Rotavirus Stability and Inactivation

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SUMMARY

The stability of the infectivity of Simian rotavirus, SA11, has been analysed and compared to the stability of reovirus type I. SA11 infectivity was stable to freezethawing, sonication, incubation at 25 °C overnight or at 37 °C for 1 h and to treatment with acid, ether, chloroform and Genetron. In contrast to reovirus, the infectivity of SA11 was more rapidly inactivated by heating at 50 °C. SA11 infectivity was inactivated above pH 10.0 and by heating at 50 °C in 2 M-MgCl₂, but was stabilized by heating in 2 M-MgSO₄; reovirus 1 infectivity was enhanced by heating in MgCl₂. Both SA11 and reovirus 1 were inactivated by freezing in MgCl₂. These results show that rotaviruses and reoviruses can be distinguished by their patterns of inactivation by physical and chemical agents.

INTRODUCTION

Rotaviruses are ubiquitous and have been isolated from a variety of mammalian species (Wyatt et al. 1978). Rotaviruses, classified in the Reoviridae family, are believed to be a major cause of non-bacterial gastroenteritis (Dupont et al. 1977). Rotaviruses have been distinguished from viruses of the Reovirus and Orbivirus genera both morphologically, antigenically and by analysis of their nucleic acid and polypeptide compositions (Palmer et al. 1977; Wyatt et al. 1978).

The major difficulty in characterizing the rotaviruses has been the inability to propagate them to high titres in tissue culture. We have used the simian rotavirus SA11, which will grow well in cell culture and can be plaque assayed (Smith et al. 1979) to compare directly and quantitatively with the stability and inactivation of type I reovirus (strain 716). This report shows that the rotaviruses and reoviruses may be distinguished by these biological criteria. It extends a preliminary qualitative characterization of SA11 (Malherbe & Strickland-Cholmley, 1967) and details differential temperature effects on SA11 infectivity in the presence of MgCl₂, MgSO₄ and other salts.

METHODS

Viruses and cell lines. Reovirus type 1 (strain 716) was produced in Rhesus kidney LLC-MK2 cells. Simian rotavirus SA11, kindly supplied by Dr H. H. Malherbe, was produced in foetal Rhesus kidney MA104 (Microbiological Associates, Bethesda, Maryland, U.S.A.) cells. Poliovirus type I (strain LSc) was grown in Buffalo green monkey cells (Wallis et al. 1972). All cells were grown in Eagle’s minimal essential medium supplemented with 10% foetal bovine serum (FBS), 5% tryptose phosphate broth, 2% BME vitamins, 0.03% glutamine, 0.25% glucose, 100 units of penicillin, 100 μg of streptomycin and...
50 μg gentamycin per ml as well as 0.075% sodium bicarbonate. Cells were maintained in this medium containing 2% FBS. It should be noted that mycoplasma testing of MA104 cells on PPLO agar and by the Hoescht stain (Chen, 1977) revealed negative results although thin section analyses of MA104 cells have shown mycoplasma-like structures.

**Virus assays.** Virus assays were performed in MA104 cells using the plaque technique under an agar overlay (Smith et al. 1979). This assay has been shown to be sensitive, quantitative and reproducible. Briefly, the overlay medium consisted of Eagle's minimal essential medium, 1.5% agar (Difco) 0.03% glutamine, neutral red (0.02 mg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml) and 0.3% NaHCO₃ supplemented further with 100 μg/ml DEAE-dextran (Pharmacia) and 1:60 pancreatin (Oxoid Labs, London). Stock solutions of pancreatin were prepared by dissolving 1 tablet in 50 ml of distilled water; filtered sterilized samples were frozen until used by diluting 1:60 in the agar overlay. Assay cultures were incubated at 37°C and plaques counted after 4 days of incubation.

**Immunofluorescence tests.** Monolayers of cell lines grown on 15 mm round cover slips were washed three times with tris (hydroxylmethyl) aminoethane buffer (TBS), pH 7.2, which contained 20 mM-tris, 140 mM-NaCl, 5 mM-KCl, 0.4 mM-Na₂HPO₄, 6 mM-dextrose, 0.5 mM-MgCl₂ and 0.7 mM-CaCl₂. Monolayers were then inoculated with 2 p.f.u./cell of SAll. At various intervals after infection, coverslip cultures were washed with TBS (4°C), fixed for 10 min at −20°C in ethanol, and stained by the indirect immunofluorescence (IF) tests as previously described (Estes & Butel, 1977). Stained cultures were mounted in Elvanol and observed with a Zeiss u.v. fluorescence microscope.

Rabbit antisera to the Nebraska calf diarrhoea virus (NCDV; kindly donated by Norden Laboratories, Lincoln, Nebraska) or hyperimmune guinea pig sera to SAI1 were employed as the primary antisera followed by fluorescein-labelled goat anti-rabbit IgG or fluorescein-labelled goat anti-guinea pig sera (Hyland Laboratories, Costa Mesa, California). The fluorescein labelled antibody was absorbed with mouse liver powder and with SAI1-infected cell extracts to remove non-specific fluorescence. The hyperimmune guinea pig sera to SAI1 was prepared by footpad inoculation of 25 μg of purified SAI1 followed by two intramuscular boosts on days 14 and 28. Guinea pigs were bled on days 35, 38 and 42 and a typical serum exhibited a neutralization titre of 1 : 25,000, a complement fixation titre of 1 : 512 and an immunofluorescent titre of 1 : 60. The specificity of reactivity of the antisera was established by (i) absorption with SAI1-infected cells which resulted in a loss of specific immunofluorescence, and (ii) agglutination of purified simian SAI1 particles by immune electron microscopy. The antiserum showed no reaction by immunofluorescence against uninfected cells, reovirus type 1-infected cells, or poliovirus types 1-, 2- or 3-infected cells.

**Inactivation and stability studies.** Inactivation studies on reovirus 1 and rotavirus were performed and assayed simultaneously on MA104 cells to allow direct comparison of the results.

**Salt effects.** Virus was diluted two- to tenfold in distilled water or the salts indicated; the salts were prepared so that the concentrations given are final. Samples were equilibrated at pH 6.9 to 7.1 with 0.1 N-HCl or -NaOH and samples placed at the temperatures indicated. After the specified incubation, virus was plunged into an ice-water bath for dilution in ice-cold TBS.

**Temperature inactivation.** Virus was diluted tenfold in TBS and sonicated at 10 kHz for 30 s at 4°C (Ratheon Sonic Oscillator, Waltham, Mass.). Immediately after sonication samples were incubated for heat inactivation studies as previously described (Estes & Butel, 1977). For freeze-thawing experiments, samples were quick frozen in a dry ice ethanol bath, stored at −20°C and thawed at 25°C.
### Table 1. Effect of physical and chemical treatments on SA11 infectivity*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Titre (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$3.5 \times 10^7$</td>
</tr>
<tr>
<td>Freeze-thawing (3 ×)</td>
<td>$3.4 \times 10^7$</td>
</tr>
<tr>
<td>Sonication</td>
<td>$3.0 \times 10^7$</td>
</tr>
<tr>
<td>25 °C, 24 h</td>
<td>$3.2 \times 10^7$</td>
</tr>
<tr>
<td>37 °C, 1 h</td>
<td>$3.1 \times 10^7$</td>
</tr>
<tr>
<td>Ether</td>
<td>$2.9 \times 10^7$</td>
</tr>
<tr>
<td>Genetron</td>
<td>$2.8 \times 10^7$</td>
</tr>
<tr>
<td>Chloroform</td>
<td>$2.0 \times 10^7$</td>
</tr>
<tr>
<td>Trypsin,† (100 µg/ml; 1:4 units/ml)</td>
<td>$9.0 \times 10^7$</td>
</tr>
<tr>
<td>Trypsin,* (10 µg/ml; 4.0 units/ml)</td>
<td>$12 \times 10^7$</td>
</tr>
<tr>
<td>5 mM-EDTA</td>
<td>$3.7 \times 10^6$</td>
</tr>
<tr>
<td>5 mM-EGTA</td>
<td>$5.3 \times 10^6$</td>
</tr>
</tbody>
</table>

* SA11 infectivity was assayed by plaque titration after treatment of virus as described in Methods.

† Tissue culture grade trypsin.

‡ Twice crystallized trypsin.

**pH stability.** Virus was diluted 1:100 into 0.05 M-glycine buffer adjusted to the indicated pH prior to virus addition. Samples were shaken, incubated at 22 °C for the specified times, neutralized and diluted in pH 7.0 glycine buffer for assay. All pH experiments were compared to a control at pH 7.0 and the pH of each buffer was confirmed at the end of each experiment.

**Extraction with organic solvents.** Virus was diluted two- to tenfold in ether, Genetron or chloroform. After incubation for 30 min at 25 °C, the aqueous phase containing the virus was separated from the Genetron or chloroform phase by centrifugation at 5000 g for 15 min. Ether was removed by gentle bubbling of air through a sterile pipette.

**Trypsin treatment.** Virus was diluted tenfold in TBS alone or in TBS containing the indicated concentrations of ICN tissue culture or Worthington (Freehold, New Jersey) 2 × crystallized trypsin and incubated for 30 min at 37 °C. The virus was then diluted in TBS containing trypsin or TBS alone and used for assay. Trypsin activity was determined as previously described (Graham, 1977). One unit equals 1 µM of substrate hydrolysed per min.

**Treatment with chelating agents.** Virus was diluted twofold in 0.02 M-tris (hydroxymethyl) aminomethane buffer (pH 7.2) containing saline (0.15 M-NaCl) alone or in this buffer containing 10 mM-EDTA or 10 mM-ethyleneglycobis (β-aminoethyl-ether)- N,N′ tetra-acetic acid (EGTA). Virus infectivity was assayed after incubation at 37 °C for 30 min.

**RESULTS**

**Stability of SA11 infectivity to various physical and chemical treatments**

As shown in Table 1, SA11 infectivity was stable to ether, Genetron and chloroform treatments. In addition, SA11 infectivity was stable to repeated freeze-thawing, to sonication and to incubation at 37 °C for 1 h or to ambient temperature (25 °C) for at least 24 h. SA11 infectivity was also enhanced by trypsin treatment. In contrast to the stability of SA11 infectivity with the previous treatments, SA11 infectivity was significantly reduced after incubation with the chelating agents EDTA or EGTA.

The comparative survival of infectivity of SA11 and reovirus-1 after heating at 50 °C...
are shown in Fig. 1. SA11 infectivity was more rapidly inactivated at 50 °C than was reovirus. Only 20% of the SA11 infectivity remained after 5 min of incubation and this infectivity decreased to less than 1% survival after 30 min at 50 °C. In contrast, approx. 50% of the reovirus-1 infectivity survived after 30 min.

It is noteworthy that the buffer used for these heat inactivation studies greatly influenced SA11 inactivation but did not markedly affect reovirus-1. Heating at 50 °C for 15 min in tris-buffered saline (Fig. 1) or in phosphate-buffered saline (data not shown) resulted in losses of greater than 95% of SA11 infectivity while heating in water resulted in losses of only 70 to 80% (see Table 2). Reovirus-1 titres were consistently lowered only 20 to 30% whether heated in TBS, PBS or water.

Incubation temperatures of 33°, 37° or 39 °C used during the plaque assay did not affect virus titres for either SA11 or reovirus-1. Analysis of both SA11 virus and reovirus infections by immunofluorescence revealed that although the infection proceeded more rapidly at 39 °C, the percentage of cells infected was not different at 34°, 37° or 39 °C. These results suggest that although SA11 virus replication is not naturally temperature sensitive, SA11 virions may contain a heat-labile capsid protein required for infectivity.

The effects of pH on SA11 and reovirus 1 infectivity

The effect of pH on infectivity of SA11, reovirus 1, and poliovirus 1 is shown in Fig. 2. SA11 and reovirus 1 were less stable than poliovirus 1 to acid treatment, and SA11 and reovirus were also more sensitive than poliovirus to alkaline pH conditions.
Rotavirus inactivation

Table 2. Effects of salts and temperature on SA11 and type 1 reovirus infectivity

<table>
<thead>
<tr>
<th>Final salt concentration</th>
<th>Titre (p.f.u./ml) after incubation at*</th>
<th>% Survival after incubation at†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 °C</td>
<td>50 °C</td>
</tr>
<tr>
<td>Virus</td>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>SA11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td>3.1 × 10^7</td>
<td>7.8 × 10^6</td>
</tr>
<tr>
<td>2 M-MgCl₂</td>
<td>3.0 × 10^7</td>
<td>1.0 × 10^4</td>
</tr>
<tr>
<td>2 M-MgSO₄</td>
<td>3.2 × 10^7</td>
<td>3.9 × 10^7</td>
</tr>
<tr>
<td>2 M-NaCl</td>
<td>5.4 × 10^7</td>
<td>4.7 × 10^7</td>
</tr>
<tr>
<td>2 M-CaCl₂</td>
<td>3.0 × 10^3</td>
<td>1.0 × 10^3</td>
</tr>
<tr>
<td>Reovirus 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td>4.2 × 10^7</td>
<td>2.6 × 10^7</td>
</tr>
<tr>
<td>2 M-MgCl₂</td>
<td>2.6 × 10^7</td>
<td>5.5 × 10^7</td>
</tr>
<tr>
<td>2 M-MgSO₄</td>
<td>2.0 × 10^7</td>
<td>1.0 × 10^7</td>
</tr>
</tbody>
</table>

* Virus was diluted tenfold in H2O or in the salts indicated. Samples were either held at 4 °C or heated at 50 °C for 15 min, then diluted and assayed.
† Calculated relative to the infectivity in H2O at 4 °C.

Fig. 2. Survival of infectivity of rotavirus, reovirus and poliovirus at various pHs. ■■■, SA11; ●●●, reovirus 1; ▲▲▲, poliovirus 1.
The effects of salts on SA11 and reovirus I infectivity

As shown in Table 2, SA11 infectivity was rapidly inactivated by heating for 15 min at 50 °C in 2 M-MgCl₂, CaCl₂ and NaCl. Less than 2 % of the infectivity remained after these treatments. In contrast, MgSO₄ stabilized SA11 such that the starting infectivity remained after heating for 15 min. The effects of these salts on reovirus I was also determined (Table 2). In contrast to SA11, reovirus I infectivity was slightly enhanced by heating in MgCl₂ but not by MgSO₄. Both SA11 and reovirus infectivity were inactivated by freezing in MgCl₂, but were not affected by freezing in MgSO₄.

DISCUSSION

Preliminary characterization of rotaviruses from several species, based on limited quantal analysis of the infectivity of SA11 (Malherbe & Strickland-Cholmley, 1967) or neonatal calf diarrhoea virus (Welch & Thompson, 1973) and on morphological observations by electron microscopy (Palmer et al. 1977) have suggested that these viruses are relatively stable entities. In this study, comparative quantification of the stability of SA11 and reovirus infectivity confirmed that while the infectivity of both viruses is stable, they can be distinguished by their patterns of inactivation and stabilization by heating in water or in salts. It is of interest that the differential stability of SA11 in MgCl₂ and MgSO₄ more closely resembles the pattern observed for myxoviruses and SV40 (Wallis et al. 1965) than the typical reovirus pattern. The chemical basis for the greater thermolability of SA11 infectivity remains undetermined, but these studies support the classification of the rotaviruses as a separate genus within the Reoviridae family (Melnick, 1977). Another finding of interest is that SA11 but not reovirus I infectivity is significantly reduced by treatment with either 5 mM-EDTA or 5 mM-EGTA. This observation is consistent with the reports of others (Cohen, 1977; Elias, 1977; Hruska et al. 1978) suggesting that double-shelled rotavirus particles are infectious and EDTA treatment converts these rotavirus particles to non-infectious single-shelled particles. The effect with EGTA suggests that calcium may be a critical cation required to maintain virus integrity.

The observation that trypsin treatment enhances SA11 infectivity (Table 1) is consistent with the recent reports that proteolytic enzymes (trypsin or pancreatin) may be required for the growth of rotaviruses (Babiuk et al. 1977; Theil et al. 1977; Almeida et al. 1978); and with our own studies showing that these enzymes are required for the development of a reproducible virus plaque assay for SA11 (Smith et al. 1979). The results described here could suggest that trypsin enhancement of infectivity may be the consequence of direct proteolytic action on the virus particles in agreement with the findings of Theil et al. (1977) with the porcine rotavirus. However, because pancreatin is also included in our assay system, further experiments are in progress to determine the basis of enzyme enhancement of infectivity. It might involve direct alteration of the virus particles or virus proteins, or it might affect virus penetration into cells, cell to cell transfer of virus between infected cells or inactivate virus interfering substances present in the virus preparations.

Knowledge of the stability of the rotaviruses is a pre-requisite to the development of methods for the detection and concentration of these enteric viruses from sewage-contaminated water supplies. These data are also of potential importance in the development of a vaccine against rotavirus gastroenteritis.

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REFERENCES


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