absorption during disk preparation or an interaction between betaine and KBr. To examine whether or not an interaction between betaine and KBr exists, a betaine disk was prepared using KCl, and the IR spectrum was measured. The result is shown in Fig. 3. It is clear from Figs. 2 and 3 that the spectrum of betaine in the KBr disk is quite different from that in the KCl disk throughout the entire region measured.

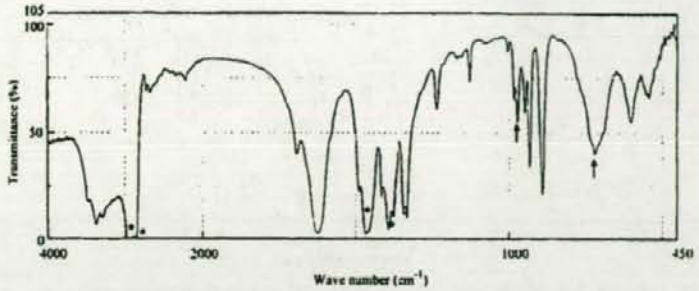

Fig. 4. IR spectrum of a 1 : 10 mixture of dried betaine and KBr (the paste method)
*Bands due to liquid paraffin.
† extra bands observed by the paste method.

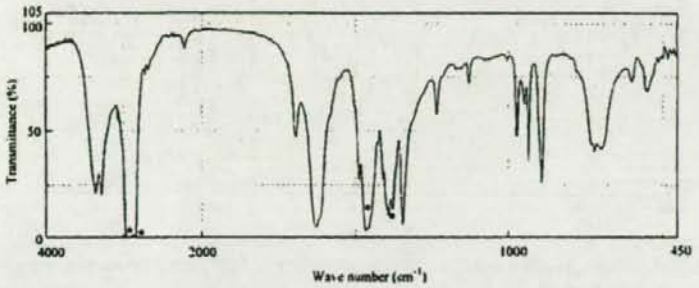

Fig. 5. IR spectrum of a 1 : 10 mixture of dried betaine and KCl (the paste method)
*Bands due to liquid paraffin.

Thus, there was an interaction between betaine and KBr during the procedures of mixing and grinding, or compressing, which resulted in a change of the IR spectrum. In the KBr method, it is well known that a sample may form a solid complex with KBr[8], or may interact with KBr through hydrogen bonding or ion-exchange[9, 10]. To elucidate the causes of the altered spectrum, the IR spectrum of betaine was measured by the paste method using a paste prepared by mixing betaine and alkali metal halide (KBr or KCl) at the mixing ratio of about 1 : 10 (betaine : alkali metal halide), and grinding in agate mortar. The measurement was carried out using a portion of the resultant paste, with a greater thickness than usual. The results are shown in Figs. 4 and 5.

The IR spectrum measured by the paste method in the presence of KBr (Fig. 4) and that of the KBr method (Fig. 2) are in good agreement in the region between 3,500 and 3,000 cm^{-1}, and almost in agreement in the region of 1,000–800 cm^{-1} although extra bands (marked with †) exist. From these results, it was presumed that the spectrum of betaine is easily changed even by very mixing and grinding betaine with KBr. When the KBr disk was prepared by very brief grinding, the IR spectrum of betaine was again different from that shown in Fig. 2.

It has been reported that the spectrum of betaine measured by the KBr method is influenced by the relative humidity in the measuring room[2]. It has also been reported that the IR spectrum of betaine anhydride, which was obtained by crystallization from ethanol, coincided with that of betaine hydrate when measurement was conducted by the KBr method under the condition of 12% or higher relative humidity[2]. We measured the IR spectrum at a humidity level as low as possible (23℃, relative humidity 30%), but could not obtain the IR spectrum of betaine anhydride. The relative humidity varies greatly within Japan, and also among the cold districts (Hokkaido, North Europe, *etc.*) where betaine is mainly produced. Such regional differences in humidity may be linked to the variations observed in the IR spectra provided by the manufacturers in different districts, since test laboratories are not necessarily environmentally controlled. It was therefore considered that the KBr method does not necessarily give an IR spectrum with good reproducibility.

On the other hand, the IR spectrum measured by the paste method in the presence of KCl (Fig. 5) is in agreement with that measured by the KCl disk method (Fig. 3) in the region between 1,000 and 800 cm^{-1}. In addition, these IR spectra are almost in agreement with the IR spectrum of betaine hydrate measured by the paste method described below, and, also are similar to the IR spectrum provided by NIST. These facts indicate that the KCl method is preferable, and that betaine tends to absorb moisture to form a hydrate. Therefore, further investigation into the IR spectrum was conducted by the paste method, which was hardly affected by humidity.

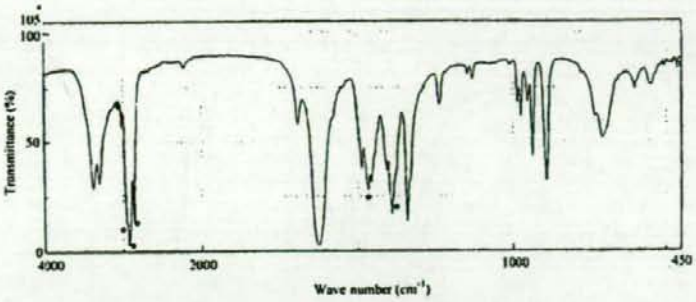

Fig. 6. IR spectrum of commercial betaine (the paste method)
*Bands due to liquid paraffin.

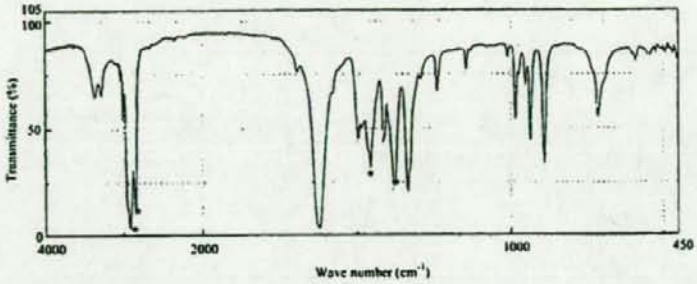

Fig. 7. IR spectrum of betaine dried over phosphorus pentoxide at 105℃ for 3 hours (the paste method)
*Bands due to liquid paraffin.

2. Examination by the paste method

In the paste method, a target substance is coated with liquid paraffin when preparing a paste for measurement. Therefore, the IR spectrum should be littel affected by humidity, even if the substance is hygroscopic. Figure 6 shows the IR spectrum of a betaine product (commercially available bulk powder) measured by the paste method without drying treatment. The high similarity of the IR spectrum in Fig. 6 to that of betaine hydrate in The Sigma Library[6] indicates that the bulk powder contained predominantly hydrate. Because the water content generally varies from one betaine product to another, the IR spectrum of a single product cannot serve as a standard IR spectrum. Another bulk powder also gave a similar IR spectrum to that shown in Fig. 6.

We then examined suitable drying conditions for obtaining the anhydride in a reproducible manner. Drying commercial betaine at 105℃ for 3 hours did not affect the IR spectrum according to the General Method of "Loss on Drying Test" in the Voluntary Specifications of Existing Food Additives: 3rd Ed.[1] Thus, drying was conducted over phosphorus pentoxide at 105℃ for 3 hours. The IR spectrum of this dried betaine is shown in Fig. 7. The IR spectrum in Fig. 7 agreed with that of betaine anhydride provided by The Sigma Library[6]. Further, the spectrum in Fig. 7 was in agreement with that of betaine anhydride measured by the KBr method[2], except that the former also contains absorption bands due to liquid paraffin.

When drying was conducted at different temperatures, 120, 140, 170 or 200℃, over phosphorus pentoxide for three hours, the IR spectra of the dried betaine samples were identical. We, therefore, concluded that the IR spectrum shown in Fig. 7 could be available as a standard IR spectrum. Needless to say, when this spectrum is used as a standard in the identification of a betaine product, the spectra must be compared in the region other than that containing the absorption bands marked by "*", which are due to liquid paraffin.

As mentioned above, the IR spectra of betaine hydrate measured by the KBr and paste methods were different. On the other hand, the IR spectrum of betaine anhydride measured by the KBr disk method at low humidity (relative humidity; less than 12%)[2] was in agreement with that of betaine anhydride measured by the paste method. Accordingly, when using the IR spectrum measured by the KBr method in the identification test of betaine, it is necessary to confirm that the spectrum is identical to the spectrum measured by the paste method in order to ensure the reliability of the test results. The identification method based on the IR spectrum has various advantages, including energy saving, and wide applicability, and is a convenient alternative to chemical identification methods. It is therefore important to ensure good reproducibility of the spectrum.

Conclusion

We examined suitable conditions and methods to obtain a reliable and reproducible IR spectrum applica-

ble to identification of betaine as a standard spectrum. It was found that a reproducible spectrum can be obtained by drying betaine at 105℃ for 3 hours over phosphorus pentoxide and then measuring the IR spectrum by the paste method. The IR spectrum thus obtained is suitable to be used as a standard reference IR spectrum.

Acknowledgments

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Effects of Possible Endocrine Disrupting Chemicals on Bacterial Component-Induced Activation of NF-κB

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Endocrine disrupting chemicals (EDCs) have a possibility to exacerbate infectious diseases because EDCs disturb the human immune system by interfering with endocrine balance. To assess the influence of EDCs on the innate immune function of macrophages, we investigated the effects of thirty-seven possible endocrine disruptors on lipopolysaccharide (LPS)- or bacterial lipopeptide (Pam_3CSK_4)-induced activation of nuclear factor kappa B (NF-κB). Alachlor, benomyl, bisphenol A, carbaryl, kelthane, kepone, octachlorostyrene, pentachlorophenol, nonyl phenol, *p*-octylphenol and ziram inhibited both LPS- and Pam_3CSK_4-induced activation of NF-κB. Simazine inhibited only LPS-induced activation. A strong inhibitory effect was observed with ziram and benomyl. On the other hand, diethylhexyl adipate and 4-nitrotoluene tended to enhance the activation induced by Pam_3CSK_4 and LPS, respectively. Aldicarb, amitrole, atrazine, benzophenone, butyl benzyl phthalate, 2,4-dichlorophenoxy acetic acid, dibutyl phthalate, 2,4-dichlorophenol, dicyclohexyl phthalate, diethylhexyl phthalate, diethyl phthalate, dihexyl phthalate, di-*n*-pentyl phthalate, dipropyl phthalate, malathion, methomyl, methoxychlor, metribuzin, nitrofen, permethrin, trifluralin, 2,4,5-trichlorophenoxyacetic acid and vinclozolin had no significant effects at 100 μM. These results indicate that some agrochemicals have the potential to inhibit macrophage function and suggest that endocrine disruptors may influence the development of bacterial infections.

Key words endocrine disruptor; nuclear factor kappa B (NF-κB); lipopolysaccharide; bacterial lipoprotein; macrophage

Endocrine disrupting chemicals (EDCs) are exogenous substances that mimic, antagonize, impair, enhance, or inhibit the actions of endogenous hormones and in turn cause abnormalities of growth, reproduction, development, behavior, and immune function, or cause malignant tumors.[1—3] EDCs act at multiple sites *via* multiple mechanisms of action. Receptor-mediated mechanisms have attracted the most attention, but other mechanisms including hormone synthesis, transport, and metabolism are also involved.[4,5] However, the secondary effects of EDCs, such as the influence on infectious diseases, have been poorly investigated.

Infectious diseases are caused by microbial invasion. To overcome this invasion, the human body utilizes a sophisticated and complicated immune system. Disturbance of this well-organized immune system may result in the development of serious infectious diseases. It is, therefore, plausible to consider that EDCs may exacerbate infectious diseases because disruption of endocrine function seriously disturbs the immune system. It is well known that macrophages play an important role in the defense mechanisms of the host immune system. Macrophages are activated by microbial components such as lipopolysaccharide (LPS) or bacterial lipopeptides.[6] Previously, we studied the effects of various possible EDCs on macrophage activation and found that some EDCs strongly inhibit LPS-induced TNF-α and nitric oxide production by macrophages.[7] Since the activation of the transcriptional factor NF-κB is essential for the production of TNF-α and nitric oxide, in the present study we investigated the effects of possible EDCs on bacterial component-induced activation of NF-κB.

MATERIALS AND METHODS

Reagents Benomyl and permethrin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). 2,4-Dichlorophenoxy acetic acid was obtained from Kanto Chemical (Tokyo, Japan). The other thirty four chemicals used as EDCs were purchased from Wako Pure Chemical Industries (Osaka, Japan). Their abbreviations are shown in Table 1. These chemicals were selected from the SPEED'98 list (http://www.env.go.jp/en/chemi/ed.html; SPEED'98 has been revised and "Perspectives on Endocrine Disrupting Effects of Substances—ExTEND 2005—" has been issued during the preparation of this manuscript) depending on their availability and water solubility. Chemicals that show higher cellular toxicity were excluded. LPS prepared from *E. coli* O111 was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) and re-purified as described previously.[8] Bacterial lipopeptide Pam_3CSK_4 was obtained from Bachem (Bubendorf, Switzerland). An NF-κB-dependent luciferase reporter plasmid pcELAM-L was constructed by inserting the *Not*I (blunt ended)–*Sal*I fragment of pELAM-L[9] into the *Nru*I–*Xho*I site of pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.).

Cell Culture The NF-κB reporter cell line stably carrying an NF-κB-dependent luciferase reporter plasmid pcELAM-L was established as follows. After linearizing with *Bgl*II, pcELAM-L was transfected into a mouse macrophage cell line RAW 264 (obtained from the Riken Cell Bank, Tsukuba, Japan) cells by using FuGene6 transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany). Stable transfectants were selected for G418 resistance at a concentration of 0.5 mg/ml. The cell line was maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 0.5 mg/ml G418, 10% (v/v) heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA, U.S.A.), penicillin (100 U/ml), and streptomycin (100 μg/ml).

NF-κB Reporter Assay The NF-κB-dependent luciferase reporter assay was performed as described elsewhere.[10] Briefly, the NF-κB reporter cells ($1—3\times10^5$/well) were plated in 12-well plates and on the following day stimulated for 6 h with 10 ng/ml of either LPS or Pam_3CSK_4 in the absence or presence of EDCs, which had previously been

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Table 1. Effects of Possible EDCs on LPS- or Pam_3CSK_4-Induced Activation of NF-κB

Chemicals	Abbreviation	LPS stimulation	Pam_3CSK_4 stimulation
Alachlor	ACL	56.2±8.18*	51.4±2.65**
Aldicarb	ACB	113.1±9.52	105.8±4.23
Amitrole	ATA	107.0±9.50	105.5±9.68
Atrazine	ATZ	79.4±10.71	86.5±4.67
Benomyl	BML	3.2±0.31**	3.2±1.01**
Benzophenone	BZP	85.6±8.94	94.9±9.39
Butyl benzyl phthalate	BBP	108.6±7.72	82.9±9.77
Bisphenol A	BPA	70.5±4.37**	55.7±6.32**
Carbaryl	NAC	43.4±6.68*	55.7±2.37**
2,4-Dichlorophenoxy acetic acid	2,4-D	96.7±10.41	111.6±7.20
Dibutyl phthalate	DBP	92.3±9.60	91.9±6.20
2,4-Dichlorophenol	DCP	91.4±8.13	96.7±6.17
Dicyclohexyl phthalate	DCHP	90.2±7.02	83.4±6.44
Diethylhexyl adipate	DOA	106.4±8.10	108.3±2.84*
Diethylhexyl phthalate	DOP	103.6±9.13	98.9±8.67
Diethyl phthalate	DEP	86.4±4.93	94.5±3.23
Dihexyl phthalate	DHP	97.7±7.15	101.1±7.23
Di-*n*-pentyl phthalate	DPP	96.4±10.90	101.7±4.47
Dipropyl phthalate	DPrP	92.1±8.75	88.2±13.68
Kelthane	KLT	17.2±8.65*	25.8±9.85*
Kepone	KPN	8.0±5.01**	10.8±7.28**
Malathion	MAT	89.5±12.50	84.3±3.74
Methomyl	MTM	92.2±7.81	108.3±9.26
Methoxychlor	DMDT	94.9±5.63	77.2±9.98
Metribuzin	MTB	92.0±9.36	110.8±9.70
Nitrofen	NIP	100.5±5.61	102.8±8.49
4-Nitrotoluene	NTT	115.1±4.30**	108.5±10.75
Octachlorostyrene	OCS	74.1±4.85**	97.4±11.29
Pentachlorophenol	PCP	70.6±6.52*	50.5±7.20*
Nonyl phenol	NNP	58.6±4.12**	49.3±8.24*
p-Octylphenol	OTP	59.6±5.82**	44.5±6.34*
Permethrin	PMT	116.1±9.33	88.2±12.00
Simazine	CAT	79.9±7.24*	97.8±9.86
Trifluralin	TFR	110.5±4.77	115.1±9.84
2,4,5-Trichlorophenoxyacetic acid	2,4,5-T	92.7±7.91	114.1±10.87
Vinclozolin	VCZ	92.9±8.51	102.6±4.23
Ziram	ZRM	0.0±0.02**	0.0±0.01**

Values are expressed as percent (mean±S.E.M.) of respective control where no EDC treatment was performed. Data are from at least three independent experiments. All chemicals were used at 100 μM. * $p<0.05$, ** $p<0.01$, compared with the respective control by paired Student's *t*-test.

dissolved in DMSO. The final concentration of DMSO was adjusted to 0.1% and this concentration of DMSO was added to wells without EDCs as a control. Reporter gene activity was then measured according to the manufacturer's (Promega, Madison, WI, U.S.A.) instructions. The protein concentrations of the cellular extracts were determined by the Bradford method (Bio-Rad, Hercules, CA, U.S.A.), and reporter activity was normalized to the protein concentration for the compensation of difference in cell numbers and viabilities between wells.

Statistical Analyses The paired Student's *t*-test was used to evaluate statistical significance. A *p*-value of less than 0.05 was considered to be significant.

RESULTS

We selected thirty seven chemicals (described as EDCs in this study) that are suspected of having endocrine disrupting effects from among agrochemicals and resin-related chemicals (see Materials and Methods). To systematically compare the effect of each chemical on bacterial component-induced activation of NF-κB, the mouse macrophage cell line RAW 264 stably carrying an NF-κB-dependent luciferase reporter gene was incubated with 100 μM of each chemical followed by either LPS or Pam_3CSK_4 and the reporter activity was measured. Table 1 summarizes the results. Ten chemicals, ACL, BML, BPA, NAC, KLT, KPN, PCP, NNP, OTP and ZRM, inhibited both LPS- and Pam_3CSK_4-induced activation of NF-κB. OCS and CAT inhibited only LPS-induced activation. On the other hand, DOA and NTT slightly enhanced the activation induced by Pam_3CSK_4 and LPS, respectively. Cellular toxicity assessed from protein concentrations of cellular extracts was observed with KLT, KPN and ZRM at the concentration (100 μM) used (data not shown).

We next examined the concentration-dependency of the fourteen chemicals that affected LPS- or Pam_3CSK_4-induced activation of NF-κB (Fig. 1). All of the EDCs tested, except for DOA and NTT, inhibited the activation in a concentration-dependent manner. The Pam_3CSK_4-induced activation of NF-κB was not affected by 100 μM OCS (Table 1) but was inhibited by 200 μM OCS (Fig. 1B). An increase in reporter activity was observed at 100 μM DOA and NTT (Table 1) but no significant increase was observed at 200 μM of each of these chemicals. We were not able to examine the effect of 200 μM CAT because of its poor solubility. Cellular toxicity was not observed with any of the chemicals at the concentrations tested in Fig. 1 except for 100 μM of KPN and KLT (data not shown).

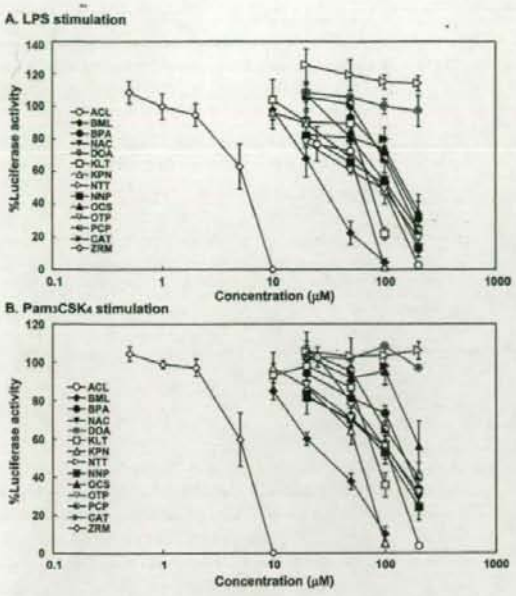

Fig. 1. Concentration-Dependent Effects of Possible EDCs on LPS- or Pam_3CSK_4-Induced Activation of NF-κB

RAW 264 cells stably carrying an NF-κB-dependent luciferase reporter gene were either left unstimulated or stimulated with 10 ng/ml of LPS (A) or Pam_3CSK_4 (B) for 6 h with or without the indicated concentrations of EDCs, and luciferase activity was then measured. Values are means±S.E.M. from three independent experiments. The reporter activity in response to LPS or Pam_3CSK_4 alone is expressed as 100%.

DISCUSSION

In order to explore the influence of EDCs on macrophage activation, in the present study we examined the effects of thirty-seven chemicals that are suspected of having endocrine disrupting effects on LPS- and Pam_3CSK_4-induced activation of NF-κB, and found that fourteen of the chemicals affected NF-κB activation in response to either stimulation (Table 1). Twelve chemicals showed inhibitory effects and two chemicals showed, if any, enhancing effects. The chemicals examined in this study are classified basically as agrochemicals and resin-related chemicals, and the stronger inhibitory activity was observed with agrochemicals. Most of chemicals exerted their effects at a concentration range of 50—200 μM, which may be higher than the levels that possibly occur in the environment. In actual circumstances, human bodies are exposed to multiple chemicals chronically, and bioaccumulability and synergistic effects of these chemicals have not been well studied. Therefore, it can not be ruled out that chronic exposure to even low levels of these chemicals may have some impacts.

LPS and bacterial Pam_3CSK_4 utilize Toll-like receptor (TLR)4 and TLR2, respectively, to stimulate macrophages. Since the chemicals that had inhibitory activity affected both LPS- and Pam_3CSK_4-induced activation of NF-κB, these chemicals probably affect the signaling component common to both TLR4 and TLR2.

In this study, we found that ACL, NAC, CAT, NNP and OTP, which inhibited either LPS-induced TNF-α or nitric oxide production by macrophages in our previous study,[7] inhibited LPS-induced NF-κB activation as well. Since the activation of NF-κB is essential for the production of TNF-α and nitric oxide, the inhibition of NF-κB by these chemicals may be involved in the inhibitory effects on TNF-α and nitric oxide.

We have reported that the inhibitory activity of NAC, a carbamate agrochemical, on LPS-induced activation of NF-κB involves its alkylating reactivity.[11] In the present study, BML and ZRM, which are carbamate and dithiocarbamate agrochemicals, respectively, also showed strong inhibitory activity. In addition, ZRM and another dithiocarbamate agrochemical, mancozeb, have also been shown to inhibit LPS-induced activation of NF-κB.[12] On the other hand, we found in the present study that three other carbamate agrochemicals, ACB, MTM and VCZ showed no significant effects. Therefore, the inhibitory effect is not explained solely by carbamate and dithiocarbamate structures.

Previously, we have found that ACL inhibits the upstream process of LPS-induced degradation of IκB-α.[11] In the present study, all of the agrochemicals, excluding carbamates and dithiocarbamates, that showed inhibitory activity possess a chloromethyl ketone (or alcohol) residue, as does ACL. This may suggest that this structure is involved in the inhibitory activity. Further studies to clarify the mechanisms of the inhibitory activity of these chemicals, as well as those of resin-related chemicals, are necessary to understand the influence of EDCs on infectious diseases.

Our results demonstrated that many of the agrochemicals and resin-related chemicals which are suspected of having endocrine disrupting effects possess the ability to inhibit bacterial component-induced activation of NF-κB. Since the activation of NF-κB plays an important role in bacterial clearance, these chemicals may have the potential to exacerbate infectious diseases.

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Journal of Medical Virology 78:971–978 (2006)

Changing Distribution of Norovirus Genotypes and Genetic Analysis of Recombinant GIIb Among Infants and Children With Diarrhea in Japan

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A total of 402 fecal specimens collected during July 2003–June 2004 from infants and children with acute gastroenteritis, encompassing five localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) of Japan, were tested for the presence of norovirus by RT-PCR. It was found that 58 (14.4%) fecal specimens were positive for norovirus. Norovirus infection was detected throughout the year with the highest prevalence in December. Norovirus GII was the most predominant genogroup (98.3%; 57 of 58). The genotypes detected in this study were GI/4, GII/2, GII/3, GII/4, and GII/6. Of these, NoV GII/3 (known as the Arg320 virus cluster) was the most predominant genotype (43.9%), followed by NoV GII/4 (the Lordsdale virus cluster; 35.1%) and others. Two norovirus strains clustered with a "new variant designated GIIb" and a "new variant of GII/4" were found circulating in Japan for the first time. It was interesting to note that NoV GIIb and NoV GII/3 appeared to be the recombinant strains and the recombination site was demonstrated at the overlap of ORF1 and ORF2. The majority (96%) of the dominant norovirus strains were identified as the recombination of GII/3 capsid and GII/12 polymerase. The recombination in the NoV GIIb capsid gene at the breakpoint located at P1 domain was also identified. Obviously, NoV GIIb isolate in Japan had double recombination. This is the first report demonstrating the existence of different "new variants" co-circulating in Japanese infants and children with acute gastroenteritis. ***J. Med. Virol. 78:971–978, 2006.***

KEY WORDS: PCR; norovirus; recombination; Japan

INTRODUCTION

Norovirus (NoV) is recognized as a significant global enteropathogen, being a major cause of sporadic cases as well as of outbreaks of acute gastroenteritis in humans in various epidemiological settings, such as restaurants, schools, day-care centers, hospitals, nursing homes, and cruise ships [Chiba et al., 1979; McEvoy et al., 1996; Vinje et al., 1997; McIntyre et al., 2000]. The virus can be transmitted by food-borne, water-borne, air-borne, person-to-person spread by close contact and there might be some other unknown modes [Matson, 1994; Bon et al., 1999; Marks et al., 2000; Lopman et al., 2002; Oh et al., 2003]. NoV is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make NoV a major public health concern [Kageyama et al., 2004]. NoV is the distinct genus within the family *Caliciviridae*. The prototype strain of NoV is the Norwalk virus (Hu/NoV/Norwalk virus/1968/US), which was originally discovered from an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio, USA in 1968. NoV possesses a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The NoV genome contains three open reading frames (ORFs). ORF1

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encodes the non-structural proteins, including the RNA-dependent RNA polymerase (RdRp) while ORF 2 encodes the capsid protein (VP1) and ORF3 encodes a small capsid protein (VP2). Based on the sequence analysis of the capsid gene, NoV is divided into genogroups I and II, both known to infect humans. A recent study indicated that NoV GI and NoV GII could be classified into 14 and 17 genotypes, respectively [Kageyama et al., 2004]. The first naturally occurring recombinant NoV was the prototype Snow Mountain virus [Hardy et al., 1997]. Later several recombinant NoV strains causing sporadic cases and outbreaks of acute gastroenteritis were reported [Jiang et al., 1999a; Schreier et al., 2000; Katayama et al., 2002]. RNA recombination is one of the major driving forces of viral evolution [Worobey and Holmes, 1999]. To date, NoV is still uncultivable by standard culture methods with different cell lines. However, either VP1 alone or both VP1 and VP 2 could be expressed using recombinant baculovirus forming virus-like particles (VLPs) that are similar morphologically and antigenically to the native virion [Jiang et al., 1995]. Seroepidemiologic studies indicated a worldwide distribution of NoV. Moreover, it was found that serum antibody level to NoV was lowest in the first year of life and then rising after 2 years of age [Lopman et al., 2002; Dai et al., 2004; Peasey et al., 2004].

The objectives of this study were: to determine the incidence of NoV infections in infants and children with acute gastroenteritis in five different localities of Japan during 2003 and 2004; to characterize the genogroup and genotype of the detected NoV; and to describe the genetic diversity among them. Additionally, the age-related and seasonal distributions of NoV infection were determined.

MATERIALS AND METHODS

Fecal Specimens

A total of 402 fecal specimens were collected from infants and children with acute gastroenteritis, encompassing five different localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) of Japan during the period of July 2003–June 2004. Of these, 19 specimens were from Osaka, 22 from Sapporo, 22 from Tokyo, 45 from Saga, and 294 from Maizuru. The ages of the subjects were ranged from 2 months to 11 years with the median of 2.5 years (29 months). The majority (75%) of the affected children were aged less than 36 months and about half (54%) were male. The 10% fecal suspension was prepared in distilled water and clarified by centrifugation at 10,000*g* for 10 min. The supernatant was collected and stored at −30°C until use.

Extraction of Viral Genome

The viral genomes were extracted from 140 µl of 10% fecal suspensions using the QIAamp viral RNA Mini Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions.

Reverse Transcription (RT)

For reverse transcription (RT), 7.5 µl of extracted viral genome was added to the reaction mixture containing 2.05 µl of 5× first strand buffer (Invitrogen, Carlsbad, CA), 0.75 µl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.75 µl of 10 mM DTT (Invitrogen), 0.75 µl (200 U/µl) of superscript reverse transcriptase III (Invitrogen), 0.375 µl (1 µg/µl) of random primer (hexadeoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 µl (33 U/µl) of RNase inhibitor (Toyobo, Osaka, Japan), and 2.325 µl MilliQ water. The total volume of reaction mixture was 15 µl [Yan et al., 2003]. The RT step was carried out at 50°C for 1 hr, followed by 99°C for 5 min and then held at 4°C.

Polymerase Chain Reaction (PCR)

The NoV genogroups were identified by PCR method using specific primers as described [Yan et al., 2003]. Two pairs of specific primers G1SKF (CTGCCCGAATTYGTAAATGA) and G1SKR (CCAACCCARCCATTRTACA), and COG2F (CARGARBCNATGTTYAGRTGGATGAG) and G2SKR (CCRCCNGCATRHCCRTTRTACAT) [where B was C, G or T; H was A, C or T; N was any base; R was A or G, and Y was C or T] that amplify capsid gene of NoV were used to detect NoV GI and NoV GII, respectively. These primers were specifically generated two different sizes of amplicons of 330 and 387 bp for NoV GI and NoV GII, respectively. The RNA polymerase gene of NoV was also amplified to identify the recombinant strain of NoV using the primers as described [Jiang et al., 1999b; White et al., 2002]. The PCR was carried out with 2.5 µl of cDNA in 22.5 µl of the reaction mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI), dNTPs (2.5 mM/µl), primers (33 µM), Taq DNA polymerase (5 U/µl) (Promega, Madison, WI) and MilliQ water. The PCR was performed at 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 7 min, and then held at 4°C. The full length of capsid and polymerase regions were amplified with a newly designed specific primer NVPOLR/A (GAT GAG GTT CTG ATG AGA) and the specific primers reported by Vinje et al. [2000] and Kawamoto et al. [2001]. The PCR was performed at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and a final extension at 72°C for 7 min, and then held at 4°C.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min and then visualized under ultraviolet (UV) light. The results were recorded by photography.

Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequences of PCR products (DNA) positive for NoV were determined using the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster

City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). Phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets were generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). SimPlot software (Version 1.3) was used to compare the recombinant NoV sequences [Lole et al., 1999]. The nucleotide sequences of NoV strains 5424/03/Saga/JP and 5017/04/Maizuru/JP had been submitted to the DDBJ DNA/GenBank database and the assigned accession numbers were AB242256 and AB242257, respectively. Reference NoV strains and accession numbers used in this study were as follows: Manchester (X86560), Saitama T53GII/02/JP (AB112260), Girllington (AJ277606), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Hillington (AJ277607), Alphatron (AF195847), Toronto (U02030), Seacroft (AJ277620), Leeds (AJ277608), Lordsdale (X86557), Idaho Falls/96/US (AY054299), Fayetteville/1998/US (AY113106), Erfurt/546/00/DE (AF42118), M7/99/US (AY130761), Saitama U1 (AB039775), Camberwell (AF145896), Snow Mountain (U70059), Paris Island/2003/USA (AY652979), Oberhausen 455/01/DE (AF539440), C14/2002/AU (AY845056), Herzberg 385/01/DE (AF539439), Arg320 (AF190817), VannesL169/2000/France (AY773210), Amsterdam (AF195848), White River/94/US (AF414423), Mexico (U22498), MD145 (AY032605), Mora/97/SE (AY081134), SaitamaT29GII/01/JP (AB112221), SaitamaKU80aGII/99/JP (AB058582), Mc37 (AY237415), Stockholm/IV4348/01/SE (AJ626633), and Pont de Roide 673/04/France (AY682549).

RESULTS

Epidemiology of Norovirus Infections

A total of 402 fecal specimens collected from infants and children with acute gastroenteritis from five different localities of Japan during July 2003 and June 2004 were examined for the presence of NoV. NoV was detected in 58 out of 402 (14.4%) specimens tested. The highest prevalence of NoV was found in infants and children with the age range of 12–23 months (36.2%). No case of NoV infection was identified among infants aged less than 6 months. It was also found that infants and children younger than 3 years old had a high rate of NoV infection (79.3%). NoV was detected throughout the period of 9 months starting from October 2003 to June 2004. However, none of NoV was detected from July to September 2003. The NoV incidence was found highest in December (27.5%), followed by November (19%), and January (12.1%). The lowest NoV detection rate fell into October (5.2%).

Nucleotide Sequence and Phylogenetic Analyses of NoV Genotypes

The partial nucleotide sequences of capsid gene of NoV detected in this study were compared to each other as well as to those of NoV reference strains available in the DDBJ DNA/GenBank database by BLAST. A total of 58 NoV nucleotide sequences, including 1 of NoV GI and 57 of NoV GII were analyzed by phylogenetic grouping based on the recent NoV capsid region classification schemes described by Kageyama et al. [2004]. It was found that the NoV GI sequence (5226/04/Maizuru/JP) clustered into one distinct group with GI/4, which was represented by the Chiba/87/JP virus cluster. It was of interest to note that the 5226/04/Maizuru/JP strain showed the genetic relationship with the NoV Mie2001-U94/JP, which was previously isolated from oyster in Japan, with the nucleotide sequence identity to as high as 98%. Additionally, the 5226/04/Maizuru/JP strain revealed 90%–97% nucleotide sequence identities with those of other human NoV reference strains.

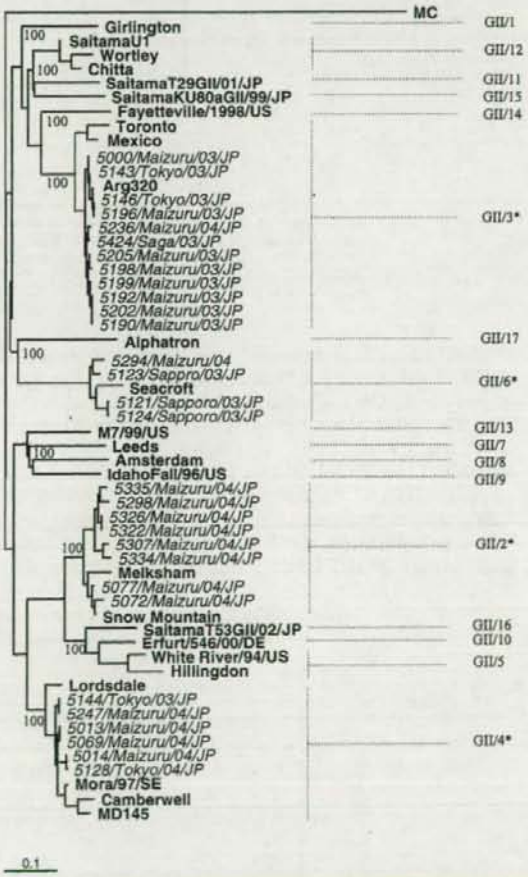

Fig. 1. Phylogenetic tree of nucleotide sequences of Japanese NoV GII. The tree was constructed from partial nucleotide sequences of capsid region of NoV GII isolates detected in Japan. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Japanese NoV was highlighted in italic. MC strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values if 100% is given. *, Genotype contains Japanese NoV detected in the study.

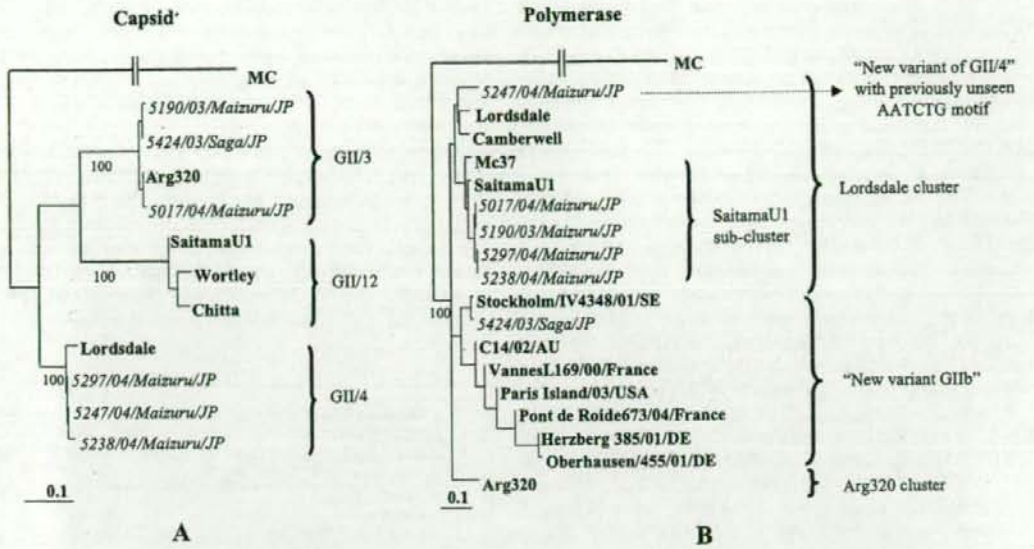

Fig. 2. Observation of changes of NoV genotypes on the basis of phylogenetic trees of nucleotide sequences. The trees were constructed from partial nucleotide sequences of capsid and polymerase regions of the Japanese representative isolates of NoV GII. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Japanese NoV was highlighted in italic. MC strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values if 100% is given.

Of 57 NoV GII sequences, four distinct genotypes, GII/2, GII/3, GII4, and GII/6 had been identified (Fig. 1). Of these, the GII/3 (known as the Arg320 virus cluster) was the most predominant genotype with the prevalent rate of 43.9%, followed by 35.1% of GII/4 (the Lordsdale virus cluster), 14% of GII/2 (the Melksham virus cluster), and 7% of GII/6 (the Seacroft virus cluster). Considering the genotype distribution by localities, GII/3 was also the most predominant in all localities, except for Osaka where none of GII/3 was identified and only one GII/4 was detected in Osaka. The nucleotide sequence identities were ranged from 58% to 99% when NoV GII strains detected in this study were compared with those the reference strains previously registered in the DDBJ DNA/GenBank database.

Nucleotide Sequence and Genetic Analyses of NoV RNA Polymerase Gene

To verify the changing epidemiology of NoV genotypes, the RNA polymerase genes of all NoV GII/3 and

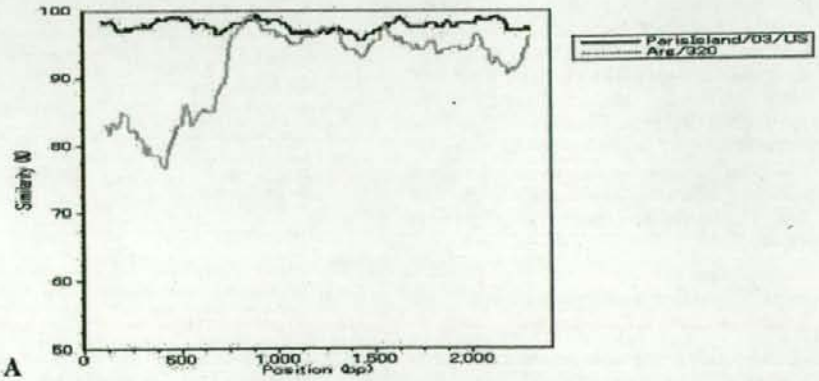

Fig. 3. Genetic characterization of recombinant NoV "new variant with polymerase GIIb." **A**: The Simplot analysis of the 5424/03/Saga/JP, the Paris Island/03/USA, and the Arg320. **B**: Evidence of recombination in NoV capsid gene.

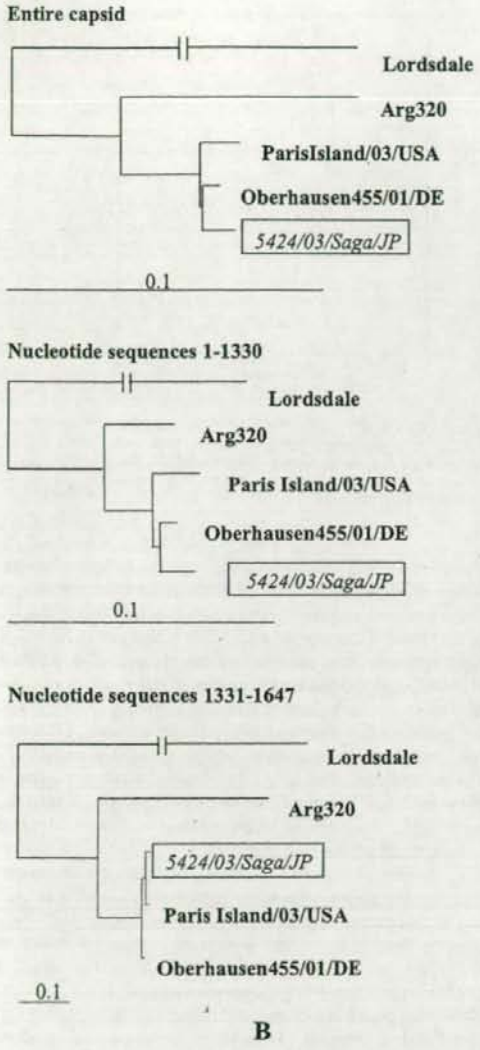

Fig. 3. *(Continued)*

NoV GII/4 were additionally amplified and sequenced. Of 25 NoV isolates with GII/3 capsid, 24 were classified into the SaitamaU1 sub-cluster (known as GII/12) but not into the Arg320 cluster when polymerase-based grouping was performed. The findings suggested that these 24 isolates were all recombinant viruses with GII/3 capsid and GII/12 polymerase. Interestingly, another NoV isolate, the 5424/03/Saga/JP, was grouped with NoV reference "new variants," which were designated as a GIIb in European countries (Fig. 2B). Taken together, the results indicated that the 5424/03/Saga/JP was also the recombinant strain.

In contrast, 20 NoV isolates belonging to GII/4 (the Lordsdale virus cluster), the genotype remained the same no matter the polymerase or capsid regions, was analyzed. Of these, 19 isolates shared significantly high identity of polymerase nucleotide sequences ranging from 98% to 100%. However, they shared only 93% sequence identity with those of the 5247/04/Maizuru/JP. It should be noted that the 5247/04/Maizuru/JP contained the previously unseen AATCTG motif starting at position 4,820 in the polymerase region (referring to the Norwalk virus, M87661). Obviously, this isolate was recognized as a "new variant of GII/4" according to the definition of Lopman et al. [2004]. Furthermore, as shown in Figure 2A, the majority (77.6%, 45 of 58) of NoV isolates were classified into GII/3 and GII/4 based on the partial capsid region, however, they were grouped into a SaitamaU1 sub-cluster based on the partial polymerase region (Fig. 2B).

Genetic Characterization of Recombinant Strain With GIIb Polymerase

To localize the potential recombination site and to understand a possible recombination mechanism of the "new variant" GIIb, the full-length nucleotide sequences of capsid and polymerase regions were determined and analyzed. When the nucleotide sequence of the 5424/03/Saga/JP was compared with those of the Arg320 and the Paris Island/03/US using the SimPlot software, region of genetic recombination was found between nucleotides 1,514 and 1,533 (the overlap of ORF1 and ORF2) (Fig. 3A). Up stream to this junction the nucleotide homology was notably different, and the SimPlot analysis showed a sudden drop in the nucleotide identity for the Arg320 but not for the 5424/03/Saga/JP and the Paris Island/03/US. The results demonstrated that the nucleotide sequences of capsid genes among these three strains were almost identical, but the polymerase sequences of the 5424/03/Saga/JP and the Paris Island/03/US were distinctly different from that of the Arg320.

Within the 5424/03/Saga/JP capsid sequence, the recombination at the breakpoint located at the beginning of P1 domain (position 1,330 nt in the capsid region) was identified. The capsid sequence of the recombinant 5424/03/Saga/JP showed alternate identities to the Oberhausen455/01/DE (nucleotides 1–1,330) and the Paris Island/03/USA (nucleotides 1,331–1,647) (Fig. 3B). The Oberhausen455/01/DE was detected in 2001 in Germany, whereas the Paris Island/03/USA was detected in 2003 in the United States. Quite possibly, the Paris Island/03/USA and the Oberhausen455/01/DE were putative parental strains of the 5424/03/Saga/JP. Taken together, the findings indicated that the 5424/03/Saga/JP had a double recombination.

Genetic Characterization of Recombinant Strain With SaitamaU1 Polymerase

As mentioned above, 24 isolates of GII/3 had high homology (98%–100%) at the nucleotide level of capsid

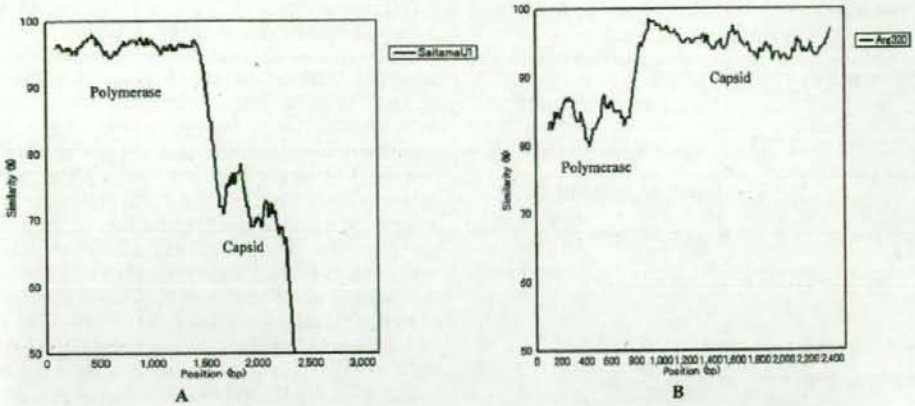

Fig. 4. Genetic characterization of recombinant virus with GII/3 capsid. **A**: The Simplot analysis of the NoV representative isolate, the 5017/04/Maizuru/JP, and the reference strain SaitamaU1. The high and low homologies with the polymerase and capsid regions among them, respectively, were found. **B**: The Simplot analysis of the 5017/04/Maizuru/JP and the reference strain Arg320. The low and high homologies with the polymerase and capsid regions among them, respectively, were found.

and polymerase. The findings demonstrated that they came from the same source of infection and represented the same strain. Furthermore, they were also suspected to be recombinant NoV based on their partial capsid and polymerase sequence. To further analyze this finding, the complete nucleotide sequences of the capsid and polymerase regions of one representative isolate, the 5017/04/Maizuru/JP, were determined. The 5017/04/Maizuru/JP shared a consistently low level of sequence identity (84%) in the RNA polymerase region but consistently high identity (95%) in the capsid region with the Arg320. In contrast, the 5017/04/Maizuru/JP shared consistently high level of sequence identity (96%) in the polymerase region and consistently low identity (70%) in the capsid region with those of the SaitamaU1. A recombinant site was also observed at the overlap of ORF1 and ORF2 (Fig. 4).

DISCUSSION

In this study, the prevalence of NoV infection among infants and children with acute gastroenteritis in five different localities of Japan was reported. Overall, the prevalence rate was 14.4% in all age groups of the subjects included in this study. However, the prevalence rate was increased up to 79.3% in infants and young children with the ages of less than 3 years old. These results were consistent with previous reports on NoV epidemiology worldwide in which the prevalence was ranged from 10% to 60% or more [Marks et al., 2000; Iritani et al., 2002; Lopman et al., 2002; Oh et al., 2003; Phan et al., 2004]. The findings suggested that approximately 14.4% of the etiologic agents of acute gastroenteritis cases in infants and children in Japan might be due to NoV and 85.6% might be responsible by other pathogens. The result also confirmed that NoV is one of the important enteropathogens responsible for viral gastroenteritis among infants and children in Japan. In some reports, NoV was prevalent in cold season, and several studies did not find a seasonal correlation [Vinje et al., 1997; Lopman et al., 2002; Phan et al., 2004]. The findings in this study are in agreement with the surveillance on pediatric cases of viral gastroenteritis in Japan, which demonstrated that the main peak of NoV infection was in the period of November, December, and January [Iritani et al., 2003; Inouye et al., 2000].

The results of this study showed that all Japanese NoV isolates belonged to two distinct genogroups, GI and GII, and these represented 1.7% and 98.3%, respectively. The results indicated that NoV GII was the dominant group causing acute gastroenteritis among Japanese pediatric population. It was interesting to note that the NoV GI 5226/04/Maizuru/JP, which was recovered from one 6-year-old-female patient with diarrhea, was closer genetically to the NoV Mie2001-U94/JP isolated from Japanese oyster than to human NoV reference strains available in the DDBJ DNA/GenBank database. This finding supported a view of possible NoV transmission to human through the contaminated oyster, which known as a reservoir of NoV. According to other reports published by different groups of investigators, NoV belonging to the Lordsdale cluster (GII/4) represented the most predominant genotype detected in sporadic gastroenteritis among infants and children not only in Japan but also in many other countries who run NoV surveillance [Chiba et al., 1979; McEvoy et al., 1996; Vinje et al., 1997; McIntyre et al., 2000]. However, it is surprising to note that in the present study NoV GII/3 was the most predominant, followed by NoV GII/4, NoV GII/2, and NoV GII/6. To verify this unusual observation, the polymerase regions of all NoV GII/3 and NoV GII/4 were further characterized. Remarkably, all NoV GII/3 except the 5424/04/Saga/JP were identified as the recombinant viruses that

related genetically to the SaitamaU1-like polymerase and the Arg320-like capsid. More interestingly, the SaitamaU1-like polymerase of NoV GII/3 was identical with those of NoV GII/4. The recombination of the NoV GII/4 polymerase and the Arg320-like capsid leading to an appearance of novel recombinant virus in the present study is postulated. Recently, NoV capsid protein was demonstrated to contain the determinants that are important for the immune recognition [Nilsson et al., 2003; Kirkwood, 2004]. Therefore, the emergence of recombinant virus with GII/3 capsid could be explained by a lack of acquired immunity for NoV GII/3 in Japanese infants and children. Interestingly, these recombinant strains suddenly appeared in a short period of 4 months (October 2003–January 2004) (data not shown). This sudden appearance and disappearance of strains might indicate that the virus appeared at the time that pediatric population lack antibody protection to these strains, and the virus disappeared by the time that the population began to acquire viral immunity. However, several studies reported that dominant strains could persist in one region over a number of years, which suggests that some other uncommon strains could be more virulent [Noel et al., 1999; Phan et al., 2004].

Another interesting finding of this study was the detection of "new variant with GIIb polymerase" 5424/03/Saga/JP in Japan. This isolate was isolated from a male patient with the age of 2 years old who developed a symptom of acute gastroenteritis in Saga, Japan. Surprisingly, based on the genetic analysis, this strain appeared to be an intratypic double recombinant. More interestingly, "new variant of GII/4 with unseen AATCTG motif" was also detected for the first time in a 2-year-old male patient with acute gastroenteritis in Maizuru, Japan in 2004. This motif was not present in any of the GII/4 sequences analyzed worldwide before 2002 from the food-borne viruses in European database and from the DDBJ DNA/GenBank database. This variant was first noted in Germany and the Netherlands in 2002 and become the predominant cause of NoV outbreaks throughout Europe [Lopman et al., 2004].

In conclusion, this is the first report on the existence of different "new variants" co-circulating in Japanese infants and children with acute gastroenteritis. This is also the first, description to the best of our knowledge, of the emergence and the importance of a novel recombinant virus causing acute gastroenteritis in Japan and warns of the threat it poses. Further epidemiologic studies should be conducted to determine whether this recombinant strain continues to be dominant in Japan in the coming year.

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ORIGINAL ARTICLE

Viral Gastroenteritis and Genetic Characterization of Recombinant Norovirus Circulating in Eastern Russia

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SUMMARY

From November 2003 to March 2004 a total of 100 fecal specimens from infants and children with acute gastroenteritis in the city of Birobidzhan, Eastern Russia were tested for the presence of diarrheal viruses by RT-multiplex PCR. Of these, 74 fecal specimens were positive for diarrheal viruses and this represented 74%. Among the diarrheal viruses detected, group A rotavirus was the most prevalent (67%; 67 of 100), followed by norovirus (4%; 4 of 100), group C rotavirus (1%, 1 of 100), sapovirus (1%; 1 of 100), and hepatitis A virus (1%; 1 of 100). It was found that 86.6% (58 of 67) of group A rotavirus were serotyped as G3. Sapovirus and hepatitis A virus were genetically determined to belong to GI/1 and subgenotype 1A, respectively. Interestingly, all norovirus isolates in the study turned out to make a novel cluster when polymerase-based grouping was performed. It is noteworthy to point out that these norovirus isolates were further genetically characterized as naturally occurring recombinants, which were firstly found circulating in the Russian population studied. Breakpoint analysis of recombinant norovirus showed that the recombination site was at the open reading frame (ORF)1/ORF2 overlap. This is the first report of the existence of acute gastroenteritis caused by recombinant norovirus in Eastern Russia.
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KEY WORD

norovirus, recombination, Eastern Russia

INTRODUCTION

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality of children due to gastroenteritis is greater in developing than in the developed countries [1]. It has been well established that virtually every child becomes infected with a rotavirus at least once by 3 years of age [2]. Rotaviruses are classified into seven groups (A to G) on the basis of their distinct antigenic and genetic properties. Human infection has been reported with group A, B and C rotaviruses. Of these, group A rotavirus is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide [3]. Apart from group A rotavirus as the most common cause of gastroenteritis, norovirus is also considered to be a global enteropathogen. This virus is associated with sporadic cases and outbreaks of acute gastroenteritis in such settings as kindergartens, schools, nursing homes for the elderly, and among military recruits [4, 5]. The transmission routes of this virus are classified into foodborne, airborne, person-to-person spread and perhaps by some other unknown modes [4, 6]. However, norovirus is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make norovirus a major public health concern [7].

Norovirus (NoV, formerly known as "Norwalk-like virus") is the distinct genus within the family *Caliciviridae*. Norovirus contains a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The norovirus genome contains three ORFs (ORF1, 2 and

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