SUMMARY: In an outbreak of acute gastroenteritis which originated in a restaurant in Chiba, Japan, in December, 1987, small round structured virus (SRSV) particles were observed by electron microscopy in 14 of 16 stool specimens from patients. The particles were 30 to 35 nm in diameter, possessed amorphous surface structure surrounded by fine projections and had a buoyant density of 1.36 to 1.37 g/ml in cesium chloride. Serological responses to the SRSV were found by immune electron microscopy and Western blot (WB) assay in paired sera of 12 of 19 patients. Furthermore, WB analysis revealed that the antibody against SRSV was cross-reactive to other SRSV, Tokyo 86/510.

INTRODUCTION

Since the initial description (1) of the Norwalk agent in 1972, there have been many reports on finding small round viruses (SRVs) by electron microscopy (EM) in stool specimens from patients with acute gastroenteritis. These SRVs include Norwalk agent (1), calicivirus (2), astrovirus (3), Hawaii agent (4), Snow
Mountain agent (5), and Otofuke agent (6). These viruses have not been successfully propagated in cell cultures and therefore have not been characterized in detail biologically or biochemically.

An outbreak of acute gastroenteritis originated in a restaurant in Chiba, Japan, in December, 1987. We investigated the outbreak for the causative agent and found SRV in stool specimens from the patients by EM. We attempted to characterize the SRV by immune electron microscopy (IEM) and the Western blot (WB) assay.

**MATERIALS AND METHODS**

**Stool and sera:** Stool specimens were obtained from 14 patients on 3 or 4 days after the onset of illness. Paired serum specimens were obtained from 19 patients. Acute-phase sera were collected 4 to 5 days after the onset of illness and convalescent-phase sera 20 days later. A stool specimen containing numerous SRV particles was obtained from a 48-year-old female patient (#17 in Table II) 3 days after the onset of illness. We used the specimen as the virus material for the determination of buoyant density and IEM and WB assays.

**Virus preparation:** A 10% stool suspension homogenized in phosphate-buffered saline (PBS) was mixed with an equal volume of trichlorotrifluoroethane and centrifuged at 1,700 ×g for 20 min. The aqueous layer was collected, layered onto 30% sucrose, and centrifuged at 110,000 ×g (50.2 Ti rotor, Beckman) for 2.5 hr at 4 C. The pellet was resuspended in distilled water in a 1/50 to 1/100 volume of the initial stool suspension. This partially purified virus preparation was used for EM, IEM, and WB assays.

**Determination of buoyant density in cesium chloride (CsCl):** One milliliter of the partially purified virus preparation was layered onto 4 ml of a continuous CsCl density gradient (1.10 to 1.50 g/ml) and centrifuged at 150,000 ×g (SW 50.1 rotor, Beckman) for 4 hr, at 4 C. Twenty fractions were collected from the gradient and each fraction was examined by EM (JEM-100S, JEOL) to determine the number of virus particles.

**IEM:** IEM was performed according to the method of Kapikian et al. (1) with slight modification. Twenty-five microliters of the partially purified virus preparation was mixed with 25 μl of serum diluted to 1:20 in PBS. The mixture was incubated at 37 C for 1 hr and kept overnight at 4 C. The preparation was stained with 2% phosphotungstic acid (pH 7.2) and the reactivity of the virus particles with antibody was examined by EM. The levels of IEM antibody were rated in five classes from 0 to 4+ as follows: 0 = no aggregates; 1+ = glistening aggregates, lightly covered with antibody; 2+ = moderately glistening
aggregates, moderately covered with antibody; 3+ = non-glistening aggregates, heavily coated with antibody; 4+ = non-glistening aggregates so heavily coated with antibody that they were obscured. An increase of 1+ or more in antibody rating from acute phase to convalescent phase serum samples was considered to be significant.

**WB:** WB was performed by the procedure described by Hayashi et al. (7) and Utagawa et al (8). The partially purified virus preparation was electrophoresed in 4 to 20% linear gradient SDS-polyacrylamide gel. The gel was then electroblotted onto a nitrocellulose membrane. The membrane was cut into 2-mm-wide strips. Serum samples were diluted to 1:100 and allowed to react with the blotted strips at room temperature overnight. The immune complex of immunoglobulin G-antigen was detected by biotin-avidin enzyme immunoassay. The levels of WB antibody were graded from 0 to 4+ with the intensity of stained protein band (63 kDa). An increase of 1+ or more in antibody rating from acute phase to convalescent phase serum samples was considered to be significant.

**Virus isolation:** A 10% stool suspension in PBS was inoculated onto HeLa and RD-18S cells and cytopathic effects (CPE) were daily observed under a microscope for about 10 days. Virus isolation was attempted by two successive blind passages.

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**RESULTS**

**Outbreak**

Outbreaks of acute gastroenteritis occurred in two groups of adults who had dinner at a restaurant on December, 18 and 19, 1987. The attack rates in group A (December, 18) and group B (December, 19) were 37.0% (10/27) and 60.0% (18/30), respectively. Both groups took raw oyster and/or fish which were served at the restaurant, though the statistic analysis ($\chi^2$ test) of food consumption data failed to prove direct association of the disease with a particular raw food.

Figure 1 shows the number of patients who became ill during each of consecutive 5-hr periods after consumption of the meal. The incubation period ranged from 2 to 54 hr, showing two separate peaks. In both groups, the most prominent peak was in the range of 35 to 44 hr, comprising about 40% of the total number of patients. Among 28 patients, 13 cases were diagnosed as SRV infection, and 10 of 13 developed diarrhea between 22 to 49 hr after consumption of the meal.
Fig. 1. The incubation periods of illness.

Table I. Frequency of clinical symptoms

Each fractional number shows: patients having developed the symptom / the number examined.

<table>
<thead>
<tr>
<th>Group</th>
<th>Nausea</th>
<th>Vomiting</th>
<th>Diarrhea</th>
<th>Abdominal pain</th>
<th>Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9/10</td>
<td>3/10</td>
<td>9/10</td>
<td>8/10</td>
<td>4/10</td>
</tr>
<tr>
<td>B</td>
<td>15/18</td>
<td>6/18</td>
<td>8/18</td>
<td>11/18</td>
<td>5/18</td>
</tr>
</tbody>
</table>

Table I shows the main clinical symptoms. Nausea was the most frequent; 90% in group A and 83% in group B. Vomiting was seen in about 30% in both groups. Diarrhea was more frequent (90%) in group A, but in most cases, mild
Table II. Detection of SRSV particles in the stool specimens by EM and serological evidence of infection by IEM and WB

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>EM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IEM-determined sero-response to SRSV from patient 17*</th>
<th>WB-determined sero-response to SRSV from patient 17*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acute</td>
<td>Convalescent</td>
</tr>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>0</td>
<td>+2</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>0</td>
<td>+3</td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
<td>0</td>
<td>+3</td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
<td>0</td>
<td>+2</td>
</tr>
<tr>
<td>5</td>
<td>NA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>NA</td>
<td>+1</td>
<td>+3</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
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<td>+1</td>
<td>+2</td>
</tr>
<tr>
<td>11</td>
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<td>+1</td>
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<td>+1</td>
</tr>
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<td>15</td>
<td>+</td>
<td>+1</td>
<td>+4</td>
</tr>
<tr>
<td>16</td>
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<td>+3</td>
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<tr>
<td>17*</td>
<td>+</td>
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<td>+3</td>
</tr>
<tr>
<td>18</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>+</td>
<td>0</td>
<td>+1</td>
</tr>
</tbody>
</table>

NA: Stool specimens were not available.

<sup>a</sup>: Stool specimens were examined for the presence of SRSV particles by EM.

*: SRSV from patient #17 was designated as Chiba 87/407 and used as antigen.

with soft (not watery) stool. Fever was less frequent and temperature was 37 to 38 C. The illness was generally mild and of short duration (2 to 3 days).
Detection and Characterization of SRV

Stool specimens from 14 patients were examined by EM. SRV was detected in one of two specimens in group A and five of 12 specimens in group B (Table II). As shown in Fig. 2, the virus particles are 30 to 35 nm in diameter and possess spherical shape without a complete outline and the amorphous surface structure surrounded by fine projections. These are the typical characteristics of small round structured virus (SRSV).

We examined the buoyant density in CsCl of SRSV obtained from the specimen of patient 17 and designated as Chiba 87/407 virus. The number of virus particles in each fraction from CsCl gradient is shown in Fig. 3, which exhibits the peak in fraction #4. The buoyant density was estimated at 1.36 to 1.37 g/ml in CsCl.

Serological Evidence of Infection

We examined paired sera for the antibody to SRSV by IEM and WB, using the Chiba 87/407 virus as antigen. The results are shown in Table II. IEM demonstrated significant serological response in five of seven patients in group A, and seven of 12 patients in group B, giving a total response rate of 63.2% (12/19).

In WB analysis, a number of nonspecific bands appeared on the blotted
strips, but an SRSV-specific protein band (63 kDa) was clearly detected and it was used for serodiagnosis. Seroconversion was observed in 12 of 19 patients. The results of WB agreed with those obtained by IEM except in patients 6 and 10 (Table II).

**Serological Tests between Two SRSVs**

Serological cross-reaction was tested by IEM and WB between two SRSVs, Chiba 87/407 and Tokyo 86/510. The latter SRSV was obtained during an outbreak of acute gastroenteritis in Tokyo in March, 1986 (7), and kindly provided by Tokyo Metropolitan Research Laboratory of Public Health for this comparative experiment. As shown in Table III, the results of WB and IEM were roughly in agreement. Chiba 87/407 antigen did not react with the paired sera from a patient infected with Tokyo 86/510 virus. In contrast, Tokyo 86/510
Table III. Serological cross-reaction between Chiba 87/407\textsuperscript{a} and Tokyo 86/510\textsuperscript{b} antigens by IEM and WB

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Paired sera from patients infected with:</th>
<th>Chiba 87/407</th>
<th></th>
<th>Tokyo 86/510\textsuperscript{c}</th>
<th></th>
<th>IEM</th>
<th>WB</th>
<th>IEM</th>
<th>WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chiba 87/407</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tokyo 86/510\textsuperscript{c}</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND: The test was not done.

\textsuperscript{a} SRSV detected in patient 17\textsuperscript{*} in Table II.

\textsuperscript{b} SRSV detected in a 39-year-old female patient in the outbreak of acute gastroenteritis in Tokyo, March, 1986.

\textsuperscript{c} The antigen and paired sera were provided by Tamie Ando, Tokyo Metropolitan Research Laboratory of Public Health.

antigen specifically reacted with the convalescent serum of the Chiba '87 patient as well as that of the Tokyo '86 patient in WB assay.

\textit{Virus Isolation}

Despite repeated trials of virus isolation, CPE was observed in neither HeLa cells nor RD-18S cells.

\textbf{DISCUSSION}

Recently, SRVs have been identified by EM in specimens obtained from outbreaks of acute gastroenteritis suspected to be associated with the consumption of contaminated food (9,10) or drinking water (11,12).

In the present study, SRV particles were detected in six of 14 stool specimens from patients in an outbreak of acute gastroenteritis among adults who had taken foods at a restaurant in Chiba Prefecture, in December, 1987.
Statistic analysis of the food consumption data failed to prove association of the disease with a particular food. Serological response to the SRV was observed by IEM and WB in paired sera from 12 of 19 patients tested. These results suggest that SRV is the etiologic agent of this outbreak. The incubation period of the disease was broadly distributed with two peaks, which ranged from 0 to 14 hr and 35 to 44 hr (Fig. 1). Since it has been reported (4,11) that the incubation period of SRV-associated gastroenteritis was about 30 to 40 hr, the late onset group in the present outbreak was considered to be due to SRV infection. In fact, most of the patients who were diagnosed as SRV infection by IEM and WB were found in the late onset group. A causative agent for the early onset group has not been identified; the culture of causative bacteria of the acute gastroenteritis (e.g. Staphylococcus aureus, Vibrio parahaemolyticus, Salmonella spp and enteropathogenic Escherichia coli) of stools from all patients was negative.

The SRV (Chiba 87/407) observed in this outbreak was 30 to 35 nm in diameter and had amorphous surface structure surrounded by fine projections. These morphological features are different from those of calicivirus having characteristic hollows on its surface, and also from astrovirus, which has a five- or six-pointed star appearing on its surface. Caul and Appleton (13) proposed the criteria for classification of SRVs on the basis of morphological and/or physical differences among them. According to their criteria, the Chiba 87/407 virus is closest to the small round structured virus (SRSV) which includes Norwalk, Hawaii, and UK viruses. The buoyant density of the Chiba 87/407 virus was 1.36 to 1.37 g/ml in CsCl, the value being in agreement with the densities reported for other SRSVs (6,14).

In general, IEM or RIA (15) has been used for serological diagnosis of SRSV or other SRV infections. Recently, the WB assay has been found to be a highly specific and sensitive method for detection of the antibody to SRSV (7,8). Hayashi et al. (7) reported that SRSV obtained from a patient with gastroenteritis in Tokyo, 1986 (Tokyo 86/510) contained a single structural protein with the molecular size of 63 kDa, which was demonstrated in WB assay as a band differentially reactive with the acute and convalescent sera from the patients infected with SRSV. Utagawa et al. (8) further studied antigenic relation of the Tokyo 86/510 virus with other viruses and found that the virus was closely related serologically with Hawaii, Otofuke, and Osaka (16) agents.

In the present experiments, we examined antigenic relationship between Chiba 87/407 and Tokyo 86/510 viruses. The Chiba 87/407 antigen clearly reacted with the convalescent serum from the patient infected with Chiba '87
virus but did not react with the sera from the patient infected with Tokyo '86 virus. On the other hand, the Tokyo 86/510 antigen differentially reacted with the paired sera of the Chiba '87 patient as well as those of the Tokyo '86 patients. These results obtained by WB analysis suggest that the two SRSVs, Tokyo 86/510 and Chiba 87/407, are antigenically related but not completely identical to each other. More detailed characterization of the Chiba 87/407 antigen by WB analysis and its antigenic relation with other known SRSVs are under study.

ACKNOWLEDGEMENTS

We thank Tamie Ando, Tokyo Metropolitan Research Laboratory of Public Health for providing us with SRSV, Tokyo 86/510, and its paired sera.

REFERENCES


