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Identification and Characterization of Carbohydrate Molecules in Mammalian Cells Recognized by Dengue Virus Type 2

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The interaction between cell surface receptors and the envelope glycoprotein (EGP) on the viral membrane surface is the initial step of Dengue virus infection. To understand the host range, tissue tropism, and virulence of this pathogen, it is critical to elucidate the molecular mechanisms of the interaction of EGP with receptor molecules. Here, using a TLC/virus-binding assay, we isolated and characterized a carbohydrate molecule on mammalian cell surfaces that is recognized by dengue virus type 2 (DEN2). Structural determination by immunochemical methods showed that the carbohydrate structure of the purified glycosphingolipid was neolactotetraosylceramide (nLc_4Cer). This glycosphingolipid was expressed on the cell surface of susceptible cells, such as human erythroleukemia K562 and baby hamster kidney BHK-21. All serotypes of DEN viruses, DEN1 to DEN4, reacted with nLc_4Cer, and the non-reducing terminal disaccharide residue Galβ1-4GlcNAcβ1- was found to be a critical determinant for the binding of DEN2. Chemically synthesized derivatives carrying multiple carbohydrate residues of nLc_4, but not nLc_4 oligosaccharide, inhibited DEN2 infection of BHK-21 cells. These findings strongly suggested that multivalent nLc_4 oligosaccharide could act as a competitive inhibitor against the binding of DEN2 to the host cells.

Key words: dendrimer, dengue virus, glycosphingolipid.

Abbreviations: Ar_4Cer, GalNAcβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer; DEN2, dengue virus type 2; DMEM, Dulbecco's modified Eagle's medium; EGP, envelope glycoprotein; FBS, fetal bovine serum; FFU, focus forming unit; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Gg_3Cer, GalNAcβ1-4Galβ1-4Glcβ1-1′Cer; Gg_4Cer, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer; Glc, glucose; GlcNAc, *N*-acetylglucosamine; HRP, horse radish peroxidase; HS, heparan sulfate; LacCer, Galβ1-4Glcβ1-1′Cer; nLc_4, lacto-*N*-neotetraose; nLc_4Cer, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer; TLC, thin layer chromatography.

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are human infectious diseases transmitted by mosquitoes (*1*). The incidences of these acute viral infections are increasing in many countries located in tropical and subtropical areas (*2*). During the 1980s, the number of patients increased at a rate of 20% per year (*3*). Currently, half the population of the earth, 2.5 billion people, reside in latent hazardous areas, and there are 50 million patients and 30 thousand deaths from DF/DHF every year (WHO http://www.who.int/topics/dengue/en/, 2002). DF/DHF, along with malaria, have very large sociopolitical effects, particularly in developing countries, and thus global countermeasures against DF/DHF are required.

Dengue virus is maintained and amplified by transmission to humans through a specific mosquito, *Aedes aegypti* (*4*). There are four serotypes of dengue virus, 1 to 4. Although immune responses acquired by infection with a certain type of dengue virus are effectively eternalized, they are not effective against other serotypes and are thought to even augment symptoms due to non-neutralizing antibodies on infection with other types of dengue virus (*5*, *6*). The mechanisms responsible for the clinical differences between DF and DHF are not yet clear. Dengue virus is classified as belonging to the same family as Japanese encephalitis and yellow fever viruses, which have been controlled successfully by specific vaccinations (*7*). However, no effective vaccines or anti-dengue agents have yet been developed.

Dengue virus, a member of the flaviviridae, has an envelope consisting of an icosahedral lipid bilayer (*8*). The virus has a positive-strand RNA genome of 10.7 kb

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in length and is translated to a single long polypeptide that is then processed by cleavage with proteases, resulting in generation of three structural (C, PrM/M, and E) and seven non-structural proteins (*9*). Envelope glycoprotein (EGP) forms a heterodimer complex with M protein converted from prM by the action of host furin-like protease and is co-expressed on the smooth surface of mature progenitor viruses (*8*, *10*, *11*). EGP, which consists of three functional domains (I, II, and III), is involved in the early events of dengue virus infection, such as attachment of the virus to the host cell surface and engagement of fusion between viral and host cell membranes (*12*, *13*). This protein is also a target of neutralizing antibodies in humans (*14–16*). Domain III of EGP is critical for virus adsorption to the receptors expressed on the host cell surface (*16*).

Infection with dengue viruses, similarly to other viruses, is initiated by the interaction of viral adhesive proteins with specific receptors expressed on the host cell surface (*17*). This initial step is not only a crucial event in infection but is also an important factor for host and tissue tropism. EGP located on the viral surface is the only viral adhesive protein in dengue viruses (*18*, *19*). On the other hand, receptor molecules specific for dengue viruses or the molecular mechanisms of infection, especially entry of dengue viruses into host cells, are not fully understood. To date, several putative receptor molecules for dengue viruses have been reported; heparan sulfate (HS)-like proteoglycan on Vero and BHK cells (*20*, *21*), 40- and 45-kDa glycoproteins on an insect cell line, C6/36 (*22*, *23*), and DC-specific ICAM3-grabbing non-integrin (DC-SIGN) expressed on human dendritic cells (*24*, *25*).

HS is the best studied of these three molecules and is often utilized when other viruses infect host cells (*26*, *27*). Although HS with unique sulfated groups has been shown to act as a high-affinity receptor for herpes simplex virus type 1, HS has been suggested to enhance the interaction of viruses with receptors rather than to bind the viruses directly, due to the concentration of viruses at locations adjacent to the receptors on the cell surface.

Recently, the three-dimensional structures of EGPs of flaviviruses, dengue virus type 2, and tick-borne encephalitis (TBE) virus have been elucidated by X-ray crystallography (*18*, *19*). To use structural information concerning EGPs for the development of effective anti-flavivirus agents, such as neutralizing agents, it is necessary to elucidate the structures of the complexes of receptor molecules and EGP. Several lines of evidence have strongly suggested that the receptor molecules on binding and entry of dengue viruses differ between cell types and virus serotypes (*28–30*). Carbohydrate residues on the surface of dengue virus-susceptible cells seem to be involved in the interaction with dengue viruses in many cases (*20*, *31–33*).

In the present study, to determine the carbohydrate determinants directly involved in adsorption of dengue virus type 2 to the host cell surface, we isolated and characterized the structures of carbohydrate molecules binding to the virus. Furthermore, we chemically synthesized compounds with carbohydrate residues related to the defined carbohydrate structures and evaluated their effectiveness in blocking dengue virus infection.

MATERIALS AND METHODS

Materials—LacCer, nLc_4Cer, and Gg_4Cer were purified from bovine brain. Gangliotriaosylceramide (Gg_3Cer) was purchased from Sigma (St. Louis, MO). Ar_2Cer (Manβ1-4Glcβ1-1′Cer), Ar_3Cer (GlcNAcβ1-3Manβ1-4Glcβ1-1′Cer), and Ar_4Cer (GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ1-1′Cer) ′Cer) were purified from larvae of the green-bottle fly, *Lucilia caesar*, as described previously (*34*, *35*).

Cell Culture and Virus—Cells of a human erythroleukemic cell line, K562, and a baby hamster kidney, BHK-21, were cultured at 37°C under 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS and Dulbecco's modified Eagle's medium (DMEM) with 5% FBS, respectively. Dengue virus type 2 (DEN2), ThNH-7/93 strain (accession no. U31949), dengue virus type 1 (DEN1), D1/Lao/03 strain, dengue virus type 3 (DEN3), D3/BD/02 isolate/1, and dengue virus type 4 (DEN4), D4/(D4-17 strain), were propagated in C6/36 cells as described previously (*36*).

Extraction and Purification of Reactive Glycosphingolipids with DEN2 from K562 and BHK-21 Cells—Extraction and purification of a glycosphingolipid were performed as described previously (*37*, *38*). Packed K562 cells from 2.6 liters of culture were suspended with MilliQ water and mixed with chloroform/methanol/water (10:10:1, v/v/v). Whole lipids were extracted for 24 h at room temperature and filtered. Another extraction from residual materials was performed for 6 h and filtered again. Whole lipids were combined and evaporated to dryness, then hydrolyzed overnight at 37°C in methanol containing 0.1 N sodium hydroxide. The whole lipid fraction was dialyzed and lyophilized, and then applied to a DEAE-Sephadex A-25 column (100 ml of column bed volume) for separation of neutral and acidic lipid fractions. The eluted neutral lipid fraction from K562 cells was concentrated and further purified by Iatrobeads column chromatography. Neutral glycosphingolipids were separated on an Iatrobeads column. The neutral lipid fraction was applied and separated by step-wise elution with different solvents as follows: (i) chloroform/methanol (8:2, v/v), (ii) chloroform/methanol/water (65:25:4, v/v/v), (iii) chloroform/methanol/water (65:35:8, v/v/v), and (iv) chloroform/methanol/water (50:40:10, v/v/v). A glycosphingolipid reactive with DEN2 on TLC plates was further purified by a preparative TLC method. For extraction of the neutral lipid fraction from BHK-21 cells, confluent monolayers of BHK-21 cells on 20 tissue culture dishes were harvested and suspended with MilliQ water. Whole lipids were extracted for 2 h at room temperature with chloroform/methanol/water (5:5:1, v/v/v) and filtered. Residual materials were extracted further for 1 h with the same solvent and filtered. Both extracts were combined and evaporated to dryness, then hydrolyzed overnight at 37°C in methanol containing 0.1 N sodium hydroxide. The extracts was dialyzed and lyophilized. Dried extracts was dissolved in chloroform/methanol/water (30:60:8, v/v/v) and applied to a DEAE-Sephadex A-25 column (25 ml of column bed volume) for separation of the neutral lipid fraction. The obtained neutral lipid fraction from BHK-21 cells was concentrated and subjected to analyses.

TLC/Virus-Binding Assay—TLC/virus-binding assay was carried out as described previously (*39*). Briefly, plastic

TLC plates were developed using chloroform/methanol/12 mM $MgCl_2$ (50:40:10, v/v/v) or isopropanol/water/25% ammonium hydroxide (75:25:5, v/v/v). Glycosphingolipids resolved on TLC plates were soaked for 1 h at room temperature in phosphate-buffered saline (PBS) containing 1% ovalbumin and 1% polyvinylpyrrolidone (PVP) (solution A). The plates were incubated overnight at 4°C in ice-cold solution A containing DEN [3.8×10^7 focus forming units (FFU)]. Virus solution was removed carefully and human anti-dengue antiserum from patients with dengue hemorrhagic fever was added as the primary antibody (1:1,000), and the plates were incubated at room temperature for 1 h. After washing three times, they were incubated at room temperature for 1 h with horseradish peroxidase (HRP)–conjugated goat anti-human immunoglobulin as the secondary antibody (1:1,000). After washing three times, the plates were visualized with a KONICA immunostaining HRP-1000 kit (KONICA, Tokyo, Japan).

TLC-IMMUNOSTAINING

TLC-immunostaining of the purified glycosphingolipids and lipid fractions from K562 cells and BHK-21 cells was performed as described previously (*37*, *38*). Plastic TLC plates were soaked at room temperature for 1 h in PBS containing 1% bovine serum albumin (BSA). The plate was then incubated at room temperature for 1 h in a mixture containing mouse monoclonal anti-nLc_4Cer antibody. After washing with PBS containing 0.05% Tween20, the plate was incubated at room temperature for 1 h with HRP-conjugated goat anti-mouse immunoglobulin (Jackson ImmunoResearch, West Grove, PA). After washing with PBS, the plate was visualized with a KONICA immunostaining HRP-1000 kit.

DEN2 Binding to Glycosphingolipids—The binding activity of DEN2 for glycosphingolipids on ELISA-based virus-binding assay was evaluated. Briefly, each glycosphingolipid in ethanol (50 μl per well) was dried and immobilized on wells of plastic plates (Nunc, Roskilde, Denmark). Wells were blocked overnight at 4°C with 200 μl of PBS containing 1% BSA. After washing with PBS, the plates were incubated for 1 h at 28°C with virus solution diluted with PBS containing 0.1% BSA. After washing five times, the plates were incubated for 1 h at 28°C with human anti-DEN antiserum as the primary antibody, followed by HRP-conjugated Goat anti human immunoglobulin as the secondary antibody. After washing four times, the complexes were detected by incubation with *o*-phenylenediamine as a substrate. The reaction was terminated by addition of 1 N HCl. The absorbance was measured at 492 nm.

Synthesis of Dendrimers—Three carbosilane dendrimers carrying lacto-*N*-neotetraose (nLc_4) [or lactose (Lac)], Fan(0)3-nLc_4 (or Lac), Ball(0)4-nLc_4 (or Lac), and Dumbbell(1)6-nLc_4 (or Lac) were synthesized according to the following method (A. Yamada *et al.*, accepted for publication in *Carbohydrate Research*). 4-Pentenyl-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1-4)-(3,6-di-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1-3)-(2,6-di-*O*-benzoyl-β-D-galactopyranosyl)-(1-4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside was synthesized by glycosylation of 4-pentenyl-*O*-(2,6,-di-*O*-benzoyl-β-D-galactopyranosyl)-(1-4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside and *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1-4)-3,6-di-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl chloride. Conversion of the protecting groups followed by thioacetylation resulted in an nLc_4 derivative, 5-(acetylthio)-pentenyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1-4)-(3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1-3)-(2,4,6-di-*O*-acetyl-β-D-galactopyranosyl)-(1-4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside. Briefly, carbosilane dendrimers (25.0 mg, 0.053 mmol) and the nLc_4 derivative (301.2 mg, 0.222 mmol) dissolved in 0.6 ml of dimethylformamide/methanol (1:1, v/v) were treated at room temperature for 11 h with methanol containing 1 M sodium methoxide (224 μl) under an argon atmosphere. After addition of acetic acid (0.2 ml), the mixture was evaporated. The residue was dissolved in pyridine (4 ml), and acetic anhydride (2 ml) was added. The mixture was stirred at 30°C for 3 h. The resultant solution was poured into ice-water, extracted with ethyl acetate, and subjected to the usual work-up process. The acetylated products were purified by recycling preparative HPLC on a column of JAIGEL-2.5H and 3H with a single solvent system of chloroform. The yields of acetylated Fan-shape, Ball-shape, and Dumbbell-shape compounds were 142.0 mg (64%), 121.3 mg (55%), and 110.0 mg (44%), respectively. Acetylated compounds were dissolved in 1.0 ml of methanol and 0.1 M sodium methoxide was added under an argon atmosphere. The reaction mixture was stirred for 30 min. The progress of reaction was monitored by TLC. When the reaction was complete, the mixture was treated with Amberlite IR120B (H^+) resin to neutralize the base. The resin was filtered off and the filtrate was concentrated. The products were purified by gel exclusion chromatography (Sephadex G50) with 5% acetic acid as eluent. The yields of Fan-shape, Ball-shape, and Dumbbell-shape dendrimers were 71.2 mg (quantitative), 39.1 mg (68%), and 42.4 mg (96%), respectively. The carbosilane dendrimers with nLc_4 tetrasaccharides synthesized in this study were identified by ^1H and 13C NMR spectra and by high-resolution mass spectra. The molecular masses of Fan(0)3-nLc_4, Ball(0)4-nLc_4, and Dumbbell(1)6-nLc_4 were 2,657.89, 3,431.69 and 5,303.89, respectively. All dendrimers were chemically stable and neutrally charged in aqueous solution.

Focus-Forming Assay—Virus titers were determined by focus-forming assay using BHK-21 cells. BHK-21 cells were seeded onto 96-well plates and cultured for 24 h at 37°C in DMEM supplemented with 2% FBS. After removal of medium, virus solutions serially diluted with serum-free DMEM were inoculated onto the plates, and the cells were incubated for 2 h at 37°C. After removing the virus solution, overlay medium (DMEM containing 1% FBS and 0.5% methyl cellulose) was added, and plates were incubated at 37°C for 44 h. The cells were fixed and permeabilized with PBS containing 5% paraformaldehyde and 1% NP-40, respectively. Infectious foci were detected with human anti-dengue antisera as the primary antibody and HRP-conjugated goat anti-human immunoglobulin as the secondary antibody. Virus infectivity was determined as focus-forming units (FFU).

Inhibition of Virus Infection by Dendrimers—DEN2 (1,800 FFU/ml as final concentration) was premixed with Fan, Ball, and Dumbbell-shape dendrimers at the indicated concentrations. The virus-dendrimer premixtures

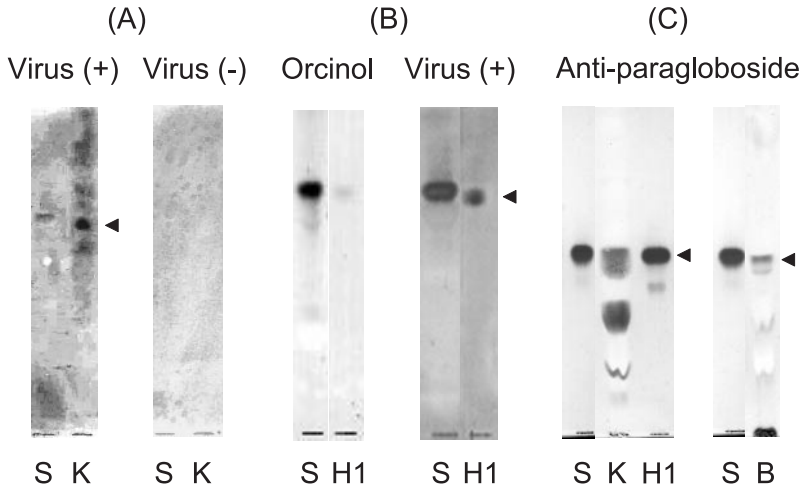

Fig. 1. **Reactivity of dengue virus type 2 with glycosphingolipids in human K562 cells and BHK-21 cells.** TLC/virus-binding and TLC-immunostaining assays were carried out as described in "MATERIALS AND METHODS." A and B, TLC/virus-binding assay with DEN2; C, TLC-immunostaining with anti-nLc_4Cer antibody. S, nLc_4Cer from bovine erythrocytes; K, whole lipids from K562 cells; H1, purified glycosphingolipid from K562 cells; B, neutral glycosphingolipid fraction from BHK-21 cells. Plates were developed with chloroform/methanol/water (5:4:1, v/v/v). The arrowhead shows glycosphingolipids reactive with dengue virus type 2.

(125 μl) were then inoculated for 2 h at 37°C onto BHK-21 cells grown in 48-well plates. After washing three times with serum-free DMEM, overlay medium was added and plates were incubated at 37°C for 43 h. Infectious foci of cells were then visualized by focus-forming assay as described above and counted under a light microscope. The optimal titer of inoculated virus was predetermined such that more than 50 foci appeared per well.

RESULTS

A Glycosphingolipid from a Human Cell Line, K562, Bound to DEN2—Dengue viruses are maintained and amplified by transmission to humans through *Aedes* mosquitoes. The adsorption of viruses to the host cell surface is the initial and critical step for viral infection. We explored the molecules in human cells which involved in binding of DEN2 to the host cells. To investigate these molecules, glycosphingolipids and glycoproteins were obtained from the cells and tested for viral binding activity. We examined human erythroleukemia K562 cells, which are sensitive to DEN2 infection (*40*). First, whole proteins were extracted from K562 cells as described previously (*22*), and subjected to virus (DEN2)-overlay assay. Several positive signals were observed on a blotted membrane. DEN2 predominantly bound to a protein with a molecular mass of 100 kDa and some minor proteins with molecular masses of around 30–40 kDa (data not shown). Next, whole lipids from the cells were extracted with organic solvents. Neutral and acidic glycosphingolipid fractions were prepared from whole lipids by DEAE-Sephadex A-25 column chromatography. Both fractions were examined for virus-binding activity. Dengue virus type 2 significantly bound to one component in the whole lipid fraction with similar mobility to authentic nLc_4Cer on TLC (arrowhead in Fig. 1A). The virus did not significantly bind to any acidic glycosphingolipids (data not shown). Thus, reactive glycosphingolipid was isolated from the neutral glycosphingolipid fraction by Iatrobeads column chromatography and characterized. The isolated glycosphingolipid, termed H1, showed similar mobility to authentic nLc_4Cer on TLC plates and was clearly recognized by DEN2 (arrowhead in Fig. 1B). Anti-nLc_4Cer monoclonal antibody (clone H11) equally detected the reactive glycosphingolipid in

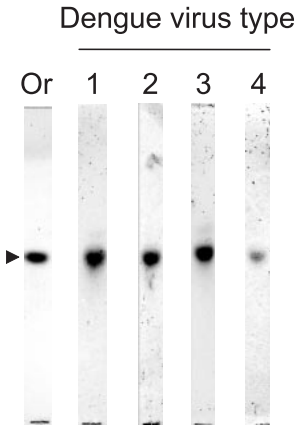

Fig. 2. **Binding of different types of dengue virus to nLc4Cer.** TLC/virus-binding assay was performed using DEN1 to DEN4. Or, staining with orcinol/H_2SO_4 reagent. Plates were developed with isopropanol/water/25% ammonium hydroxide (75:25:5, v/v/v). The arrowhead shows nLc_4Cer reactive with dengue viruses.

K562 whole lipid fraction, purified H1 and authentic nLc_4Cer from bovine erythrocytes (*41*), strongly suggesting that the carbohydrate structure of the reactive glycosphingolipid, H1, is identical to that of authentic nLc_4Cer, Galβ1-4GlcNAcβ1-3Galβ1–4Glcβ1-1′Cer (Fig. 1C). Additionally, we investigated whether nLc_4Cer is present in another susceptible cell line, BHK-21. This cell line also contained nLc_4Cer (Fig. 1C). We next examined whether nLc_4Cer is recognized by the all serotypes of dengue viruses, DEN1 to DEN4. As shown in Fig. 2, dengue viruses commonly bound to nLc_4Cer, suggesting that this glycosphingolipid was involved in the binding of all serotypes of dengue viruses to the host cell surface.

BINDING ACTIVITY OF DEN2 TO GLYCOSPHINGOLIPIDS

Dengue virus particles clearly bound to nLc_4Cer from K562 cells on TLC plates. To clarify the carbohydrate determinant responsible for the interaction with DEN2, we examined DEN2 binding activity to glycosphingolipids by TLC/virus-binding assay and ELISA-based virus-binding assay, and the results are shown in Fig. 3, A and B, respectively. Dengue virus type 2 showed the strongest

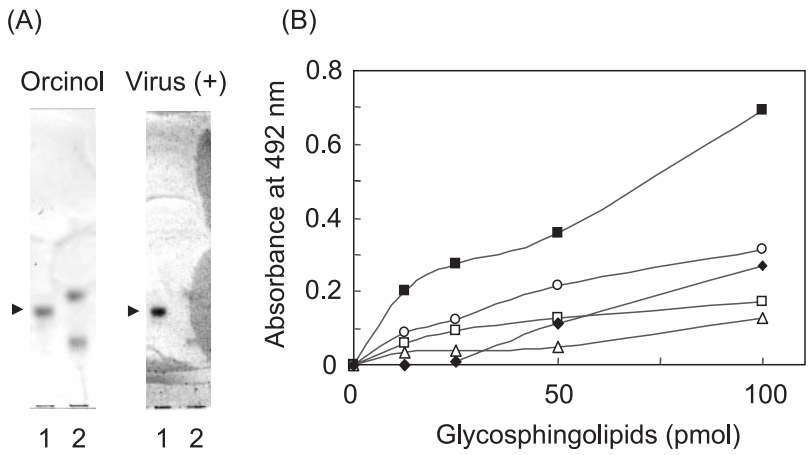

Fig. 3. **Binding activity of DEN2 to glycosphingolipids.** (A) Binding of DEN2 to nLc_4Cer. nLc_4Cer (lane 1), Gg_3Cer (lane 2, upper band) and Gg_4Cer (lane 2, lower band) were resolved on a TLC plate. Each glycosphingolipid was applied at 0.22 nmol on plates. (B) Dose response of DEN2 binding to glycosphingolipids immobilized on plastic plates by ELISA-based virus-binding assay. Wells were coated with varying amounts of nLc_4Cer (filled square), Gg_4Cer (open circle), LacCer (open square), Gg_3Cer (open triangle) or Ar_4Cer (filled rhombus). Values indicate the averages of duplicate wells for each sample. The results shown are representative data from three independent experiments.

binding activity to nLc_4Cer, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer, on TLC plates, and to Gg_4Cer, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer and Gg_3Cer, GalNAcβ1-4Galβ1-4Glcβ1-1′Cer to lesser extent (Fig. 3A). The virus did not bind to LacCer, Galβ1-4Glcβ1-1′Cer, on TLC plates (data not shown). ELISA-based virus-binding assay demonstrated that the virus significantly bound to nLc_4Cer, but not either LacCer or Gg_3Cer (Fig. 3B). The reactivity of DEN2 to glycosphingolipids on plastic microplates was the highest toward nLc_4Cer. Gangliotetraosylceramide (Gg_4Cer) showed marginal binding activity to DEN2. These results suggested that Galβ1-4GlcNAcβ- at the non-reducing terminal was involved in the interaction with DEN2. Additionally, we examined the reactivity of DEN2 to glycosphingolipids isolated from *Lucilia caesar*. Carbohydrate structures of several neutral glycosphingolipids in *Lucilia caesar* have been defined (*34*, *35*). We examined glycosphingolipids Ar_2Cer (Manβ1-4Glcβ1-1′Cer), Ar_3Cer (GlcNAcβ1-3Manβ1-4Glcβ1-1′Cer) and Ar_4Cer (GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ1-1′Cer) from *Lucilia caesar* for DEN2 binding activity. Like Gg_4Cer, Ar_4Cer showed marginal binding activity to DEN2 (Fig. 3B). This result suggested beta-GalNAc linked to GlcNAc residue at the non-reducing terminal also affected the binding activity of DEN2. Comparison of nLc_4Cer and Ar_4Cer indicates that DEN2 recognizes the beta-Gal residue more strongly than the beta-Man residue. These observations strongly suggested that a critical carbohydrate determinant for the binding of DEN2 was Galβ1-4GlcNAcβ-(Gal) at the non-reducing terminal.

Inhibition of DEN2 Infection by Dendrimers Carrying a Carbohydrate Residue Derived from nLc_4Cer—We chemically synthesized and tested compounds carrying carbohydrates reactive with DEN2. Carbosilane type compounds, termed dendrimers, were chosen as the core structures for the following reasons. Dendrimers carrying carbohydrate residues are readily soluble in water. They do not show either significant immunogenicity or toxicity in mice (*42*). Unlike polymer-type compounds, they have relatively low molecular mass, and their molecular mass can be well defined. We chose the carbohydrate structure of nLc_4Cer for synthesis of dendrimers, because nLc_4Cer was detected as one of the major glycosphingolipids in K562 cells and reacted significantly with all serotypes of dengue viruses

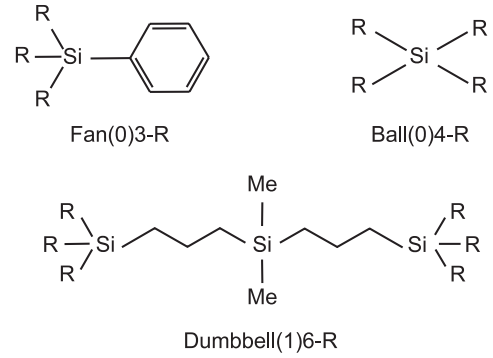

Fig. 4. **Schematic diagrams of dendrimers carrying carbohydrate used in the present study.**

on TLC plates. In addition, nLc_4Cer was detected in hamster BHK-21 cells, which are widely used for infection experiments (Fig. 1C). The carbohydrate structure of LacCer was chosen as control dendrimer, because DEN2 bound much more weakly to LacCer than to nLc_4Cer. Three types of dendrimers carrying lacto-*N*-neotetraose (nLc_4, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-) or lactose (Lac, Galβ1-4Glcβ1-), termed Fan(0)3, Ball(0)4, and Dumbbell(1)6, were used for infection of BHK-21 cells with DEN2 (Fig. 4). DEN2 was premixed with dendrimers at the indicated concentrations, and the premixtures were inoculated onto BHK-21 cells. The results are summarized in Fig. 5. Although the inhibition reached 42% of control, all types of dendrimers carrying nLc_4 showed inhibitory activity against DEN2 infection in a dose-dependent manner up to 0.5 mM. On the other hand, except for Ball(0)4-Lac, dendrimers carrying Lac did not show significant inhibitory activity against DEN2 infection (Fig. 5). An oligosugar derived from nLc_4 also did not inhibit viral infection (data not shown). These results suggested that DEN2 inhibitory activity by carbohydrate residues is specific to the nLc_4 structure linked to a carbosilane backbone and requires a multivalent structure. Among the dendrimers, the effect of Dumbbell(1)6-nLc_4 on viral

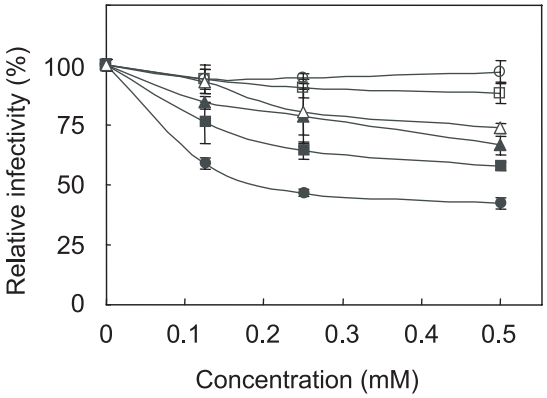

Fig. 5. **Effects of dendrimers on infection of BHK-21 cells by DEN2.** Infection experiments were performed as described in "MATERIALS AND METHODS." Graph summary describing the infectivity of BHK-21 cells by DEN2 in the presence of dendrimers, Fan(0)3-nLc_4 (filled square), Ball(0)4-nLc_4 (filled triangle), Dumbbell(1)6-nLc_4 (filled circle), Fan(0)3-Lac (open square), Ball(0)4-Lac (open triangle), and Dumbbell(1)6-Lac (open circle) at the indicated concentrations. Values indicate averages of relative infectivity of DEN2 with dendrimers relative to viral infection without dendrimers. Bars show standard deviation of triplicate measurements. The results shown are representative data from three independent experiments.

infection was greater than those of Ball(0)4-nLc_4 and Fan(0)3-nLc_4. This observation implied that the polyvalent sugar chain density may affect the inhibitory activity. None of the dendrimers examined showed cytotoxicity against BHK-21 cells at concentrations up to 2 mM (data not shown).

DISCUSSION

In the present study, we isolated and defined carbohydrate determinants associated with the interaction of dengue viruses with mammalian cells, and applied synthetic compounds carrying the determinant as anti-dengue virus agents.

To date, several putative receptor molecules for dengue viruses have been identified in mammalian cells, as follows: heparan sulfate (HS) proteoglycan, LPS/CD14-binding proteins, and dendritic cell–specific ICAM3-grabbing non-integrin (DC-SIGN) (*21*, *43*, *44*). As HS proteoglycan is the best characterized receptor for human pathogenic microorganisms, such as herpes simplex and human immunodeficiency viruses (*26*, *27*), it is difficult to fully understand the molecular mechanisms of specific interactions of dengue viruses with host cells. Putative receptor molecules reported to date are thought to involve carbohydrates. Several studies using specific lectins and glycosidases indicated that carbohydrate structures expressed on the cell surface were significantly associated with the binding of dengue viruses and another flavivirus (*20*, *32*, *45*). Thus, we hypothesized that specific carbohydrate determinants play critical roles in the initial step in dengue virus infection and focused on identification of the carbohydrate structures from host cells involved in the binding of DEN2.

We first examined whether glycosphingolipids extracted from human cells bound to DEN2. One glycosphingolipid reacted strongly with DEN2 on plastic TLC plates. Therefore, we isolated and determined the carbohydrate structure of the reactive glycosphingolipid. The glycosphingolipid reactive with DEN2 was identified as nLc_4Cer. The cell surface expression of nLc_4Cer was determined by flow cytometry using anti-nLc_4Cer monoclonal antibody (clone H11). In both K562 and BHK-21 cells, this glycosphingolipid was detected on the cell surface, indicating that nLc_4Cer can be recognized by DEN2. Previous studies demonstrated that HS is a receptor on BHK-21 cells (*20*). A neutral carbohydrate like nLc_4Cer may contribute to formation of a complex with unidentified receptor molecules on mammalian cells.

To clarify the specific interaction of DEN2 with glycosphingolipids and to elucidate the carbohydrate determinants involved in the binding of viruses with glycosphingolipids, we examined the binding activity of DEN2 to glycosphingolipids. The reactivity to nLc_4Cer was the highest among the glycosphingolipids tested in both TLC/virus-binding assay and ELISA-based virus-binding assay. These results suggested that Galβ1-4GlcNAcβ-(Gal) at the non-reducing terminal was critical to the binding activity of DEN2.

Based on the carbohydrate determinants required for binding of DEN2, we designed and chemically synthesized compounds carrying a carbohydrate reactive with DEN2. We chose a carbohydrate structure from nLc_4Cer, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-, for synthesis of dendrimers for the following reasons: (i) this structure contains the minimum carbohydrate sequence required for binding of DEN2. (ii) the reactivity of nLc_4Cer is the strongest among the glycosphingolipids tested, and only nLc_4Cer has been detected as major component in K562 and BHK-21 cells. (iii) TLC/virus-binding assay demonstrated that nLc_4Cer is recognized by all serotypes of dengue virus, DEN1 to DEN4. Three types of dendrimers carrying Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1- were synthesized and tested for their effects on viral infection of BHK-21 cells. The degrees of viral inhibition by addition of all dendrimers were marginal. Further investigation of the mechanisms on the interaction between DEN binding and carbohydrate will be required by optimization of carbohydrate structures for viral inhibition and co-crystallization or NMR analysis of DEN envelope glycoprotein with dendrimers carrying optimized sugar chains.

In the present study, we identified unique carbohydrate determinants involved in the binding of DEN2 to the host cell surface. Information on these carbohydrate residues might provide clues to the development of effective anti-dengue virus agents.

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