Improvement of the Plaque Technique for Human Rotaviruses:
Effect of Fetal Bovine Serum, Acetyltrypsin and Agar

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Summary: Dilution of viruses, which were previously treated with 10 μg acetyltrypsin/ml, in MEM supplemented with 2.5% fetal bovine serum caused little damage of cell monolayers resulting in the formation of discrete plaques. When agar and acetyltrypsin were added to the overlay medium at the concentrations of 0.8% and 5 μg/ml, respectively, clear plaques were induced. Linear relationships were obtained between virus concentration and the number of plaques, thus the procedure described in this report might be used for reliable plaque assay of rotaviruses. Moreover, these conditions for plaque formation were successful for estimating isolated viruses, as well as standard strains.

Key words: human rotavirus — plaque formation — fetal bovine serum — virus diluent — agar overlay medium

Introduction

Typing of human rotaviruses (HRV) has been performed by comparing the electrophoretic patterns of virus RNAs extracted directly from stool specimens of pediatric patients with acute gastroenteritis. Elisa with monoclonal antibodies has also been used for serotyping HRV in stool specimens.

Isolation of HRV from stools was not always successful, although a large number of virus particles (10^10—10^11 particles per gram of stool) were usually detected in stool specimens of the patients with an electron microscope. However, Sato et al. (1981) have successfully isolated HRV directly from stool specimens in MA-104 cells derived from embryonic rhesus monkey kidney. After the incorporation of MA-104 cells, some investigators have attempted to develop a plaque assay technique to measure the propagation of HRV (Urasawa et al. 1982; Aha and Sabara, 1990). Urasawa et al. (1982) reported on a plaque assay system using overlay medium containing acetylated trypsin and DEAE-dextran. However, in the current experiments, it was very difficult to form plaques with these procedures.

The purpose of the present experiments, based on the procedures of Urasawa et al. (1982), is to find the conditions that will protect cell monolayers from damage by trypsin and other factors, and to offer recommendable procedures for a plaque assay.

Materials and Methods

Cell

MA104 cells derived from embryonic rhesus monkey kidney were cultured in MEM (twice the concentration of l-glutamine) supplemented with 4% calf serum (CS) and 1% fetal bovine serum (FBS),
and passaged by 1:3 split every 5 days.

Viruses

HRV strains KU (type 1), S2 (type 2), YO (type 3), and Hochi (type 4) and five isolates (no. 10, 26, 33, 42, and 44) were propagated in MA104 cells. The cell monolayers were washed twice with phosphate-buffered saline (PBS), inoculated with the viruses that were pretreated with 10µg acetyltrypsin/ml at 37°C and incubated in MEM containing 1µg acetyltrypsin/ml. When approximately 90% of the cells yielded CPE, the cultures were stored at -80°C until use.

Plaque formation

MA104 cell cultures were prepared in 2-oz bottles in MEM supplemented with 4% FBS and 4% CS. The cell monolayers, which were formed after two days incubation at 37°C, were washed twice with PBS and inoculated with 0.2 ml of tenfold stepwise dilutions of HRV pretreated with 10µg acetyltrypsin/ml in MEM. The virus was allowed to adsorb for 90 minutes at 37°C with gentle tilting every 15 minutes. At the end of the adsorption time, 6 ml of appropriate agar overlay medium was added onto the cultures. Three ml of the second overlay medium consisting of 0.8% agar (purified agar, Oxoid), 7.5µg acetyltrypsin/ml, 200µg DEAE-dextran/ml and 1mg glucose/ml. As illustrated in Fig. 1-1, the introduction of 3% and 5% FBS in the diluent resulted in clear plaques. In contrast, 1% FBS or 3% BSA induced obscure plaques resulting in a difficult reading. Although clear plaques were observed with both 3% and 5% FBS, larger sizes and a larger number of plaques were obtained with 3% FBS (Table 1). The effects of FBS at concentrations lower than 3% on plaque formation.

Acetyltrypsin

Acetyltrypsin was purchased from Sigma. It was dissolved in PBS or water at a concentrations of 1 mg/ml or 5 mg/ml and diluted as necessary.

Results

Effect of FBS in virus inoculum on plaque formation

<table>
<thead>
<tr>
<th>Addition to inoculum</th>
<th>PFU/ml</th>
<th>Size (mm)</th>
<th>Cell damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>no</td>
<td>6.3×10^4</td>
<td>1~3</td>
<td>+</td>
</tr>
<tr>
<td>1% FBS</td>
<td>5.0×10^4</td>
<td>1~3</td>
<td>+</td>
</tr>
<tr>
<td>expt. 1 3% FBS</td>
<td>1.5×10^5</td>
<td>2~3</td>
<td>-</td>
</tr>
<tr>
<td>5% FBS</td>
<td>1.0×10^5</td>
<td>1~1.5</td>
<td>-</td>
</tr>
<tr>
<td>3% BSA</td>
<td>4.3×10^4</td>
<td>1~3</td>
<td>+</td>
</tr>
<tr>
<td>1.5% FBS</td>
<td>6.8×10^4</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>expt. 2 2% FBS</td>
<td>1.4×10^5</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2.5% FBS</td>
<td>2.1×10^5</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

The plaques, formed according to the procedures of Urasawa et al. (1982), were often difficult to read because of cell damage probably during the time of virus adsorption. To exclude the possibility of the cell damage by the enzymes used for virus activation and to stabilize the cell monolayers during the adsorption time, an attempt was made to add FBS or bovine serum albumin (BSA) to the virus inoculum. HRV strain KU was pretreated with 10µg acetyltrypsin/ml MEM for 30 minutes at 37°C and then diluted in tenfold steps with MEM containing various concentrations of FBS or BSA. After time for adsorption, the overlay medium was added onto the culture. The overlay medium consisted of MEM containing 0.8% agar (purified agar, Oxoid), 7.5µg acetyltrypsin/ml, 200µg DEAE-dextran/ml and 1mg glucose/ml. As illustrated in Fig. 1-1, the introduction of 3% and 5% FBS in the diluent resulted in clear plaques. In contrast, 1% FBS or 3% BSA induced obscure plaques resulting in a difficult reading. Although clear plaques were observed with both 3% and 5% FBS, larger sizes and a larger number of plaques were obtained with 3% FBS (Table 1). The effects of FBS at concentrations lower than 3% on plaque forma-
Fig. 1. Effects of FBS and BSA in the virus inoculum on plaque formation of the HRV strain KU

1. a. no addition,  b. 1% FBS,  c. 3% FBS,  d. 5% FBS,  e. 3% BSA
2. a. 1.5% FBS,  b. 2.0% FBS,  c. 2.5% FBS
tion were tested further. As shown in Fig. 1–2 and Table 1, the clearest plaques and the highest titers were obtained with the diluent containing 2.5% FBS. Thus, MEM supplemented with 2.5% FBS was adopted in the following experiments as the diluent for the virus pretreated with acetyltrypsin.

Effect of acetyltrypsin in the overlay medium on plaque formation

As trypsin is essential to render the virus infective, HRV cannot produce plaques on cell monolayers in the absence of the enzyme in an agar overlay medium, even if the virus has been pretreated or activated with the enzyme.

Experiments were carried out to determine the most efficient concentration of acetyltrypsin in the agar overlay medium. The results are shown in Fig. 2 and Table 2. Remarkable cell damage was observed at concentrations of acetyltrypsin over 10µg/ml in overlay medium. The plaques which formed in the presence of acetyltrypsin at concentrations of 5µg and 7.5µg/ml were very clear, but those formed at higher concentrations were not clear. Although the addition of 5µg acetyltrypsin/ml to the overlay medium yielded plaques that were smaller in size than those in 7.5µg/ml, it resulted in higher infectious titers.

<table>
<thead>
<tr>
<th>Concentration of acetyltrypsin (µg/ml)</th>
<th>PFU/ml</th>
<th>Size (mm)</th>
<th>Cell damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.4×10^5</td>
<td>1–3</td>
<td>-</td>
</tr>
<tr>
<td>7.5</td>
<td>4.3×10^4</td>
<td>1–3</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>3.7×10^4</td>
<td>1–3</td>
<td>+</td>
</tr>
<tr>
<td>12.5</td>
<td>3.1×10^5</td>
<td>1–1.5</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>6.2×10^4</td>
<td>1–3</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of acetyltrypsin in the overlay medium on plaque formation of the HRV strain KU
A. 5µg/ml, B. 7.5µg/ml, C. 10µg/ml, D. 12.5µg/ml, E. 15µg/ml
Effect of agar concentration in the overlay medium on plaque formation of the HRV strain KU

A. 0.6%, B. 0.8%, C. 1.0% D. 1.2% E. 1.5%

**TABLE 3**

*Effect of agar concentration in the overlay medium on plaque morphology and infectious titers*

<table>
<thead>
<tr>
<th>Conc. of agar Oxoid (%)</th>
<th>PFU/ml</th>
<th>Size (mm)</th>
<th>Cell damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>3.4×10^5</td>
<td>2~3</td>
<td>–</td>
</tr>
<tr>
<td>0.8</td>
<td>3.6×10^5</td>
<td>1~3</td>
<td>–</td>
</tr>
<tr>
<td>1.0</td>
<td>2.8×10^5</td>
<td>2~2.5</td>
<td>–</td>
</tr>
<tr>
<td>1.2</td>
<td>2.3×10^5</td>
<td>1~2.5</td>
<td>–</td>
</tr>
<tr>
<td>1.5</td>
<td>2.8×10^5</td>
<td>0.5~2</td>
<td>–</td>
</tr>
</tbody>
</table>

**Effect of the agar concentration on plaque formation**

Various concentrations of agar in the overlay medium were tested on plaque formation in the presence of 5μg acetyl-trypsin/ml. The morphology of the plaques formed and the infectious titers at various concentrations of agar are illustrated in Fig. 3 and Table 3. Although plaques formed at every concentration tested (0.6 to 1.5%), the higher the concentration of agar used, the smaller the plaques were. Clear plaques were produced when 0.6 to 1% agar was introduced into the overlay medium. 0.8% was adopted for plaque formation in the following experiments, as the plaques formed at this concentration were the easiest count.

**Virus concentration and number of plaques formed**

To determine if one plaque is produced by one infective particle, assays were carried out using a series of virus concentrations under the optimum conditions described above. As shown in Fig. 4, a linear relationship was obtained between virus concentration and number of plaques formed.

**Plaque formation of standard strains and isolates of HRV**

The investigation on the optimum conditions for plaque formation, described above, were performed with type 1 strain KU. An attempt was made to ascertain the applicability of these con-
Fig. 4. Linear relationships between virus concentration and number of plaques formed.

All the standard strains formed distinct plaques with sizes that were different for each type. Type 1 strain KU and type 4 strain Hochi formed larger plaques (2-3 mm) than the other types. Type 2 strain S2 formed the smallest plaques (less than 1 mm) of all the strains tested, and the plaques of type 3 strain YO had an intermediate size.

Plaque formation of clinical isolates were induced by using viruses which had been passaged eight times in MA104 cells. As illustrated in Fig. 6, the plaques were as distinct as standard viruses, and also had different sizes for different strains. Three isolates (no. 10, 26, and 44), which
were identified as type 1 by the Elisa test (Taniguchi, 1987), formed plaques of different sizes with a range that was comparable to the range between the types illustrated above (Fig. 6), demonstrating that plaque size does not correlate with virus type.

Discussion

It has been demonstrated that trypsin, a proteolytic enzyme, is essential to rotavirus for efficient growth in a cell culture (Alweida et al. 1978; Schoub et al. 1979; Sato et al. 1981). Taniguchi (1982) has observed that pretreatment of HRV with trypsin greatly enhanced the virus growth in contrast to the little growth that occurred without pretreatments. With low multiplicity of infection, efficient growth could not be achieved without trypsin in the maintenance medium, even if pretreated viruses were used for inoculation. It was noted, therefore, that the progeny viruses released from infected cells were activated by trypsin present in the maintenance medium and they became infective. So serial infections of cells could be achieved.

In plaque assays, the cells should be infected with viruses at an extremely low multiplicity. Therefore, pretreatment of the virus with trypsin before inoculation and the presence of the enzyme in the overlay medium is essential for the production of plaques. On the other hand, trypsin tends to damage the cell monolayers at the same time, and it may lead to an inhibition of virus infection and subsequently reduce the development of plaques. This probably makes the plaques more difficult to read.

Several factors affect the production of plaques. In the present experiments, some constituents in the overlay medium were examined. The most significant improvement in this study occurred with the introduction of FBS to the virus inoculum. Practically, the virus sample, which was previously treated with acetyltrypsin, was diluted in MEM supplemented with 2.5% FBS and inoculated onto MA104 cell monolayers. The major effect of FBS on plaque formation may be the stabilization of PBS-washed cell
monolayers during adsorption rather than the protection of monolayers from damage by acetyltrypsin, because the concentration of acetyltrypsin in the inoculum is considered to be too low to damage the cell monolayers. When the virus sample was pretreated with acetyltrypsin in the presence of FBS, the plaques decreased in number to approximately 1/5 (data not shown). It is, therefore, reasonable to state that the infectivity of viruses conferred by acetyltrypsin is not affected by FBS, although FBS is able to inhibit the activity of acetyltrypsin.

The linear relationship between the number of plaques and the virus concentration obtained by the plaque technique recommended, demonstrates the usefulness of this system for reliable estimation of HRV. It may also be useful for assays of neutralization antibody and in the cloning of HRV.

References


