Etiologic Agents of Acute Diarrhea

In Vitro Cultivation of Human Rotavirus in MA104 Cells

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Recently, viruses or virus-like particles have been detected by electron microscopic examination in stools from patients with acute diarrhea; they are considered the causative agents of acute diarrhea (3). Among them, the human rotavirus has been recognized as a most important pathogen of acute diarrhea in infants and young children in many parts of the world. However, these viruses associated with acute diarrhea have been identified mainly on the basis of their morphological and immunological features. Although a great deal of effort has been directed to adaptation of these viruses to continued propagation in cell cultures, only several animal viruses, including rotavirus strains, have been adapted to grow efficiently to high titer in cell culture (13). The difficulties in propagating human enteric viruses, particularly rotavirus in conventional cell culture systems, have hampered rapid progress in virological, serological, and epidemiological studies on viral diarrheal diseases.

In 1980, the Wa strain, human rotavirus type 2, was first adapted to grow efficiently in primary, green monkey, kidney cell cultures after passages in newborn gnotobiotic piglets (12). Most recently, successful in vitro cultivation systems for human rotavirus, without any passages in animals, were reported by Sato et al. (10) and subsequently confirmed by Urasawa et al. (11). On the basis of these results, it was suggested that most human rotaviruses could be cultivated in cell cultures. In view of such successful cultivation, we attempted to cultivate in cell cultures human rotaviruses that had been serotyped either subgroup 1 or subgroup 2 by immune adherence hemagglutination (IAHA) or enzyme-linked immunosorbent assay (ELISA) (5), and succeeded in isolating two tissue culture-adapted strains distinguishable from each other by IAHA (8). This chapter describes the methods for cultivating these two isolates in MA104 cell cultures.

MATERIALS AND METHODS

The fecal specimens for this study were obtained from infants with acute diarrhea and stored at −80°C. Rotavirus particles in these specimens had been previously detected by electron microscopy (EM).
The ribonucleic acid (RNA) profiles of these rotavirus strains were analyzed by polyacrylamide gel electrophoresis. For electrophoresis, the rotaviruses were purified by the method previously reported (7). The purified viruses were deproteinized with phenol, and the RNA was precipitated with ethanol. The pelleted RNA was dissolved in Laemmli’s sample buffer (9). After electrophoresis, the gels were stained with ethidium bromide solution. As shown in Table 1, the rotavirus of specimen 80SR004 was observed to have an RNA pattern with slow-moving segments 10 and 11 and was designated S type. The virus of 80SP001 exhibited an RNA pattern with fast-moving segments 10 and 11 and was designated L type. The RNA patterns, S and L type, correspond as far as segments 10 and 11 are concerned to 2s and 2l, respectively, as described by Espejo et al. (2).

Subgroup specificities were examined by ELISA and/or IAHA in collaboration with A. Z. Kapikian (National Institutes of Health, Bethesda, Maryland) (7), revealing that the rotavirus strain with an RNA pattern of S type belonged to subgroup 1 and the L type to subgroup 2.

For virus isolation in cell culture, an established cell line (MA104) derived from the kidney of an embryonal, rhesus monkey was used. Growth medium for MA104 cell culture was Eagle’s minimum essential medium (MEM), supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units penicillin and 100 μg streptomycin). Maintenance medium was MEM containing 0.5 μg/ml trypsin (Sigma IX), antibiotics, and no serum. All cultures were incubated at 37°C. The cultures for virus isolation were incubated in a roller drum.

For inoculation, 10% homogenous suspensions of fecal specimens were made in MEM and centrifuged successively at 3,000 rpm and 10,000 rpm. The clarified supernatant was mixed with an equal amount of 20 μg/ml trypsin and incubated at 37°C for 20 min. Before inoculation, the trypsin-treated fecal specimens were diluted 1:20 with MEM.

To ascertain virus growth in cell culture, every culture medium was examined by EM after staining with 1% uranyl acetate solution.

RESULTS

Isolation and Propagation of Rotaviruses in Cell Cultures

Confluent monolayer cultures of MA104 cells prepared in 10 × 110 mm culture tubes were washed with maintenance medium and then inoculated with 0.2 ml of the 1:20 diluted trypsin-treated fecal specimens. After 1 hr adsorption at 37°C, cell cultures were washed with MEM without serum, suspended in the maintenance medium containing trypsin, and then incubated in a roller drum. The media were changed every other day and checked by EM for the presence of virus particles. On the 11th day after the initial inoculation, the culture tubes were frozen and thawed three times, and centrifuged at 3,000 rpm. The supernatants of the culture lysates were treated with
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TABLE 1. Source of isolates

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Date of onset of diarrhea</th>
<th>Collection of stool</th>
<th>Rotavirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>(80SR004)</td>
<td></td>
<td></td>
<td></td>
<td>S*</td>
</tr>
<tr>
<td>Nagatsuka</td>
<td>M</td>
<td>Feb. 1, 1980</td>
<td>Feb. 6, 1980</td>
<td>++</td>
</tr>
<tr>
<td>(80SP001)</td>
<td></td>
<td></td>
<td></td>
<td>L*</td>
</tr>
</tbody>
</table>

* S: slow-moving segments 10 and 11; L: fast-moving segments 10 and 11.

Trypsin and then inoculated into fresh confluent cell monolayer cultures for the second passage.

On the second passage, the culture tubes were frozen on day 6 after inoculation and, following trypsin treatment, the culture fluids were inoculated onto fresh MA104 cell monolayers in tubes. On subsequent passages, the culture fluid for the next passage was obtained 2 or 3 days after inoculation.

Cytopathic effects were observed 10 days postinfection during the primary passage in one out of four culture tubes inoculated with stool specimens 80SR004 (Takiguchi), and 8 days postinfection in two out of four tubes, and 9 days in three out of four tubes with specimens 80SP001 (Nagatsuka). The cultivated rotavirus strain derived from 80SR004 was named KUN and that from 80SP001 was named MO. On the second passage, cytopathic effects were noticed at day 4 in one, day 5 in two, and day 6 in three out of four culture tubes with KUN; and at day 4 in two, day 5 in three, and day 6 in four out of four tubes with MO. However, distinct cytopathic effects were observed on the sixth passage with KUN, and the third passage with MO (Table 2). Thereafter, cytopathic effects were recognized 48 to 72 hr after infection. The cytopathic effects consisted of obscure cell boundary, cell fusion, cell rounding, cell detaching, and lytic foci.

Rotavirus particles in the culture media were detected by EM on the second and subsequent passages, and the number of viruses detected increased when passages progressed. Most of the virus particles found in the culture media had

TABLE 2. Occurrence of CPE and detection by EM of rotavirus particles in MA104 cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Passage in MA104 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
</tr>
<tr>
<td>KUN CPE</td>
<td>±</td>
</tr>
<tr>
<td>EM</td>
<td>−</td>
</tr>
<tr>
<td>MO CPE</td>
<td>±</td>
</tr>
<tr>
<td>EM</td>
<td>−</td>
</tr>
</tbody>
</table>
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a characteristic appearance of rotavirus with a double capsid. Empty particles or virus lacking an outer capsid were infrequently observed.

The infectious titer of the culture fluids was $10^7\text{TCID}_{50}/\text{ml}$ in KUN on the eighth passage, and $10^{7.5}\text{TCID}_{50}/\text{ml}$ in MO on the seventh passage in a roller tube culture.

**RNA Patterns of Human Rotavirus Isolates KUN and MO**

To establish the identity of the tissue culture-adapted rotavirus strains, viral RNA was examined by a polyacrylamide gel electrophoresis. In our laboratory, animal rotaviruses had never been used for investigation, but Wa strain cultivable human rotavirus had been grown and investigated. Considering the ease with which cultures could be contaminated, RNA profiles of the strains KUN and MO were compared not only with those of their original fecal viruses, but also with the RNA profile of the Wa strain that had been thoroughly investigated. Rotaviruses subjected to RNA analysis were obtained in confluent cell monolayers in roller bottles infected with the sixth passage KUN and fifth passage MO. The electrophoretic RNA patterns of the isolated rotavirus strains KUN and MO were found to be identical to those of their respective original viruses (Table 3). They were different in electrophoretic RNA pattern from the Wa strain. The isolate KUN was S type and MO was L type, as far as mobility of RNA segments 10 and 11 was concerned.

**Antigenic Specificities of the Tissue Culture-Adapted Rotavirus KUN and MO**

Subgroup specificity of the isolates grown in MA104 cell cultures was examined by IAHA in collaboration with A. Z. Kapikian, and compared with the original viruses. The KUN virus was identified as subgroup 1, and the MO virus as subgroup 2; these were the same subgroups as the rotaviruses present in the stools of the patients Takiguchi and Nagatsuka (Table 3).

**Growth in Stationary Cultures of Isolates KUN and MO**

Attempts to adapt the isolates to stationary cultures first succeeded on the 11th passage with KUN strain and on the eighth passage with MO strain.

| Table 3. Comparison of RNA patterns and subgroup specificities of isolated human rotavirus by tissue culture and their origins |
|---|---|---|---|
| **Fecal Cultivated** | **Fecal Cultivated** | **Fecal Cultivated** |
| 80SP004 (Takiguchi) | KUN (Nagatsuka) | 80SP001 (Nagatsuka) | MO |
| RNA* | S | S | L | L |
| IAHA Subgroup | 1 | 1 | 2 | 2 |

* Identical in mobility of all RNA segments between S types, and between L types.
However, their infectious titer in stationary culture was much lower than that in roller tube culture. The infectious titer of the first stationary cultures was $10^{2.5}$ TCID$_{50}$/ml with both KUN and MO strains. In addition, cytopathic changes were not as distinct as in roller cultures.

**DISCUSSION**

**Initial Isolation of the Virus**

The present study confirmed the *in vitro* cultivation method of human rotaviruses, which had been reported previously by Sato et al. and Urasawa et al. in Japan (10,11). In this experiment, however, the incubation period was prolonged until cytopathic changes appeared on the first and second passage, while in previous reports passages had been made at intervals of 3 days or 3 to 5 days (10,11). Urasawa et al. concentrated the culture fluid by centrifugation before inoculation on the second and third passages (11). The adaptation procedure adopted by different authors is therefore somewhat difficult, but the fact remains that successful *in vitro* cultivations have been made independently in three laboratories. This could suggest that most rotavirus strains could be adapted to grow efficiently and undergo serial passages with a growth yield sufficient to permit characterization of the virus.

Wa strain is the first cell culture of an adapted human rotavirus. However, serial passage of the Wa strain in cell culture was achieved only with the 11th passage material from newborn gnotobiotic piglets (12).

Sato et al. (10), having been successful in the primary isolation of human rotavirus in cell culture, emphasized the importance of using MA104 cells, pretreating the virus with trypsin, and adding trypsin in the maintenance medium. In addition, they used a roller tube culture that was supposed to produce more pronounced cytopathic effect with bovine rotavirus than a stationary culture (6).

**Appearance of Cytopathic Effects**

Although the early reports of the cytopathic effect with bovine rotavirus included a description of "flagging," where cells are partially detached from the surface and wave in the medium, as being characteristic and even diagnostic for rotavirus-infected monolayers (3), this has not been generally accepted. Cytopathic effect was also described to take the form of nondescript granular degeneration of the cell monolayer (13). In this study, cytopathic effects with KUN and MO strains included obscure cell borders, cell fusion, cell rounding, cell piling-up, cell-detaching from the surface of the tubes, and lytic foci. Thus, the appearance of cytopathic effects with the virus strains was easily recognized in the cell culture, but its true nature was not well characterized.

Because of the slow and inapparent development of cytopathic effects, the increase of the number of virus particles detected by EM in culture media provided a good aid to determine the day of virus harvest in the culture.
Although immunofluorescent staining is known to be useful for detecting virus growth in cell cultures (10,11), it was not performed in this study.

**Use of Trypsin**

The experience with isolates of animal rotavirus as well as of Wa strain has suggested that the use of trypsin is essential in the cultivation (13). In the case of human rotavirus strains adapted to grow in cell cultures by Japanese investigators, the inoculum was treated with trypsin and the latter was also added to the maintenance medium (10,11). In this study, the concentration of trypsin used was 10 μg/ml for virus treatment prior to inoculation and 0.5 μg/ml for maintenance medium; MA104 cells could tolerate this concentration. While the mechanism of action has not been elucidated, trypsin is presumed to exert an effect on the virus itself by cleaving surface polypeptide(s) and to act on progeny viruses produced in the early replication cycles (13).

**Virus Plaquing**

We attempted to propagate the roller culture-adapted rotavirus strains, KUN and MO, in a stationary cell culture that would offer the advantage of characterizing the virus. In the case of the isolate KUN, the virus was adapted to a stationary culture in MA104 cells on passage 11, and in the MO strain on passage 7. Plaque formation with these viruses is now in progress. The Wa strain and the virus strains isolated by Urasawa et al. are adapted to produce plaques in MA104 monolayer culture, which is also enhanced by the addition of trypsin in agar overlays (11,12). Efforts for plaquing should be continued to facilitate serological characterization as well as genetic manipulation of the virus.

**Electrophoretypes and Serotypes**

The electrophoretic migration patterns of tissue culture-adapted human rotavirus KUN and MO were identical to those of original fecal viruses, confirming the isolates to be human rotaviruses.

Although antigenic properties of rotavirus have not been thoroughly understood, the existence of more than two serotypes has been evidenced by a variety of methods including complement fixation, ELISA, IAHA, and neutralization (1,5,14). Recently, evidence of the dissociation of neutralization and IAHA antigenic specificities has been recognized in studies of human and bovine rotavirus reassortants (5). In addition, the existence of a correlation between the RNA patterns of human rotaviruses and their antigenic subgroup specificities has been demonstrated (4). The KUN and MO strains isolated in this study were identified by IAHA as subgroups 1 and 2, respectively, belonging to the same subgroups as the rotaviruses present in the stools. Correlation between the subgroup specificity and RNA patterns of the isolates KUN and MO was shown to be similar to that of the original stool rotaviruses.

In view of these results, neither KUN nor MO grown in MA104 cells appear
to be contaminants of tissue culture-adapted human rotavirus, Wa strain, or animal rotaviruses.

It is of interest to note that KUN strain, belonging to subgroup 1, seemed reluctant to grow in cell culture, especially on early passages, compared with MO strain, subgroup 2. Although still unproven, this may be caused by antigenic differences of human rotaviruses.

The successful cultivation of two human rotavirus KUN and MO strains with different antigenic properties in subgroup specificity correlated with electrophoretic RNA pattern might facilitate a detailed study of the viruses. The methods of culture for human rotavirus should be useful for other viruses causing acute diarrhea.

ACKNOWLEDGEMENT

The authors are indebted to Dr. A. Z. Kapikian of National Institutes of Health, USA, for his generous help with serological examinations of rotavirus strains.

REFERENCES