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# Ganglioside $GM_{1a}$ on the Cell Surface Is Involved in the Infection by Human Rotavirus KUN and MO Strains[1]

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**Rotavirus is the most common cause of severe gastroenteritis in infants and children worldwide. The cell attachment of most animal rotaviruses, which belong to the neuraminidase-sensitive strains, requires sialic acid residues on the host cell membranes. On the other hand, most human rotaviruses are classified as neuraminidase-insensitive strains. The involvement of gangliosides on the host cell surface in human rotavirus infection was investigated by immunostaining analysis of target cells, and by assaying the neutralization of infection by rotavirus and the blocking of target cellular receptors. In host cells (MA104 cells) pretreated with *Arthrobacter ureafaciens* neuraminidase, which were still infected by human rotaviruses (KUN and MO strains), $GM_3$ was hydrolyzed markedly by the neuraminidase, while $GM_{1a}$ was not hydrolyzed at all. Infection by the rotaviruses was strongly inhibited by exogenous ganglioside $GM_{1a}$, but not $GA_1$. Infection was also inhibited by pretreatment of the MA104 cells with cholera toxin B-subunit, which specifically blocked ganglioside $GM_{1a}$ on the plasma membrane. The treatment of MA104 cells with the endoglycoceramidase attenuated human rotavirus infection. From these findings, we concluded that $GM_{1a}$ on the plasma membrane of the host cells was involved in the infection by human rotavirus KUN and MO strains.**

**Key words: $GM_{1a}$, human rotavirus, MA104 cell, receptor, sialic acid.**

Rotaviruses are the major cause of severe gastroenteritis in infants and children in both developed and developing countries. Based on their ability to infect host cells treated with neuraminidase, rotaviruses are classified into two types: neuraminidase-sensitive strains and neuraminidase-insensitive strains (*1, 2*).

In the neuraminidase-sensitive strains, the cell attachment of most rotavirus strains isolated from animals requires sialic acid residues on the host cell surface (*2-8*). The infection by simian rotavirus (SA-11 strain) was prevented by preincubation of target cells with lectins specific for sialic acid, or with endoglycoceramidase, and neuraminidase (*9, 10*). Rolsma *et al.* reported that the infection by porcine rotavirus (OSU strain) was inhibited by exogenous $GM_3$ and $GM_2$, which were isolated from pooled intestines of newborn to 4-week-old piglets (*7, 11*). These studies suggested that the carbohydrate epitope for the infection of many animal rotaviruses is a sialic acid residue. Sialic acid derivatives inhibited both hemagglutination and viral binding of cells susceptible to animal rotaviruses, but not to human rotaviruses (*8, 12*).

In the neuraminidase-insensitive strains, neither the infection nor the hemagglutination of most human rotaviruses was inhibited by the pretreatment of target cells with neuraminidase from *Arthrobacter ureafaciens* (*2, 4*). The feature of carbohydrate residues through which human rotaviruses enter the host cells is still unclear. Evidence suggests that the cellular receptors for human rotaviruses contain no sialic acid, or some very special types of sialic acids that are resistant to neuraminidase treatment (*2, 4, 5*).

In the present study, we focused on identifying the molecules involved in by neuraminidase-insensitive strains of human rotavirus infection. We determined that $GM_{1a}$ on the host cell surface was involved in human rotavirus infection (KUN and MO strains) by the immunostaining analysis of target cells, and by assaying the neutralization of infection by rotavirus and the blocking of target cellular receptors.

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## MATERIALS AND METHODS

*Glycosphingolipids*—$GM_3$(Neu5Ac) was isolated from human liver (*13*). $GM_{1a}$ was isolated from bovine brain (*14*). LacCer was isolated from bovine erythrocytes (*15*). $GA_1$ was prepared from bovine brain $GM_{1a}$ (*16*). $GM_2$ was isolated from Tay-Sachs brain, and $GA_2$ was prepared from $GM_2$ by hydrolysis with neuraminidase (*17, 18*).

*Virus and Anti-Serum*—Both human (KUN and MO strains) and feline (FRV64 strain) rotaviruses were propagated in embryonic rhesus monkey kidney cells (MA104 cells) as described previously (*19, 20*). Guinea pig anti-FRV64 antiserum was prepared as follows: four-week-old pathogen-free guinea pigs were immunized with purified feline rotavirus FRV64 strain by intra-muscular injection. One month later, the guinea pigs were boosted with a similar intra-peritoneal injection. The sera of immunized-guinea pigs (anti-FRV64 antisera) were harvested and purified after one week. The antisera cross-reacted with human rotaviruses KUN and MO strains.

*Infection Assay of Rotaviruses*—MA104 cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 95% air, 5% $CO_2$ incubator. For the neutralization assay, the viruses were titrated to determine a dilution giving around 200 staining focus-forming units per well of the 96-well microtiter tray. Exogenous ganglioside $GM_{1a}$ (starting concentration, 50 $\mu$M) was serially diluted with PBS (20 mM phosphate buffer saline, pH 7.0). It was then mixed with a equal volume of trypsin-activated viruses (preincubated with 10 $\mu$g/ml of trypsin for 30 min at 37°C) and kept for 1 h at 37°C. This mixture was added to MA104 cells. After adsorption for 1 h, the inoculum was removed and replaced with the maintenance medium and left for 16 h at 37°C. Neutralization activity of exogenous $GM_{1a}$ was determined by an indirect immunostaining procedure. Rotavirus-infected cells were fixed with 80% acetone for 10 min. After washing with PBS, the fixed cells were incubated with PBS containing anti-FRV64 antiserum at 37°C for 30 min. They were then incubated with PBS containing horseradish peroxidase (HRP)-conjugated protein A (Organon Teknika N.V. Cappel Products) at 37°C for 30 min. Finally, the cells were stained with a solution containing 50 mM Tris buffer (pH 7.5), 0.5 mg/ml of 3,3′-diaminobenzidine tetrahydrochloride, and 0.01% $H_2O_2$ aqueous for 20 min at 4°C. After washing three times with PBS, the stained cells were counted under light microscopy (*8, 21*).

*Enzyme Treatment of MA104 Cells*—A confluent monolayer of MA104 cells was treated with various enzymes under different conditions. For the endoglycoceramidase (EGCaseII ACT, EC 3.2.1.123, Takara Shuzo, Kyoto), the cells were incubated with EMEM containing 5% FBS and 50 mU/ml of EGCase for 12 h at 37°C as described previously (*27, 28*). For the neuraminidase, the cells were incubated with PBS containing 100 mU/ml of neuraminidase from *A. ureafaciens* (Boehringer Mannheim GmbH, Germany; Marukin Shoyu, Kyoto) for 1 h at 37°C as described previously (*4*). The treated cells were used for the infection assay of rotaviruses, or the immunochemical analyses of glycolipid composition.

*Extraction and Immunochemical Analyses of Total Lipids from MA104 Cells*—EGCase-treated, neuraminidase-treated, and untreated MA104 cells were harvested by centrifugation at 800 rpm for 5 min and washed twice with PBS. Total lipids of the cells were twice extracted in a solvent system of chloroform/methanol (1:1, by volume) and subjected to sonication for 10 min. After centrifugation at 15,000 rpm for 5 min, the supernatant was combined and evaporated in a nitrogen stream, then dissolved in a mixture of chloroform/methanol (1:1, by volume) for analysis. Thin-layer chromatography (TLC)/immunostaining of total lipids from MA104 cells was performed by the procedure described previously (*22*). Briefly, after developing with a solvent system of chloroform/methanol/water containing 12 mM $MgCl_2$ (5:4:1, by volume), the plastic plate was dried and soaked for 1 h at room temperature in PBS supplemented with 1% polyvinylpyrrolidone (PVP) and 1% egg albumin to block non-specific antibody binding. The plate was then incubated for 1 h at room temperature in PBS supplemented with 3% PVP containing HRP-conjugated cholera toxin B subunit (CTB) (*22*), rabbit antibody, or mouse monoclonal antibodies against $GA_1$, $GM_2$, or $GM_3$, respectively. In experiments using antibodies, the plates were further incubated in HRP-conjugated protein A or HRP-conjugated goat anti-mouse IgG+IgM (H+L) (Jackson Immunoresearch Laboratories) for 1 h at room temperature. After washing several times with PBS, the plate was stained with a solution containing 100 mM citrate buffer (pH 6.0), 60 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride in acetonitrile, 100 mM 4-chloro-1-naphthol in acetonitrile, and 3% $H_2O_2$ aqueous (5:1:1:0.005, by volume) for 15 min at room temperature.

*Blocking Assay*—A confluent monolayer of MA104 cells was incubated for 1 h at 37°C with PBS containing CTB (starting concentration, 20 $\mu$g/ml) at the indicated concentration. After washing with PBS, the infection ability of rotavirus was determined by the infection assay. As a control, CTB was boiled for 10 min at 100°C.

## RESULTS

*$GM_{1a}$ Ganglioside in MA104 Cells Was Not Hydrolyzed by Neuraminidase*—To identify structural features related human rotavirus infection, we used two human rotaviruses (KUN and MO strains) and one feline rotavirus (FRV64 strain). First, the sensitivity of these strains to neuraminidase treatment of the host MA104 cells was examined. After pretreatment of MA104 cells with 100 mU/ml neuraminidase from *A. ureafaciens* at 37°C for 1 h, the infection of the FRV64 strain was strongly inhibited by 88.8±0.24% of untreated control, but that of human rotaviruses was not (Fig. 1A). This finding showed that KUN and MO strains are neuraminidase-insensitive, and FRV64 strain is neuraminidase-sensitive. This is consistent with previous studies showing that the infection of most animal rotaviruses is blocked by pretreatment with neuraminidase from *A. ureafaciens*, but human rotaviruses can still infect the cells (*4, 10*).

As shown in Fig. 1B, MA104 cells contained mainly $GM_{1a}$ and $GM_3$ as gangliosides, and lactosylceramide (LacCer) as a neutral glycosphingolipid. $GM_{1a}$ and $GM_3$ accounted for 31 and 27% of the acid glycosphingolipid fraction, respectively. $GA_1$ was not detected in MA104 cells by immunostaining. We next examined the alteration of glycosphingolipid composition of MA104 cells by treatment with

neuraminidase. After treatment with 100 mU/ml neuraminidase from *A. ureafaciens* at 37°C for 1 h, the cells were harvested, and total lipids were extracted and subjected to chemical and immunochemical analyses. Figure 1, B and C, reveals that $GM_3$ decreased markedly after hydrolysis by neuraminidase, while LacCer increased correspondingly. On the contrary, $GM_{1a}$ increased slightly after neuraminidase treatment. The longer gangliosides may be hydrolyzed to form $GM_{1a}$. This result indicated that terminal sialic acid residues of gangliosides were hydrolyzed by neuraminidase from *A. ureafaciens*: for example, $GM_3$ to LacCer. However, internal sialic acid residues of some gangliosides were difficult to hydrolyze: for example, $GM_{1a}$ was not converted to $GA_1$ at all. At neutral pH and in the absence of detergent, neuraminidase from *A. ureafaciens* could hardly remove internal sialic acid residues (*23, 29*). In Fig. 1C, TLC-immunostaining with cholera toxin B subunit (CTB) confirmed that the content of $GM_{1a}$ in MA104 cells was not reduced by neuraminidase treatment. Together, these findings indicate that $GM_{1a}$ is strongly involved in the infection by the two human rotaviruses.

*Exogenous Ganglioside $GM_{1a}$ Strongly Inhibited the Infection of MA104 Cells by Human Rotaviruses*—To clarify that ganglioside $GM_{1a}$ on the host cell surface serves as a receptor for human rotaviruses, we first examined whether exogenous ganglioside $GM_{1a}$ acts as a competitive inhibitor for human rotavirus infection. We used KUN and MO strains (human rotaviruses) and FRV64 strain (feline rotavirus). As shown in Fig. 2, the concentration of $GM_{1a}$ at 50% inhibiting dose of infected cells ($IC_{50}$) for KUN and MO strains was $12.42 \pm 2.80$ and $14.22 \pm 1.12$ $\mu$M, respectively. Exogenous $GA_1$ did not affect the infection ability of human rotaviruses. These findings showed that exogenous $GM_{1a}$ potently inhibited the infection of both KUN and MO strains in a dose-dependent manner, but not that of FRV64 strain.

*Treatment of MA104 Cells with Cholera Toxin B Subunit Strongly Inhibited Human Rotavirus Infection*—To confirm that endogenous $GM_{1a}$ on the host cell membrane acts as a functional receptor for human rotaviruses KUN and MO strains, we used CTB to block endogenous $GM_{1a}$ (*22, 24, 25*). Following pretreatment of MA104 cells with 20 $\mu$g/ml of CTB, the infection rates of human KUN and MO strains only were $19.2 \pm 4.3$ and $31.9 \pm 6.4$% of those of untreated MA104 cells, respectively, but that of feline rotavirus was not significantly reduced (Fig. 3). These findings showed that human rotavirus infection was prevented by masking endogenous $GM_{1a}$ on the host cell membrane.

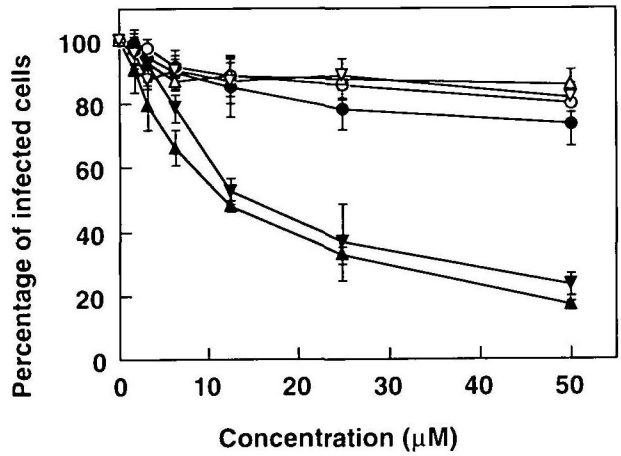


Fig. 2. **Inhibitory activity of exogenous ganglioside $GM_{1a}$ on the infection of MA104 cells by rotaviruses.** The inhibitory activity of $GM_{1a}$ (closed) and $GA_1$ (open), which are used as controls for the infections by KUN (triangle), MO (inverted triangle), and FRV64 (circle) strains of MA104 cells, was determined using a neutralization assay as described in "MATERIALS AND METHODS." The data are expressed as mean ± SD of four independent experiments. Each experiment was carried out in duplicate.

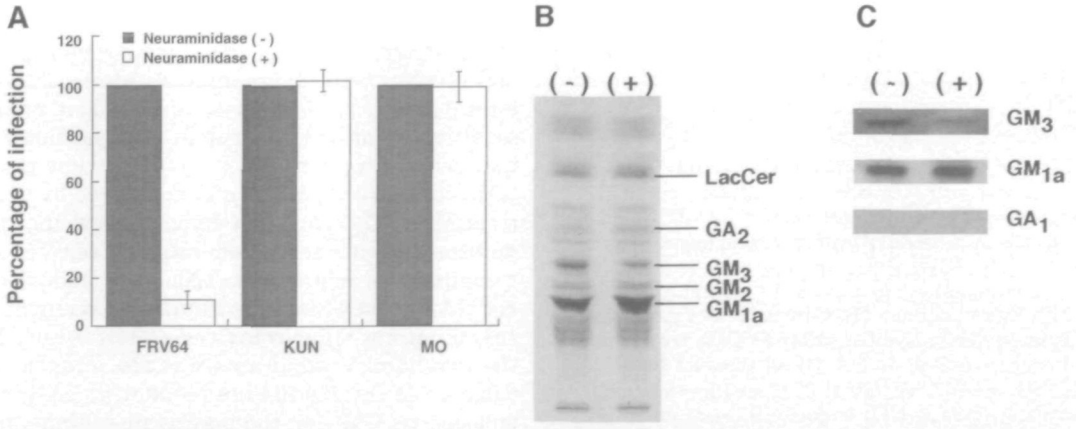


Fig. 1. **(A) Effect of neuraminidase on rotavirus infection of MA104 cells.** After incubation with 100 mU/ml neuraminidase from *Arthrobacter ureafaciens* for 1 h at 37°C, MA104 cells were inoculated with the human (KUN and MO strains) and feline (FRV64 strain) rotaviruses. Infected cells were detected by immunostaining as described in "MATERIALS AND METHODS." The ratio of infected cells of each rotavirus (open column) is expressed as a percentage of that of untreated cells (black column). **(B) Conversion of gangliosides to desialylated derivatives after neuraminidase treatment of MA104 cells.** Glycosphingolipids in MA104 cells treated with (+) or without (−) neuraminidase (100 mU/ml neuraminidase from *A. ureafaciens*) at 37°C for 1 h, were visualized by orcinol-$H_2SO_4$ reagent staining as described in "MATERIALS AND METHODS." **(C) Conversion of gangliosides to desialylated derivatives as visualized by immunochemical staining.** $GM_3$ and $GA_1$ were probed with the corresponding antibodies. $GM_{1a}$ was probed with cholera-toxin B subunit conjugated with HRP.

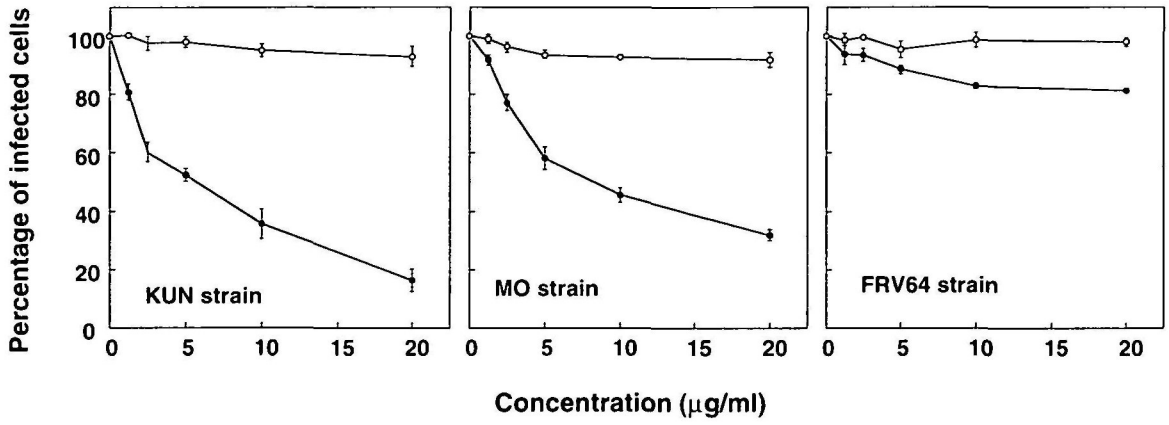


Fig. 3. **Effect of treatmet of MA104 cells with CTB on infection by human rotaviruses.** A confluent monolayer of MA104 cells was incubated for 1 h at 37°C with PBS containing CTB (closed circle) (starting concentration, 20 μg/ml). The infection ability of rotavirus was determined by the infection assay. As a control (open circle), CTB was boiled for 10 min at 100°C. The data are expressed as mean ± SD of four independent experiments. Each experiment was carried out in duplicate.

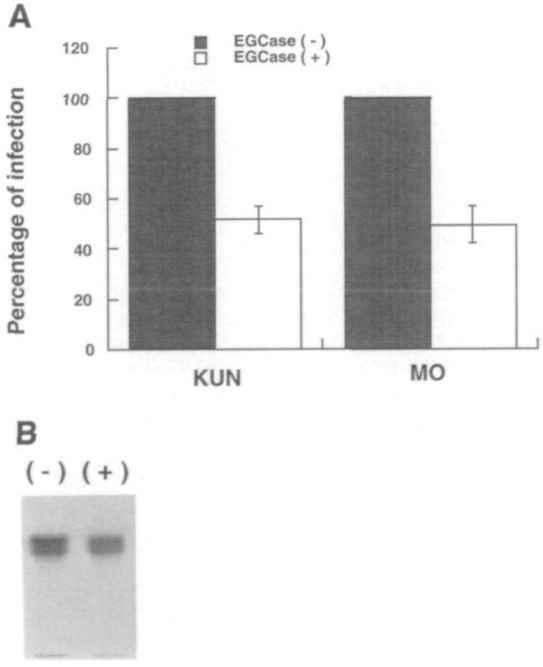


Fig. 4. **(A) Effect of EGCase on rotavirus infection to MA104 cells.** After incubation with 50 mU/ml EGCase for 12 h at 37°C, MA104 cells were inoculated with human rotaviruses (KUN and MO strains). Infected cells were detected by immunostaining as described in "MATERIALS AND METHODS." The ratio of infected cells of each rotavirus (open column) was expressed as a percentage of that of the untreated cells (black column). **(B) Conversion of ganglioside $GM_{1a}$ to desialylated derivative $GA_1$ after EGCase treatment of MA104 cells.** Endogenous $GM_{1a}$ in MA104 cells treated with (+) or without (−) the EGCase (50 mU/ml) at 37°C for 1 h, was immunochemically visualized by using CTB to detect $GM_{1a}$ as described in "MATERIALS AND METHODS."

*Endoglycoceramidase Attenuated the Infection Ability of Human Rotaviruses*—We also tested whether the removal of glycosphingolipids from the plasma membrane of MA104 cells with endoglycoceramidase (EGCase) influences rotavirus infection. After treatment of MA104 cells with 50 mU/ml EGCase at 37°C for 12 h, the infection rates of human rotaviruses (KUN and MO strains) were reduced by 52.4±5.4 and 49.6±3.7%, respectively (Fig. 4A). We examined the composition of glycosphingolipids in MA104 cells after EGCase treatment. The EGCase-treated and untreated cells were harvested, and total lipids were extracted twice with a solvent system of chloroform/methanol (1:1, by volume). The extracted lipids were subjected to immunochemical analysis with CTB to detect $GM_{1a}$. In Fig. 4B, we found that half of the endogenous $GM_{1a}$ on the surface of MA104 cells was hydrolyzed by the treatment (45.7%), since the optimum pH for EGCase was quite acidic (pH 5.0-5.5). At neutral pH, the hydrolytic activity of EGCase was reported to be only 60-70% of that at pH 5.0 (*26*). The findings from this experiment indicated that the infection of human rotaviruses is associated with EGCase-sensitive glycosphingolipids on the host cell surface, including $GM_{1a}$.

## DISCUSSION

In this study, we showed that human rotaviruses (KUN and MO strains) were neuraminidase-insensitive strains, and feline rotavirus (FRV64 strain) was a neuraminidase-sensitive strain in the previous classification (*1, 2*). TLC-immunostaining with CTB revealed that the content of $GM_{1a}$ in MA104 cells was also unaffected by neuraminidase treatment. Therefore, we hypothesized that $GM_{1a}$ was a putative receptor for human rotaviruses. We found that, as a competitive inhibitor, exogenous ganglioside $GM_{1a}$, but not $GA_1$, strongly inhibited the infection by human rotaviruses, but not feline rotavirus FRV64 strain. Infection by the two human rotaviruses was also prevented by preincubation of the MA104 host cells with CTB, which blocks endogenous $GM_{1a}$ on the plasma membrane, but infection by the feline rotavirus was not affected. Since CTB specifically binds $GM_{1a}$ (*22, 24, 25*), it inhibited the attachment of human rotaviruses to the host cell membrane. Conversely, gangliosides other than $GM_{1a}$ may be involved in feline rotavirus infection. Previous studies demonstrated that some gangliosides inhibited infection by neuraminidase-sensitive strains. Infection by porcine rotavirus (OSU strain) was inhibited by the addition of $GM_3$ and $GM_2$,

which were isolated from pooled intestines of newborn to 4-week-old piglets (*7, 11*).

We here examined the involvement of endogenous gangliosides in infection by two human rotaviruses. We consider that these neuraminidase-insensitive strains utilize internal sialic acid residues, since these are hardly hydrolyzed by neuraminidase treatment. On the other hand, neuraminidase-sensitive strains may utilize the terminal sialic acid residues of glycosphingolipids in the infection of host cells.

We noted that the infection by rotavirus was not completely prevented by treatment of the host cells with EGCase, since the optimum pH for EGCase was quite acidic (pH 5.0-5.5). At neutral pH, the hydrolytic activity of EGCase was reported to be only 60-70% of that at pH 5.0 (*26*). The inhibitory rate of human rotavirus infection was 45-50%. In our experiment, EGCase hydrolyzed only 60% of glycosphingolipids in the MA104 host cells. We conclude that the hydrolytic ability of EGCase is correlated with the infection rate of rotaviruses.

Our findings also confirmed the previous hypothesis that neuraminidase-insensitive strains use gangliosides that are resistant to neuraminidase treatment (*2, 4, 5*): for example, gangliosides containing an internal sialic acid residue, such as $GM_{1a}$. Therefore, sialic acid residues on the cell surface play an important role in rotavirus infection for both the neuraminidase-sensitive and -insensitive strains. Further investigation is required concerning the use of different molecular species of sialic acid (*N*-acetylneuraminic acid and *N*-glycolylneuraminic acid), different sialic acid-galactose linkages (alpha 2-3 and alpha 2-6 linkage), and positions of the linked sialic acid (terminal and internal sialic acid).

We also examined the binding activity of rotaviruses to highly purified immobilized $GM_{1a}$ by the TLC or ELISA/virus-binding assays, but found no significant binding activity of rotaviruses (KUN, MO, and FRV64 strains) to either $GM_{1a}$ or other gangliosides. One possible explanation is that endogenous $GM_{1a}$ may be a part of the cell receptors for rotaviruses, and consist of coreceptors together with other components, such as proteins or lipids. We detected only faint signals of the neutral glycosphingolipids containing a beta-Gal (GalNAc) terminal residue on the TLC plates. These signals may be false positives. Previous studies reported that some strains of rotaviruses (group A porcine rotavirus NVCD strain or simian rotavirus SA-11 strain) recognized neutral glycosphingolipids ($GA_1$, $GA_2$) but not gangliosides (*2, 9, 10, 30*). To further investigate reactive glycosphingolipids immobilized on TLC plates or ELISA is required. Endogenous $GM_{1a}$ may be involved in other processes of human rotavirus infection, such as a fusion step.

Finally, we concluded that endogenous $GM_{1a}$ on host cellular membranes is involved in human rotavirus infection (KUN and MO strains) of target cells.

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