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Inhibition of influenza-virus-induced cytopathy by sialylglycoconjugates

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Abstract—The anti-viral activity of gangliosides such as SPG (sialylparagloboside), GD1a, GM3, and GM4 was assessed by inhibition of the cytopathy of MDCK cells due to infection with the influenza virus A/PR/8/34. The inhibitory effect was in the following sequence: SPG > GD1a > GM3 > GM4. The IC_{50} of SPG and GD1a was 7 and 70 μM, respectively, indicating that they are more effective than the representative inhibitor amantadine. Although 3′-sialyllactose (3′-SL) and 3′-sialyllactosamine (3′-SLN), which are identical to the terminal trisaccharides of GM3 and SPG, respectively, did not show any inhibitory effect, introduction of an amino group to the reducing end of 3′-SL following amidation with lauroyl chloride gave the inhibitory potency, which was comparable to that of GM3. These results suggest that the viral hemagglutinin recognizes exogenous sialyloligosaccharides rather than inherent sialyloligosaccharides expressed on MDCK cells, since introduction of the hydrophobic moiety to oligosaccharides might cause micelle formation.

Keywords: Ganglioside; Influenza virus; Infection process; Competitive inhibitor; Receptor sugar chain; Antiviral agents

1. Introduction

Extensive efforts have been undertaken to identify and develop anti-influenza virus drugs.[1] Influenza A viruses express two representative glycoproteins, hemagglutinin (HA) and neuraminidase (NA), on the envelope. Viral HA molecules play a pivotal role in binding to sialic acid residues expressed on target endothelial cells. NA molecules act as an enzyme to release newly formed viruses from the HA receptors by cleaving off sialic acid.[1–4] Compounds capable of interacting with either HA or NA molecules should inhibit binding of the influenza virus, and the release of a daughter virus from infected cells.[5–10]

Abbreviations: MDCK cell, Madin–Darby canine kidney cell; PBS, phosphate buffered saline; CPE, cytopathic effect; IC_{50}, 50% inhibitory concentration; $TCID_{50}$, 50% tissue culture infection dose; HA, hemagglutinin; NA, neuraminidase; Neu5Ac, *N*-acetylneuraminic acid; GM4, I^3Neu5Acα2–4GalCer; GM3, II^3Neu5Acα-LacCer; GM2, II^3Neu5Acα-Gg^3Cer; GM1, II^3Neu5Acα-Gg^4Cer; GD3, II^3Neu5Acα^2-LacCer; GD1a, IV^3Neu5Acα, II^3Neu5Acα-Gg^4Cer; GD1b, II^3Neu5Acα^2-Gg^4Cer; SPG (sialylparagloboside), IV^3Neu5Acα-nLc^4Cer; Sul (sulfatide), I^3SO^3-GalCer; AMD, 1-aminoadamantane hydrochloride; 3′-SLN (3′-sialyllactosanine), Neu5Ac(α2–3)Gal(β1–4)GlcNAc; 3′-SL (3′-sialyllactose), Neu5Ac(α2–3)Gal(β1–4)Glc

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Gangliosides, belonging to a family of glycosphingolipids, contain one or more sialic acid residues and are important constituents of cell membranes. Specific recognition of gangliosides by external ligands causes signaling to the cytoplasm.[11] Influenza viruses can recognize sialylsugar chains of cellular gangliosides by HA molecules: this identification response is the characteristic for viral entry to cells.[12] It is clear that if binding of viral HA to sialylsugar chains of gangliosides is inhibited by exogenous materials, the following viral replication and release of a daughter virus are downregulated. However, it is unclear yet whether gangliosides and their sialyloligosaccharides competitively inhibit viral replication following cytopathy of infected cells. Here, we report that treatment of MDCK cells with exogenous gangliosides and a newly synthesized GM3 analogue (*N*-3′-sialyllactosyl-laurinamide), induced significant inhibition of the cytopathy induced by infection with the influenza virus A/PR/8/34.

2. Materials and methods

2.1. Influenza A virus

The influenza virus A/PR/8/34 (H1N1) was reinfected more than five times in MDCK cells with trypsin (2 μg/mL). The virus stock had a titer of 10^{-6} $TCID_{50}$/mL and was stored at −80 °C until required.

2.2. Virus titration assay in vitro

MDCK cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 100 μg/mL of streptomycin, 100 U/mL of penicillin G, and 10% heat-inactivated fetal bovine serum. After infection with A/PR/8/34 at a multiplicity of infection (MOI) of 0.01, the medium was changed to a maintenance medium containing 1 μg/mL folic acid, 1 μg/mL biotin, 0.2% bovine serum albumin, and 2 μg/mL trypsin. To determine infectivity levels, infected cells were incubated in the growth medium for 2 days. Aliquots of 10-fold serial diluents of culture supernatants were distributed into fresh MDCK cell cultures. After incubation at 35 °C for 4 days, CPE was assessed by a rounding up of infected cells and their subsequent detachment from the tissue culture dish. Based on the observation of CPE, the $TCID_{50}$ was calculated using the methodology reported by Behrens-Kärber.[13]

2.3. Glycolipids and oligosaccharides

Gangliosides (GM4, GM3, GM2, GM1, GD1a, and GD1b) and sulfatide (Sul) were isolated from the whole brain of a minke whale; SPG was prepared from human erythrocytes as reported by Momoi et al.[14] Sialic acid derivatives, 3′-sialyllactosamine (3′-SLN) and 3′-sialyllactose (3′-SL), were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.4. Preparation of *N*-3′-sialyllactosyl-laurinamide (*N*-3′-SL-NH-C12)

N-3′-SL-NH-C12 was prepared as reported by Kobayashi et al.[15] In brief, 3′-SL (50 mg, 79 μmol) was dissolved in 1.5 mL of a supersaturated soln of ammonium hydrogen carbonate, and the mixture was stirred at 37 °C for 4 days. Ammonium hydrogen carbonate was added at intervals so as to ensure saturation. The reaction progress was monitored by TLC (4:3:3:2 EtOAc–AcOH–MeOH–water) and visualized by the resorcinol reagent.[16] After removal of ammonium hydrogen carbonate by repeated evaporation, aliquots of 0.8 mL of sodium carbonate soln (40 mg/mL) and 0.8 mL of MeOH were added to a crude soln (0.2 mL) of sialyl-lactosylamine (3′-SL-NH_2). The soln was stirred magnetically at 0 °C for 2 h and lauroyl chloride (Aldrich) in 0.2 mL of tetrahydrofuran was added at fivefold the molar quantity to the initial 3′-SL. The progress of the reaction was monitored by TLC (6:4:1 $CHCl_3$–MeOH–0.2% $CaCl_2$ soln). A new component,

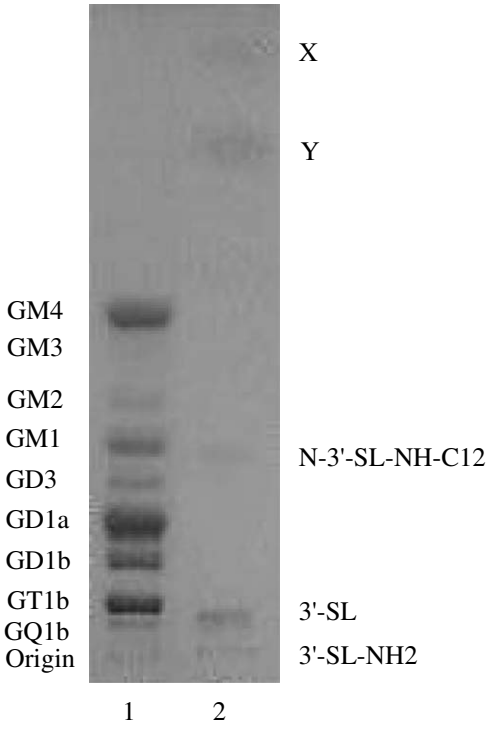

Figure 1. TLC of the synthesized GM3 analogue *N*-3′-SL-NH-C12. Lane 1, ganglioside mixture. Lane 2, a reaction mixture during the acylation of 3′-SL-NH2 with lauroyl chloride. Three resorcinol positive spots were 3′-SL-NH2, 3′-SL, and *N*-3′-SL-NH-C12. Spots X and Y were unreacted lauroyl chloride and the byproduct (laurinamide). TLC was developed with 6:4:1 $CHCl_3$–MeOH–0.2% $CaCl_2$ and visualized by the resorcinol reagent.[16]

migrating just below GM1 on a TLC plate, was observed in the reaction mixture (Fig. 1). After 5 h, the mixture was washed three times with an equal vol of $CHCl_3$ in order to remove unreacted lauroyl chloride. The final product was purified by a combination of a Sephadex LH-20 column (1.0 × 25 cm) using MeOH as eluent, and an Iatrobead (6RS-8060, Iatron, Tokyo) column (1.0 × 10 cm) by successive elution with 4:1, 7:3, 3:2, 2:1, 1:1, and 1:2 $CHCl_3$:MeOH. The purity of the resultant product was more than 98% by TLC and HPLC, and the overall yield was 35% (Fig. 2A). The pseudo-molecular ion ($[M+H]^+$) of the product obtained by LC/MS/MS was found at *m/z* 815.2, which is a value consistent with the theoretical molecular weight of *N*-3′-SL-NH-C12 (Fig. 2B).

The comparative ^{1}H NMR data of the synthesized GM3 analogue along with 3′-SL and GM3 are shown in Table 1. Signals of 3′-SL observed at δ 5.22, 4.66, and 4.53 ppm were anomeric protons assignable to αH-1 and βH-1 of Glc and βH-1 of Gal. In the case of the GM3 analogue (*N*-3′-SL-NH-C12), a signal at 4.71 ppm was βH-1 of Glc but αH-1 was not observed as in GM3. Furthermore, signals of alkyl methyl (1.24 ppm), methylene (0.85 ppm), and amide proton (8.51 ppm), which appeared after the modifications of 3′-SL indicate an *N*-linked β-anomeric bond between glycosyl and laurylamide residues. From the above results we conclude, therefore, that the synthesized GM3 analogue is *N*-3′-sialyllactosyl-laurinamide (*N*-3′-SL-NH-C12).

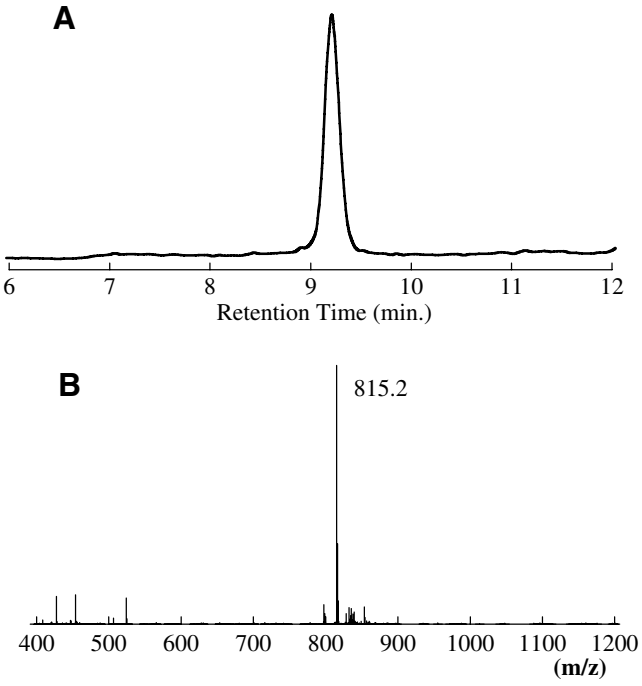

Figure 2. LC/MS/MS of the synthesized GM3 analogue *N*-3′-SL-NH-C12. Panel A, chromatogram. Panel B, mass spectrum of the peak in panel A. The pseudo-molecular ion ($[M+H]^+$) at *m/z* 815.2 was consistent with the theoretical molecular weight of *N*-3′-SL-NH-C12. The analytical conditions are given under Materials and methods.

Table 1. ^{1}H NMR chemical shifts (ppm) of 3′-SL, 3′-SL-NH-C12, and GM3

	Gal		Glc	CONH
	αH-1	βH-1	βH-1	
3′-SL	5.22	4.66	4.53	—
N-3′-SL-NH-C12	—	4.73	4.20	8.51
GM3	—	4.20	4.16	7.50

2.5. Assay for anti-influenza A virus activity

MDCK cells were infected with 10-fold serial dilutions from a stock virus in 96-well flat-bottomed microplates for 1 h. In order to assess the anti-viral activity of each ganglioside in the range of 1–200 μM, the ganglioside was treated at different time periods: (1) MDCK cells were incubated in the growth medium containing each ganglioside for 1 h before infection; (2) the cells were infected with the virus in the maintenance medium containing the ganglioside for 1 h; (3) after infection, the cells were treated with the ganglioside in the growth medium; (4) the cells were infected with the virus in a solution that contained the ganglioside, and subsequently incubated in the growth medium containing the ganglioside; (5) the ganglioside was added at different time periods (1–8 h) after infection. CPE was estimated by microscopic observation during incubation for 4 days.

2.6. Determination of Neu5Ac released from the ganglioside by influenza virus NA in the culture medium

MDCK cells were incubated with 100 μM of ganglioside after infection. At different time periods, a 5-μL aliquot of the culture medium was collected from each well and mixed with an equal vol of MeOH in order to quench the activity of influenza NA. Aliquots (5 μL) of mixtures were applied on an HPTCL plate (Silica Gel 60, E. Merck) and developed with a 6:4:1 $CHCl_3$–MeOH–0.2% $CaCl_2$ soln. After visualization with the resorcinol reagent,[16] free Neu5Ac and ganglioside were determined with a dual wavelength TLC scanner.

2.7. Electron microscopy

For electron microscopic analysis, infected cells were incubated for 48 h in a 4-well removable polystyrene chamber (Lab-Tek Chamber Slide, Nalge Nunc International, Rochester, NY), washed twice with PBS, and fixed with 2% glutaraldehyde. After post-fixation with 2% osmium tetroxide, the monolayers were dehydrated in EtOH, then propylene oxide, and embedded in an epoxy resin mixture. Epoxy resin filled with resupinate

gelatin capsules was used for embedding purposes, thin sections of which, were stained with uranyl acetate and lead citrate, and examined on a JEOL 100-S electron microscope (JEOL, Tokyo).

2.8. NMR measurement

Sialyloligosaccharides were dissolved in D_2O; gangliosides and *N*-3′-SL-NH-C12 were dissolved in 1:4 D_2O–Me_2SO-d_6. Each soln was lyophilized to remove exchangeable protons: the procedure was then repeated a second time. Then, the sialyloligosaccharide was dissolved in D_2O containing acetone as an internal standard. Gangliosides and *N*-3′-SL-NH-C12 were dissolved in 1:49 D_2O–Me_2SO-d_6 containing Me_4Si as an internal standard. The one- and two-dimensional (COSY) proton NMR spectra were obtained on an EX-400 NMR spectrometer (JEOL).

2.9. LC/MS/MS

LC/MS/MS analysis was performed using a Shiseido binary pump system, coupled online to a quadruple ion trap LCQ mass spectrometer Thermoquest LCQ-DECA (Thermo Finnigan, San Jose, CA, USA) through its electrospray interface. The HPLC condition was as follows: 1.0×10 mm C8 column (Inertsil WP300 C8 5 μm, GL Science Inc. Tokyo), 0.05% formic acid with 0–54% MeCN gradient over 4 min at a flow rate of 0.2 mL/min. The mass range recorded in survey acquisitions was m/z 340–2000.

3. Results

The anti-influenza activity of gangliosides was estimated by inhibition of the cytopathy of MDCK cells induced by infection with the influenza virus A/PR/8/34. Among gangliosides, SPG, GD1a, GM3, and GM4 demonstrated significant inhibitory activities while other gangliosides, GM2, GM1, GD3, GD1b, and Sul did not (Fig. 3). Inhibition levels, expressed as $\log_{10}(S_{TCID_{50}}/C_{TCID_{50}})$, were dependent on the timing of treatment of cells with gangliosides. Treatment with gangliosides before infection was not found to be effective (Fig. 3a). A significant inhibitory effect of gangliosides was observed in both cases: the initial phase during infection (Fig. 3b), and the expansion phase after infection (Fig. 3c). During and after infection, treatment with gangliosides was found to be more effective in the expression of inhibitory effects (Fig. 3d). With regard to the dose effects of SPG, a 100% survival rate was obtained at a concentration of 66 μM (Fig. 4). No significant difference was observed between the virus-infected cells treated with 66 μM SPG, and the virus-free control cells during the incubation for 4 days. No evidence of cytotoxicity of

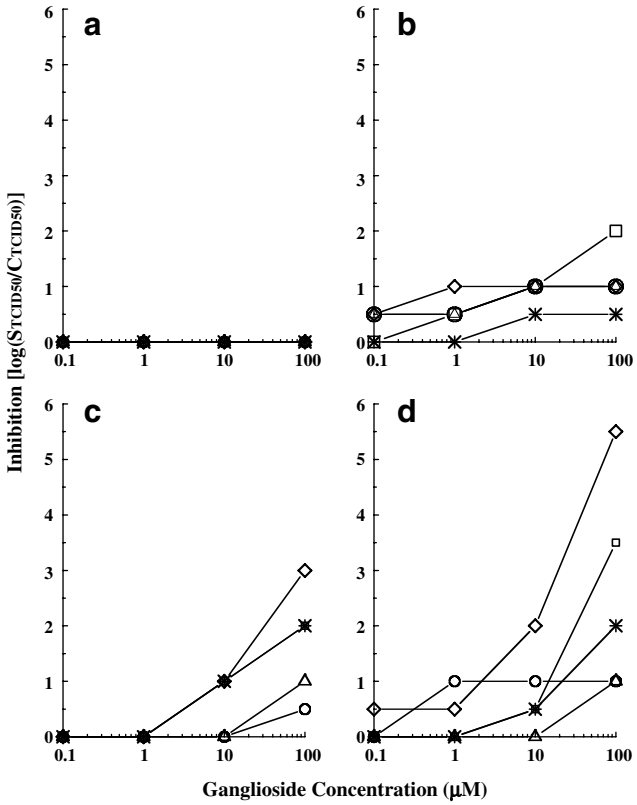

Figure 3. Anti-influenza A virus activities of gangliosides. Inhibition levels are expressed as $\log_{10}(S_{TCID_{50}}/C_{TCID_{50}})$ at concentrations of 0.1, 1.0, 10, and 100 μM. $S_{TCID_{50}}$ and $C_{TCID_{50}}$ mean the $TCID_{50}$ value of the samples tested, and of controls (in the absence of any ganglioside or AMD), respectively. A $C_{TCID_{50}}$ value was $10^{-6.5}$. (a) Cells were treated with gangliosides 1 h before infection; (b) cells were infected with the virus in the presence of ganglioside, and test samples were washed out; (c) after infection, cells were treated with ganglioside; and (d) cells were infected with the virus in the presence of gangliosides and incubated together. All gangliosides showed no evidence of toxicity to MDCK cells even at 200 μM: GM4 (△), GM3 (○), GD1a (□), SPG (◇), and AMD (∗).

SPG was observed up to 500 μM. In the case of GD1a, the 100% survival value was at 130 μM. The 50% inhibitory concentration (IC_{50}) of SPG and GD1a were 7 and 70 μM, respectively. The IC_{50} of the representative antiviral compound AMD was found to be more than 130 μM, however, at this concentration level, cytotoxicity of AMD was observed without infection.

Electron microscopic observation revealed that SPG inhibited the viral replication of A/PR/8/34 in MDCK cells, when cells were infected with a 10^{-3} dilution of A/PR/8/34. Treatment of cells with 100 μM SPG did not cause any change as a result of infection at 10^{-3} dilution (Fig. 5a). In the case of 100 μM AMD, some virus particles were identified at the apical projection; virus replication was also detected (Fig. 5b). Without SPG and AMD, a control infection at 10^{-5} dilution induced necrocytosis and some granular structures at the apical projection (Fig. 5c). The infection was also

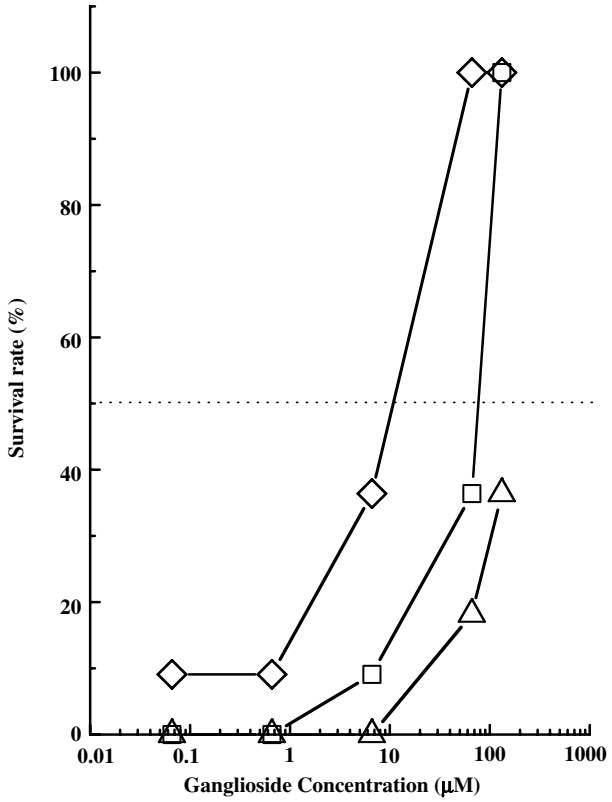

Figure 4. Survival rates of MDCK cells infected with influenza virus A/PR/8/34. Inhibition of viral-induced cytopathy by SPG (◇), GD1a (□), and AMD (△) was assayed. At 66 μM of SPG, the survival rate was reached 100%. At this concentration, no difference was observed in the aspects of virus-infected cells, virus-free cells (in the control experiment), and inhibitor- and virus-free cells throughout the incubation period of over 4 days. In this experiment, 100% survival (S_{100}) corresponds to $TCID_{50}$ of SPG more than 66 μM, and 0% survival (S_0) is $TCID_{50}$ in the absence of any inhibitor. The survival rate (S_r) was calculated as follows: $S_r = (\log S_{100} - \log S_{TCID_{50}} / \log S_0) / (\log S_{100} - \log S_{100} / \log S_0)$. Here, $S_{TCID_{50}}$ is $TCID_{50}$ value of the samples tested at each concentration. A dotted line shows a 50% survival rate.

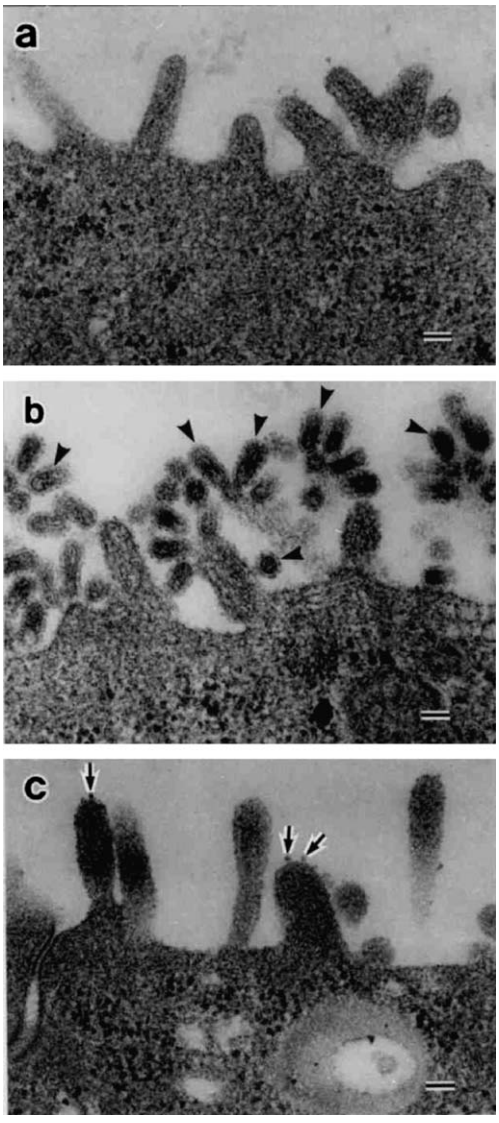

Figure 5. Electron microscopic observation of MDCK cells infected with influenza virus A/PR/8/34. (a) 100 μM of SPG, (b) AMD, and (c) controls without SPG or AMD. Concentrations of virus are 10^{-3} dilution in (a) and (b), and 10^{-5} dilution in (c). Panel (b) shows the presence of some virus particles (arrowheads) on the apical portion of the MDCK cells. Panel (c) shows some granular structures (arrows) at the apical projection of the MDCK cells. The scale bar in each panel indicates 0.1 μm.

prevented by SPG and AMD at virus dilutions of 10^{-4} and 10^{-5}.

In order to analyze the timing effects of gangliosides, SPG was added to cultures at 1-h intervals after the infection procedure (Fig. 6). The inhibitory effect of SPG was observed up to 5 h after infection, but its addition after 6 h led to reduced activity. The appearance of free Neu5Ac in culture supernatants was detected 16 h after infection (Table 2). Generally, the virus release began from 4 to 6 h after infection and reached a maximum by 16–24 h.[17,18] Considering the time lag between the eclipse period and the latent period, most of the newly synthesized viruses might be expected to be released after 8 h. It is clear that detection of free Neu5Ac at 16 h in the culture supernatants is due to the action of NA, of which the A/PR/8/34(H1N1) virus produces a daughter virus as the second generation.

4. Discussion

This study clearly demonstrates that exogenous gangliosides such as SPG, GD1a, GM3, and GM4 had significant inhibitory effects on CPE caused by influenza A virus. SPG belong to a neolacto-series ganglioside containing Neu5Acα2–3Galβ1–4GlcNAc sugar chain. However, oligosaccharides of Neu5Acα2–3Galβ1–3GalNAc (ganglio-series) and Neu5Acα2–3Galβ1–4Glc, identical to the terminal trisaccharides of GD1a and GM3, respectively, showed no inhibitory activity. The A/PR/8/34 virus bound most effectively to lacto- and

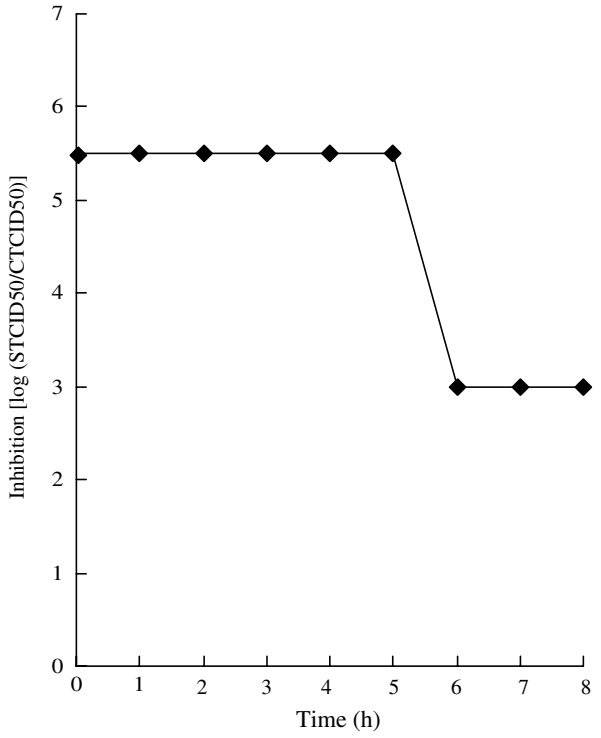

Figure 6. Inhibitory effect of sialylparaglobosides (SPG) on virus-induced cytopathy of MDCK cells. After infection, the culture medium was replaced with a fresh medium containing SPG (100 μM) at 1 h intervals.

neolacto-series gangliosides, followed by the ganglio-series gangliosides and GM3.[12] It is worth noting that the order of the potency for the anti-viral effect of gangliosides coincides well with the order of affinity of gangliosides to HA.[12]

In the infection process, the initial stage is recognition of the sialyl sugar chain by the viral HA. Competitive binding to the sialyl sugar chain between the viral HA and exogenous gangliosides resulted in a lowering of the viral infectious ability. Indeed, SPG acted as a competitive inhibitor against binding of the viral HA to the receptor sugar chain during the infection process (Figs. 3 and 4). However, sialyloligosaccharides, 3′-SL and 3′-SLN showed no evidence of inhibitory effect. It is suggested that this might be due to the lack of hydrophobic moiety of ceramide, unlike gangliosides, since many studies, using synthesized sialosides containing various hydrophobic aglycons, have indicated that the hydrophobic moiety plays an important role in the binding affinity for viral HA.[19–21] Therefore, in order to introduce hydrophobicity to 3′-SL, *N*-3′-SL-NH-C12 was synthesized. As expected, this analogue had a comparable activity to that of GM3. The analogue is classified into a single-tailed amphiphile, which contains 3′-SL as a polar head and the compound easily forms micelle in aqueous solution. The chromatographic behavior on a Sephadex G-75 column indicated micelle formation of *N*-3′-SL-NH-C12, as gangliosides including GM3.[22–24] From the micelle particle, Neu5Ac residues of 3′-SL portions may protrude toward the outside. The state of high-density epitope formed on the surface provides a multivalent binding site for HA. It has been reported that a higher binding affinity was achieved through multivalent attachment of Neu5Ac groups to HA.[25–29] In the aqueous solution, however, the Neu5Ac residue in free 3′-SL does not face toward HA because of either the rotational or translational behaviors at the incubation temperature. Thus the GM3 analogue (*N*-3′-SL-NH-C12) may act as an inhibitor against the infection.

Table 2. Neu5Ac released from SPG by neuraminidase of influenza A/PR/8/34 (H1N1) virus

MDCK cells	Time (h)					
	0	8	16	24	48	96
Infected	0	0	10	20	80	90
Control	0	0	0	2	3	6

Values are % of the total sialic acid.
MDCK cells were incubated in the culture medium containing 100 μM of SPG after the infection procedure. During the incubation, Neu5Ac released from SPG by influenza virus neuraminidase was determined at an appointed time as described in Materials and methods.
From the start (0 h) to 8 h, the determination was performed at 1-h intervals.
No Neu5Ac release was detected in the period.

The fact that the attachment of a hydrophobic portion to 3′-SL generates the inhibitory potency suggests another possible mechanism of inhibition. It has been reported that amphiphilic glycosphingolipids are taken up by endocytosis of cells.[30] The endocytosed gangliosides have an opportunity to encounter newly synthesized HA before viral assembly during the time lag between the eclipse period and the latent period, resulting in interference with the intracellular replication of the PR/8/34 virus.

The effective sites of anti-viral action might involve adsorption, penetration, uncoating of virus, synthesis of virus-specific nucleic acids, synthesis of virus-specific proteins, virus assembly, maturation, and release.[4] The anti-influenza A virus drug, AMD, and its structural analogue rimantadine, have been already used clinically.[1] It has been reported that the drugs block the ion channel activity of the influenza A virus M2 protein, resulting in inhibition of uncoating.[31] However, the clinical use of AMD has been limited by its side effects, which may be due to inhibition of endogenous ion channel activity,[32] and by the rapid emergence of resistant viral strain.[33] Two neuraminidase inhibitors (4-guanidino-Neu5Ac2en and 4-amino-Neu5Ac2en), which were designed on the basis of the crystal structure of influenza virus neuraminidase,[6] have been developed as anti-influenza virus reagents. The inhibitors plug the active site of viral neuraminidase to inactivate the enzyme activity,

and restrict the release, or the elution of newly synthesized virions from infected cells.

Within a few decades, X-ray studies have revealed the structure of the receptor-binding sites and the receptor-binding specificity of HAs of the influenza A virus.[34] The amino acid residues taking part in binding with sialic acid in the site are conserved in all subtypes of influenza virus, throughout their antigenic variation.[21,35–37] The linkages between sialic acid and the penultimate Gal play an important role in the recognition specificities of HAs from various infecting virus species; viruses from humans recognize the α-(2→6) linkage, those from avians and equines recognize α-(2→3) linkage, and those from swine appear to recognize both linkages.[38–42] Furthermore, using the binding assay on a TLC plate, Suzuki et al.[12] have shown that the sugar chain containing GlcNAc as the third sugar linking to the penultimate Gal is recognized more potently than that containing GalNAc or Glc.

Emerging influenza pandemics have been accompanied by the evolution of receptor-binding specificity, from the preference of avian viruses for sialic acid receptors in α-(2→3) linkage to the preference of human viruses for α-(2→6) linkage. A recent study[43] on the H5 avian and the H9 swine influenza virus HAs from viruses closely related to those that caused outbreaks of human disease in Hong Kong in 1997 and 1999 suggests that there is a strong risk that the α-(2→3) receptor of avian virus evolves into the α-(2→6) receptor of human virus via the α-(2→3)/α-(2→6) receptor of swine viruses, which is an evolutionary intermediate structure of the receptor-binding site.

As discussed above, the inhibition mechanism of SPG is clearly different from those of AMD and the neuraminidase inhibitors. The binding of HA with the epitope sugar chain on the receptor is an intrinsic property of influenza viruses and is essential for viral propagation. In this context, gangliosides, especially SPG, are proposed to be an important model for the development of drugs for the prophylaxis and treatment of infection by various strains of influenza A viruses, including unexpectedly generated pandemic viruses.

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