Gangliosides as binding sites in SA-11 rotavirus infection of LLC-MK2 cells

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The chemical nature of receptors involved in the attachment of simian rotavirus (SA-11) to a monkey kidney cell line (LLC-MK2) was investigated. Enzymic treatment of cells before virus infection indicated that membrane proteins and phospholipids are not involved in virus attachment, whereas sialic acid and galactose participate in the receptor structure to differing extents. Incubation of SA-11 with bovine brain gangliosides before infection strongly reduced its ability to bind to cell membranes. Similar experiments with individual purified gangliosides from bovine brain showed that virus infection was prevented by preincubation with GM1. Moreover, desialylated cells regained susceptibility to virus infection when coated with whole gangliosides or GM1 immediately after Clostridium perfringens neuraminidase treatment. The binding of SA-11 to whole gangliosides or GM1 was quantified by an ELISA procedure. The results suggest that gangliosides, mainly GM1, are part of the receptor structure for SA-11 of susceptible LLC-MK2 cells.

Introduction

Rotaviruses are non-enveloped dsRNA viruses belonging to the Reoviridae family, and have been identified as the most important viral pathogen causing gastroenteritis in infants and young animals world-wide (Woode, 1982; Kapikian & Chanock, 1990). In the human or animal host, rotavirus attaches to and infects mature enterocytes of the intestinal villi, but the identity of the intestinal receptors is still not known. It has been hypothesized to be lactase of the enterocyte brush border (Holmes et al., 1976), molecules on the M cells (as for reoviruses), or Fc receptors in the intestine (Bartlett et al., 1987). Little is known about the chemical structure of cellular components involved in rotavirus binding to the cell membrane. It has been reported that pretreatment of fresh erythrocytes from various species with receptor-destroying enzyme prevents their agglutination by simian rotavirus SA-11, suggesting that the virus interacts with neuraminic acid-containing receptors (Bastardo & Holmes, 1980; Fukudome et al., 1989). Recently, it has also been shown that SA-11 binding to susceptible cells is inhibited in vitro and in vivo by sialic acid glycoproteins (Yolken et al., 1987; Keljo & Smith, 1988; Willoughby & Yolken, 1990). The participation of cellular sialic acid in binding of virus to the plasma membrane has been confirmed by the inhibitory activity of lectin from Limulus polyphemus (Yolken et al., 1987).

In this paper we report results relating to the role of protein, lipid and carbohydrate membrane components in cellular binding sites for simian rotavirus SA-11. For this purpose susceptible LLC-MK2 cells were treated with different proteases, lipases and glycosidases, and the resulting changes in virus replication were analysed. To characterize the cellular sialic acid–virus interaction further, the cells were treated with different neuraminidases capable of releasing neuraminic acid from membrane glycoproteins and glycolipids, and characterized by a specific action on defined ketosidic linkages. Moreover, experiments were carried out to determine whether sialoglycolipids (gangliosides) can act as host cell receptors for rotaviruses.

Methods

Cells. LLC-MK2 cells, a monkey kidney cell line, were grown at 37 °C in 1:1 mixture of MEM (Flow Laboratories) and 199 medium (Flow Laboratories) containing 1.2 g/l NaHCO3, and supplemented with 10% inactivated foetal calf serum (Flow Laboratories), 2 mM-glutamine, penicillin [100 international units (IU)/ml] and streptomycin (100 μg/ml).

Virus. Simian rotavirus SA-11 was grown in LLC-MK2 cells. Virus was pre-activated with 20 μg/ml trypsin (type IX; Sigma) for 30 min at 37 °C, diluted 10-fold in 199 medium and then inoculated onto confluent cell monolayers grown in roller bottles at an m.o.i. of 10 p.f.u./cell. After 90 min at 37 °C, the inoculum was removed, and the monolayers were washed once with PBS pH 7.4 and then incubated at 37 °C in 199 medium containing 1 μg/ml trypsin. When extensive c.p.e.

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was observed, infected cultures were frozen and thawed three times, centrifuged (3000 g, 10 min), and supernatants were stored at −70 °C.

**Enzymes.** Stock solutions of enzymes were made up in PBS: trypsin from bovine pancreas (10¹ U/ml), pronase E from *Streptomyces griseus* (12 U/ml), subtilisin from *Bacillus amyloliquefaciens* (12:2 U/ml), proteinase K from *Trichactium album* (32-6 U/ml), phospholipase A₂ from bee venom (10³ U/ml), phospholipase C from *Clostridium perfringens* (10¹ U/ml) and phospholipase D from cabbage (10² U/ml) (all from Sigma), β-galactosidase from *Escherichia coli* (16 U/ml) and α-mannosidase from *Canavalia ensiformis* (13 U/ml) (both from Boehringer Mannheim), α-fucosidase from bovine epididymis (1-5 U/ml) and neuraminidase from *C. perfringens* (20 U/ml) (both from Sigma), and neuraminidase from *Arthrobacter ureafaciens* (1 U/ml) (Calbiochem).

Stock solutions of neuraminidase from *Vibrio cholerae* (Berhinger-werke) and neuraminidase from influenza virus (Calbiochem) were made up in PBS with 2 mm-CaCl₂ at concentrations of 1 U/ml and 100 µU/ml, respectively.

**Sialic acid determination.** Sialic acid released was determined in neuraminidase-treated cell supernatant by the method of Aminoff (1959) using N-acetylneuraminic acid (NANA) as a standard.

**Ganglioside preparation.** Mixtures of gangliosides from bovine brain material (MBPG) were extracted and purified as described by Svennerholm & Fredman (1980). This mixture contained predominantly GM₁, GD₁a, GD₁b and GT₁b, accompanied by GD₃, GT₁a and traces of QG₁b. The individual gangliosides used in this work (GM₁, GD₁a, GD₁b and GT₁b) were isolated from bovine brain and purified according to Ghidoni *et al.* (1980). The gangliosides were suspended in PBS and vortexed for 2 min before use.

**Enzyme cytotoxicity.** To establish the maximal non-cytotoxic dose of an enzyme, LLC-MK₂ cell monolayers were treated with different concentrations of proteases or phospholipases for 20 min at 26 °C, or glucosidases for 30 min at 37 °C. After treatment, cells were washed three times with 199 medium and incubated with the same medium for 24 h at 37 °C in 5% CO₂. After this time, cell morphology, number and viability (neutral red uptake in dispersed cells) were determined. Enzyme dilutions that did not affect any of these parameters were considered to be non-cytotoxic and were utilized for enzymic treatments as described below.

**Effect of enzymic treatments on virus replication.** LLC-MK₂ cells grown in tissue culture chamber slides (Lab-Tek; Miles Laboratories) for 24 h at 37 °C in 5% CO₂ were treated with each enzyme at different non-cytotoxic concentrations. After incubation, monolayers were washed three times with 199 medium to remove traces of enzyme and incubated with pre-activated SA-11 virus at an m.o.i. of 5, 0-5 or 0-3 p.f.u./cell, which infect approximately 100%, 50% and 25% of a control cell culture. The infection was monitored 5 h later by indirect immunofluorescence.

Briefly, cell monolayers were washed with PBS pH 7-4, dried and incubated for 10 min at −20 °C in acetone. The cells were then covered with hyperimmune rabbit anti-rotavirus serum, produced by using the immunization schedule previously described by Bastardo *et al.* (1981), and incubated at 37 °C for 45 min. After incubation, cells were washed with PBS and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit gammaglobulin antibodies (Kallestad). After 45 min at 37 °C, cells were washed with PBS, mounted in glycerol and viewed through a Leitz Dialux fluorescence microscope. The results were expressed as the percentage of infected cells in treated as opposed to untreated cultures.

**Effect of enzymic treatments on virus binding.** LLC-MK₂ monolayers were mechanically detached and cells were washed three times with PBS. Enzymic treatments were performed as above on 2 x 10⁶ cells suspended in the same buffer. Then, cells were washed three times with PBS and infected with SA-11 at an m.o.i. of 50 for 90 min at 4 °C. After infection, cells were washed three times in PBS at 4 °C and virus binding was examined by flow cytometric analysis after fluorescent staining.

Briefly, all steps were carried out at 4 °C by utilizing pre-cooled reagents. Infected cells were incubated with a rabbit anti-rotavirus serum for 60 min and after washing five times with PBS, cells were stained with FITC-conjugated anti-rabbit gammaglobulin antibodies (Kallestad). After 60 min, cells were washed again, fixed in PBS containing 1% paraformaldehyde, and flow cytometric analysis was performed using a Becton Dickinson Fac Scan.

By this analysis either the fluorescence intensity of cells (shown in the figures) or the percentage of fluorescent cells (reported in the text) was obtained.

**Effect of gangliosides on virus replication.** Mixtures of virus and different MBPG concentrations were prepared. The mixtures were added to the cells immediately or after a 2 h incubation at 25 °C. Control cultures were infected without gangliosides. Viral antigen synthesis was examined 5 h later by indirect immunofluorescence (see above). The same experiments were carried out with individual purified gangliosides.

**Effect of gangliosides on SA-11 attachment to LLC-MK₂ cells.** A mixture of virus (10⁵ p.f.u./ml) and MBPG (40 µg/ml) was incubated for 1 h at 25 °C. LLC-MK₂ cells, suspended in PBS at a concentration of 2 x 10⁶ cells/ml, were infected with the mixture or with the same virus preparation without MBPG for 90 min at 4 °C. After infection, cells were washed three times in PBS at 4 °C and virus binding was detected by flow cytometric analysis (see above). The same experiments were carried out with GM₁.

**Ganglioside coating of enzyme-treated LLC-MK₂ cells.** After treatment with neuraminidase from *C. perfringens*, the LLC-MK₂ cell monolayers were washed three times with 199 medium and incubated with different concentrations of MBPG for 20 min at 37 °C. Unadsorbed gangliosides were removed by washing the cells three times with 199 medium. Cells were then inoculated with virus to assess their susceptibility to infection and virus replication was detected 5 h later by indirect immunofluorescence (see above). The same experiments were carried out with GM₁.

**ELISA.** The binding of SA-11 virus to plastic-adsorbed gangliosides was examined by an ELISA method. MBPG or GM₁ were attached to the inner bottom surface of the wells of a Linbro microtitration plate (Flow Laboratories). For the assay, 100 µl of each ganglioside dilution in carbonate buffer (15 mM-Na₂CO₃, 35 mM-NaHCO₃ pH 9-6) were applied to the wells in quadruplicate. After incubation at 4 °C overnight, the plate was rinsed three times with PBS containing 10 mg/ml bovine serum albumin (PBS-BSA). Unoccupied binding sites were blocked by incubation for 30 min at 37 °C with PBS-BSA. The plate was rinsed with the same buffer and then incubated for 3 h at room temperature with 10⁷ p.f.u. of SA-11 suspended in PBS-BSA (100 µl/well). After washing three times with PBS-BSA, virus binding was evaluated by means of an anti-rotavirus rabbit serum (1 h at 37 °C) and a peroxidase-linked swine anti-rabbit antibody (1 h at 37 °C). The substrate reaction with o-phenylenediamine (20 min at room temperature) (Sigma) was read in a Titertek Multiskan (Flow Laboratories) at 492 nm. Thorough washing with PBS between assays was included.
Results

Effect of enzymes on the interaction of SA-11 with LLC-MK2 cells

The effect of enzymic treatment on viral antigen synthesis was monitored by indirect immunofluorescence 5 h after infection (Fig. 1a, b).

(i) Proteases

Trypsin, pronase E, subtilisin and proteinase K were used at various concentrations to inhibit SA-11 infection. No decrease in the percentage of infected cells was observed at the concentrations used (pronase E, $6 \times 10^{-3}$ to $6 \times 10^{-2}$ U/ml; subtilisin, $6 \times 10^{-3}$ to $1.2 \times 10^{-2}$ U/ml; proteinase K $6 \times 10^{-2}$ to $6 \times 10^{-1}$ U/ml). Susceptibility to SA-11 infection was enhanced by treatment with 250 and 2500 U/ml trypsin, resulting in a two- and threefold increase in the number of fluorescent cells, respectively.

(ii) Lipases

When LLC-MK2 cells were incubated with different concentrations of phospholipases, no significant reduction in the percentage of infected cells was observed; 30% fluorescence inhibition was obtained with 10 U/ml phospholipases A$_2$ and C, and 20% inhibition was observed after treatment with 5 U/ml phospholipase C.

(iii) Glycosidases

Various concentrations of glycosidases were used for the pretreatment of LLC-MK2 cells before the virus binding step. The results in Table 1 show that $\alpha$-fucosidase and $\alpha$-mannosidase treatment was not significantly effective, whereas $\beta$-galactosidase and neuraminidase treatment affected SA-11 attachment to susceptible cells to differing extents. As for the action of neuraminidases, treatment with $V$. cholerae and influenza virus neuraminidases did not cause significant fluorescence inhibition, whereas a strong reduction in the percentage of infected cells was noticed following treatment with $C$. perfringens and $A$. ureafaciens neuraminidases. The effects observed with neuraminidases from various sources, releasing comparable amounts of neuraminic acid, might be due to their different substrate specificity. In fact, $V$. cholerae

Fig. 1. Indirect immunofluorescence of SA-11 rotavirus-infected LLC-MK2 cells. Intracytoplasmic fluorescence 5 h post-infection at 37 °C (0-5 p.f.u./cell): (a) mock-infected cells; (b) infected cells. Membrane fluorescence 90 min post-infection at 4 °C (50 p.f.u./cell): (c) mock-infected cells; (d) infected cells. Bar marker represents 10 μm.
and influenza virus neuraminidases are ineffective against some gangliosides because of the steric hindrance produced by their terminal carbohydrate structures (Rauvala, 1979; Suzuki et al., 1980). When the enzymes were added after infection in control experiments, both treated and untreated cultures exhibited a similar percentage of infected cells.

**Effect of enzymes on virus binding**

Results obtained with enzyme suggested that virus infection was affected only by trypsin, neuraminidase and β-galactosidase treatment. A possible effect of these enzymes on the attachment of SA-11 to LLC-MK2 cells was investigated by indirect immunofluorescence 90 min after infection at 4 °C (Fig. 1c, d). For quantitative evaluation of virus binding, flow cytometric analysis was carried out (Fig. 2).

Fig. 2(a) shows the difference in fluorescence intensity observed between mock-infected and infected cells, and the results obtained after enzymic treatment are shown in Fig. 2(b, c and d). The maximum difference between the summation curves of the histograms is indicated as D; D/s (n) represents the degree of similarity between the two summation curves (the closer this value is to zero, the more similar are the curves). These parameters were significant only for β-galactosidase- and neuraminidase-treated cells (Fig. 2b, c). Moreover, D and D/s (n) values obtained for neuraminidase-treated cells (Fig. 2c) were very close to those for mock-infected cells (Fig. 2a). The analysis also indicated that β-galactosidase and neuraminidase treatments caused a 58% and 97% reduction in the number of fluorescent cells respectively (data not shown), suggesting that both galactose and sialic acid are involved in virus binding.

The small reduction (11%; data not shown) in the percentage of fluorescent cells after trypsin treatment

Table 1. **Effect of glycosidase treatment on the attachment of SA-11 to LLC-MK2 cells**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Concentration (U/ml)</th>
<th>Fluorescence* (%)</th>
<th>NANA released (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>2</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>(C. perfringens)</td>
<td>10</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>2</td>
<td>70</td>
<td>12</td>
</tr>
<tr>
<td>(V. cholerae)</td>
<td>0.05</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>0.25</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>(A. aerogenes)</td>
<td>1</td>
<td>80</td>
<td>2</td>
</tr>
<tr>
<td>(influenza virus)</td>
<td>10</td>
<td>70</td>
<td>10</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>0.8</td>
<td>60</td>
<td>–</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>8</td>
<td>40</td>
<td>–</td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td>0.65</td>
<td>80</td>
<td>–</td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td>6.5</td>
<td>70</td>
<td>–</td>
</tr>
<tr>
<td>α-Fucosidase</td>
<td>0.02</td>
<td>80</td>
<td>–</td>
</tr>
<tr>
<td>α-Fucosidase</td>
<td>0.2</td>
<td>80</td>
<td>–</td>
</tr>
</tbody>
</table>

* Results were obtained utilizing an m.o.i. of 5 p.f.u. SA-11/cell.
suggested that proteins sensitive to this enzyme do not participate in virus binding.

As in previous experiments, trypsin treatment resulted in an increase in rotavirus antigen synthesis and additional control experiments were carried out to verify whether the increase in fluorescence was due to residual trypsin activity causing a further activation of the rotavirus inoculum. The inoculum was either activated for a longer period of time (2 h) or with a greater concentration of enzyme (40 μg/ml); under these conditions we were unable to detect any variation in the percentage of fluorescent cells. This finding may indicate a trypsin-mediated permeabilization of cell membranes leading to a higher rate of virus internalization.

**Competitive binding between gangliosides and LLC-MK2 cells for SA-11**

The results reported suggested that sialic acid and galactose are involved in the cellular receptor structure for SA-11. To obtain additional information about the role of these carbohydrates in virus infection, various concentrations of MBPG and major components of MBPG (GM1, GD1a, GD1b and GT1b) were mixed with SA-11 suspensions and applied to LLC-MK2 cells directly or after 2 h preincubation at 25 °C. Cell cultures were examined 5 h after infection to determine the percentage of infected cells. Viral antigen synthesis was not affected significantly (90% fluorescent cells at the highest concentrations) when the different ganglioside preparations were applied directly to the cells together with the virus (data not shown). A dose-dependent inhibition of cellular infection was observed after preincubation of SA-11 with MBPG or isolated gangliosides (Fig. 3). Viral antigen synthesis was strongly affected by the action of MBPG and GM1. However, inhibition was never complete and a few infected cells were always observed. In these experiments, controls were included which consisted of LLC-MK2 cells incubated with the different ganglioside preparations for 1 h before and after infection. All these controls gave results similar to those obtained with untreated cells.

**Effect of gangliosides on virus attachment to LLC-MK2 cells**

The effect of MBPG and GM1 on virus binding was investigated by flow cytometric analysis (Fig. 4). At a concentration of 40 μg/ml, both MBPG and GM1 were able to reduce virus binding (Fig. 4b, c), and the reduction in the number of fluorescent cells was 50% and 76%, respectively (data not shown).

![Figure 3](image-url)  
**Fig. 3.** Effect of gangliosides on SA-11 attachment to LLC-MK2 cells. The competitive binding of MBPG or isolated gangliosides and LLC-MK2 cells for SA-11 was tested by infection of cells with virus-ganglioside mixtures preincubated for 2 h at 25 °C. Gangliosides were used at concentrations of 0 μg/ml (□), 8 μg/ml (■), 40 μg/ml (□) or 200 μg/ml (□).  

![Figure 4](image-url)  
**Fig. 4.** Effect of gangliosides (40 μg/ml) on SA-11 binding to LLC-MK2 cells determined for 2500 cells by flow cytometric analysis. Parts (i). (a) Fluorescence intensity of virus-infected (solid line) and mock-infected (dotted line) cells. (b) Fluorescence intensity of virus-infected (solid line) and MBPG-virus mixture-infected (dotted line) cells. (c) Fluorescence intensity of virus-infected (solid line) and GM1-virus mixture-infected (dotted line) cells. Parts (ii) show summation curves.
Table 2. Effect of gangliosides on neuraminidase-treated LLC-MK2 cells*

<table>
<thead>
<tr>
<th>Neuraminidase (U/ml)</th>
<th>Ganglioside (µg/ml)</th>
<th>Fluorescent cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus control</td>
<td>-</td>
<td>- 100</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>1</td>
<td>- 10</td>
</tr>
<tr>
<td>Restoration of susceptibility to infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBPG treatment</td>
<td>1 8</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>1 40</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>1 200</td>
<td>65</td>
</tr>
<tr>
<td>GM1 treatment</td>
<td>1 8</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>1 40</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>1 200</td>
<td>75</td>
</tr>
</tbody>
</table>

* LLC-MK2 cells were treated with 1 U/ml of neuraminidase (type V; Sigma) from C. perfringens for 30 min at 37 °C, coated with various concentrations of MBPG or GM1 for 20 min at 37 °C and infected with SA-11 at an m.o.i. of 5.

Restoration of susceptibility to infection by gangliosides and GM1

Restoration of receptors for specific myxovirus binding has been demonstrated by incorporation of sialic acid into asialylated erythrocytes (Paulson et al., 1979). Exogenous gangliosides are known to insert in the plasma membrane of cells (Holmgren et al., 1975; Markwell et al., 1981; Superti et al., 1986a, b). Our working hypothesis was based on the recovery of susceptibility to rotavirus infection by the insertion of gangliosides into the plasma membranes of neuraminidase-pretreated cells. In this series of experiments, the cells were treated with neuraminidase from C. perfringens for 30 min at 37 °C and, after removal of enzyme, were treated further with MBPG or GM1. Desialylated LLC-MK2 cells were resistant to infection by SA-11, as shown by immunofluorescence (Table 2). MBPG added exogenously partially restored the susceptibility of the cells to infection in a dose-dependent relationship, this being more evident with GM1 (Table 2). In other experiments in which ganglioside-coated LLC-MK2 cells were treated with neuraminidase before being inoculated with SA-11, their susceptibility to infection was reduced to neuraminidase control values (data not shown).

SA-11 binding to plastic-adsorbed gangliosides

The binding of SA-11 to MBPG or GM1 was investigated by an ELISA procedure. In this test, plastic-adsorbed gangliosides were incubated with either virus or PBS-BSA. Control experiments in which SA-11 was incubated in the absence of gangliosides were also carried out. Binding of SA-11 to 8 µg/ml MBPG (A_{492} 0.31 ± 0.04) or GM1 (A_{402} 0.34 ± 0.02) was clearly stronger than to BSA-treated plastic surfaces (A_{492} 0.05 ± 0.02).

Discussion

Our results suggest that protease-sensitive membrane proteins and phospholipids are not involved in the attachment of SA-11 to LLC-MK2 cells because the proteases and lipases tested fail to inhibit virus infection. β-Galactosidase and neuraminidase treatments are both able to prevent virus replication. As the inhibition has been shown to occur at the adsorption step, it is conceivable that gangliosides, mainly GM1, participate in the receptor structure for SA-11.

It is well known that the action of neuraminidases from different sources can vary on the basis of their specificity. Both neuraminidases from V. cholerae and influenza virus hydrolyse α,2-6' and α,2-3' ketosidic linkages (Gottschalk & Drzeniek, 1972); neuraminidase from C. perfringens hydrolyses GM1 and GM2 gangliosides when these glycolipids are monomeric, whereas this action is sterically hindered when gangliosides are aggregated (Rauvala, 1979); neuraminidase from A. ureafaciens hydrolyses GM1 and GM2 gangliosides when these glycolipids are monomeric, whereas this action is sterically hindered when gangliosides are aggregated (Rauvala, 1979); neuraminidase from A. ureafaciens hydrolyses α,2-3', α,2-6' and α,2-8' linkages of NANA residues with a highly specific action on ganglioside GM1 (Sugano et al., 1978).

Treatment of cells with neuraminidases from V. cholerae or influenza virus causes only a slight inhibition of SA-11 attachment to LLC-MK2 cells, whereas a low percentage of infected cells is observed after treatment with C. perfringens or A. ureafaciens neuraminidases. It must be emphasized that these latter enzymes have a specific action on GM1 ganglioside (detected in LLC-MK2 cells by thin-layer chromatography; data not shown) which is believed to be resistant to various neuraminidases of viral, bacterial and mammalian origin (Sugano et al., 1978; Suzuki et al., 1980). The role of GM1 in SA-11 binding to LLC-MK2 cells was investigated (i) by incubating MBPG or different, purified gangliosides isolated from MBPG with the virus and measuring their effect on virus infection, (ii) by measuring the effect of MBPG and GM1 on virus binding, (iii) by coating C. perfringens neuraminidase-treated cells with MBPG or GM1 and determining whether they recover susceptibility to SA-11 infection and (iv) by evaluating the specific binding of SA-11 to MBPG and GM1 attached to a plastic surface. The data obtained in the first and second series of experiments demonstrate the ability of MBPG and GM1 to bind the virus, and prevent virus infection and attachment, respectively. Among the major components of MBPG, only GM1
induces a marked reduction in virus replication. The two cell populations of different fluorescence intensity detected by flow cytometric analysis of MBPG (Fig. 4b) might be accounted for by the presence of less active components (GD1a, GD1b or GT1b) in the ganglioside mixture.

In the third set of experiments, the coating of desialylated cells with MBPG or GM1 results in a marked restoration of the susceptibility of the cell to virus infection.

It is generally accepted that exogenous gangliosides can induce a variety of cellular modifications, such as alterations in differentiation (Dimpfel et al., 1980; Leon et al., 1982; Roison et al., 1981), membrane dynamics, by modifications of Na÷-, K+-ATPase activity (Leon et al., 1981), adenylate cyclase activity (Partington & Daly, 1979), cell surface sialylation-desialylation processes (Hakomori et al., 1980) and nerve sprouting (Gorio et al., 1980). Thus, in our experiments gangliosides might have acted through an indirect effect on the cell membrane. To verify this hypothesis, sialylated LLC-MK2 cells were incubated with gangliosides before SA-11 infection. No modification of infectivity is observed under these conditions.

Finally, binding of SA-11 to MBPG and GM1 has been confirmed by means of polystyrene-adsorbed gangliosides. The amphipathic ganglioside molecules attach spontaneously with strong hydrophobic bonds to plastic surfaces via their ceramide moiety, which leaves the oligosaccharide portion free to react with virus or other ligands. This procedure reduces the risk of any non-specific influence of the ceramide on the binding reaction and has the great practical advantage that bound ligand can then be conveniently detected by ELISA or other methods (Holmgren et al., 1980). In the experiments with MBPG or GM1, full inhibition of virus attachment to the cell surface or 100% recovery of susceptibility to SA-11 infection after coating of desialylated LLC-MK2 cells was never observed. This is not surprising if components other than GM1 are also involved in SA-11 attachment to LLC-MK2 cells. Sialoglycoproteins may play an active role in the binding process. Indeed, sialoglycoproteins, such as fetuin and bovine submaxillary mucin, have been shown to be potent inhibitors of rotavirus binding (Yolken et al., 1987; Keljo & Smith, 1988). Thus, different glycoconjugates might be an integral part of the receptor for SA-11.

We presume that this ‘receptor’ is a multicomponent complex structure, although it is curious that mucin should inhibit the binding of a pathogen that invades a mucosa ordinarily covered by mucin, as previously discussed (Keljo & Smith, 1988). Notwithstanding, V. cholerae toxin has been shown to bind certain glycoproteins, such as fetuin or thyroglobulin, even though it is well known that GM1 is its receptor in the small intestine (Cuatrecasas, 1973).

We have demonstrated, by various independent techniques, that SA-11 interacts with GM1. Our results are in conflict with recent data (Willoughby et al., 1990) indicating a possible role for asialo-GM1 as the ligand for rotaviruses. At present we have no obvious explanation for this discrepancy. However, it should be remembered that GM3, GM1 and GD1a are the major gangliosides in the small intestine of 2-week-old mice; they increase in amount during the suckling period and decrease after weaning, when they are replaced by neutral glycosphingolipids (Sato et al., 1982). The appearance of asialo-GM1 and the disappearance of GM1 correlate with temporal development of relative resistance to rotavirus infection (Kraft, 1957). The role of GM1 in the attachment of rotavirus to susceptible cells should be investigated further, as the characterization of rotavirus receptors may be useful in the prevention of infection.

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