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Binding of human cytomegalovirus to sulfated glucuronyl glycosphingolipids and their inhibitory effects on the infection

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Interactions between human cytomegalovirus (HCMV) and various carbohydrate structures were analysed using sulfated glucuronyl glycosphingolipids (SGGLs) and the structurally related glycosphingolipids (GLs). A thin-layer chromatography-overlay assay and a solid-phase binding assay revealed that HCMV strongly bound to sulfated glucuronyl lactosaminylparagloboside, one of the SGGLs having the repeating lactosamine structure (3Galβ1–4GlcNAc1-)$_2$ in addition to the 3-*O*-sulfated glucuronyl moiety. The virus bound less strongly to other 3-*O*-sulfated GLs, which included sulfated glucuronyl paragloboside and cerebroside sulfate ester, and also to (3Galβ1–4GlcNAc1-)$_2$-containing GLs that included nLc$_6$Cer. Thus, a (3Galβ1–4GlcNAc1-)$_2$ and a 3-*O*-sulfated saccharide seem to be important structures for the binding by HCMV. When virus particles were preincubated with these GLs, inhibitory effects were observed both on expression of the viral immediate-early gene and on plaque formation by HCMV. These effects were very well correlated with the abilities of the GLs to bind to the virus. Pretreatment of host cells with HNK-1 monoclonal antibody, which specifically recognizes SGGLs, resulted in partial inhibition of plaque formation by HCMV. These results clearly show that HCMV recognizes and binds to the sulfated carbohydrate structure in SGGL and also suggest that binding of HCMV to the specific sugar structure may play an important role in HCMV infection.

Introduction

The first step in virus infection is the attachment to the host cell surface. In this event, a specific interaction between viral proteins and cell surface molecules enables the virus to enter target cells. There are several examples in which the specific recognition of cell surface glycoconjugates by virus plays an important role in the initial process of virus infection (Willoughby *et al.*, 1990; Lycke *et al.*, 1991). Human cytomegalovirus (HCMV) is a betaherpesvirus that causes disease in both normal and immunocompromised hosts. The initial mechanism whereby HCMV enters the host cells has not been completely elucidated. As with other herpesviruses, it has been considered that HCMV binds to the host cells by multiple steps and that cell surface glycoconjugates may be involved in these events. Heparan sulfate on the cell surface has been implicated as the initial attachment molecule of HCMV (Kari & Gehrz, 1992; Neyts *et al.*, 1992; Compton *et al.*, 1993). As for the subsequent binding receptor, several proteins have been reported to be involved (Tayler & Cooper, 1990; Keay & Baldwin, 1992; Soderberg *et al.*, 1993; Wright *et al.*, 1994), and most of them are glycoproteins. These reports strongly suggest that glycoconjugates are important in the binding of HCMV to the host cells (Balzarini *et al.*, 1991). Thus far, however, a precise structure of the carbohydrate sequence responsible for binding with HCMV has not been analysed.

Glycosphingolipids (GLs) are mainly localized on the outer leaflet of the plasma membranes and are considered to play important roles in cellular recognition in various systems. Over the last decade much interest has been directed towards the potential immunological role of GL antigens. In a previous report, we showed that the sera of HCMV-infected patients

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contain elevated titres of antibodies against sulfated glucuronyl glycosphingolipids (SGGLs), which carry a sulfated carbohydrate epitope recognized by the monoclonal antibody HNK-1 (Ogawa-Goto *et al.*, 1994). While the titres of the SGGL antibodies in the sera of patients were positively correlated with those of anti-HCMV antibodies, they did not bind to HCMV-infected host cells and were distinguishable from the anti-HCMV antibodies, indicating that the anti-SGGL antibodies were unlikely to be produced against either the virus itself or HCMV-infected host cells (Ogawa-Goto *et al.*, 1994). Another possible explanation is that the production of anti-SGGL antibodies may represent anti-idiotypic antibodies and mimic the cell surface structure which interacts with HCMV. This leads us to consider the possibility that the sulfated carbohydrate epitope in SGGLs plays an important role in the binding of HCMV to host cells. In the present study, we analysed whether HCMV recognized the sulfated carbohydrate epitope of SGGLs in a series of binding experiments. We also investigated the details of carbohydrate structures responsible for the HCMV binding using various kinds of GLs.

Methods

■ **Cell and virus.** Human embryonic lung fibroblasts MRC-5 (Riken Cell Bank, Tsukuba, Japan) were used throughout the study between passages 25 and 35. The cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) (Gibco BRL) and were maintained in MEM supplemented with 2% FBS. The AD169 strain of HCMV (Rowe *et al.*, 1956) was used in the study. Infectivity titre of the virus was determined as plaque-forming units by the agarose overlay method (Wentworth & French, 1970).

HCMV was purified according to the previously described method (Tayler & Cooper, 1989) with a slight modification. In brief, confluent cell monolayers were infected with HCMV at an m.o.i. of 1–2 p.f.u. per cell at 37 °C. For purification of unlabelled virions, the infected cells were incubated until the maximum cytopathic effects (CPE) were observed. After removal of cell debris by a low speed centrifugation (4000 ***g***, 20 min), virus particles in the culture medium were collected as pellets by a high speed centrifugation (32000 ***g***, 1 h). The virus particles re-suspended in MEM were further purified by a D-sorbitol gradient (30–70%) centrifugation at 90000 ***g*** for 1 h, pelleted by centrifugation at 85000 ***g*** for 30 min and re-suspended in an appropriate volume of PBS.

■ **Biotinylation and radiolabelling of HCMV.** Biotinylation of HCMV was carried out by incubating the purified virion suspension (about 1 mg protein/ml) with biotin-3-sulfo-*N*-hydroxysuccinimide ester (final concentration 90 μg/ml) (Sigma) for 30 min at room temperature (Inghirami *et al.*, 1988). Free biotin was removed by dialysis against PBS at 4 °C and the HCMV was stored at −80 °C until use.

To obtain radiolabelled virions, cells were infected with HCMV as described above. When 30–50% of cells in the culture exhibited CPE, the culture medium of the infected cells was replaced with Dulbecco's modified MEM containing a reduced level of methionine, 2% FBS and 10 μCi/ml [^{35}S]methionine (Tran^{35}S-labelling mix; ICN). After an additional incubation period of 4 days, virus particles in the culture medium were purified as described above. Specific activities of the virus particles ranged from 0·02 to 0·06 c.p.m. per p.f.u.

■ **Glycosphingolipids (GLs).** Three groups of GLs were used in this study, (i) sulfated, (ii) neutral and (iii) sialic acid-containing GLs (Ogawa-Goto *et al.*, 1990, 1993). Nomenclature of GLs was based on the system of IUPAC-IUB Commission on Biochemical Nomenclature (1977).

(i) Sulfated glucuronyl paragloboside (SGPG) and sulfated glucuronyl lactosaminylparagloboside (SGLPG) were chemically synthesized as described (Nakano *et al.*, 1993). Cerebroside sulfate ester (CSE) was purified from rat brain. SM3, SM2, SB2 and SB1a were generous gifts from I. Ishizuka (Teikyo University, Tokyo, Japan). (ii) GalCer was purified from rat brain. LacCer, Gb_3Cer, Gb_4Cer and nLc_4Cer were purified from human erythrocyte membrane. GA1 was purchased from Wako chemicals. nLc_6Cer was prepared by a sialidase treatment (*Clostridium perfringens*; Sigma) of sialyl-nLc_6Cer ganglioside. (iii) LM1 was purified from human peripheral nerves; GD1a and GD1b were from bovine brain. GM3, GD3 and sialyl-Le^x gangliosides were purchased from Wako chemicals. GM1 was obtained from Fedia. Sialyl-nLc_6Cer ganglioside was a generous gift of T. Taki (Tokyo Medical Dental University, Japan). (iv) In addition, glucuronyl paragloboside (GPG) was chemically synthesized as described (Nakano *et al.*, 1993) and cholesterol sulfate was obtained from Sigma.

For inhibition experiments with GLs, GL solutions in chloroform–methanol were placed in glass tubes and the solvents were evaporated. After the addition of PBS, ultrasonication was performed to produce a micellar solution. The amount of GLs in the micellar solution was determined by the anthrone–sulfuric acid method and adjusted to the appropriate concentration by adding PBS.

■ **Antibodies.** Monoclonal antibody HNK-1 hybridoma clone was obtained from the ATCC. Antibodies were purified from murine ascites fluids by affinity chromatography on a column conjugated with mannose binding protein (IgM purification kit) according to instructions from the manufacturer (Pierce). Mouse control IgM was purchased from Zymed Labo. Monoclonal antibody against GA1 was obtained from Seikagaku Kogyo Ltd.

■ **Thin-layer chromatography (TLC)-overlay assay.** GLs were spotted on aluminium-backed high-performance TLC plates (Silica gel 60; Merck) and developed with chloroform–methanol–0·2% $CaCl_2$ (55:45:10, by vol.). After development, the plate was dipped in hexane containing 0·4% polyisobutylmethacrylate for 3 min and air-dried. Nonspecific binding was blocked by incubating the plate in PBS containing 1% BSA (PBS–BSA) at 37 °C for 2 h. Then the plate was overlaid with a solution of ^{35}S-labelled HCMV (about $1–3 \times 10^5$ c.p.m./ml) and incubated at 4 °C overnight. After extensive washing with buffer A (150 mM NaCl, 1 mM EDTA, 1% gelatin, 10 mM Tris–HCl pH 7·0), radioactive bands were detected with an image analyser (Bas 1000; Fuji Photofilm).

■ **Solid-phase binding assay.** The binding of biotinylated HCMV to GLs that had been immobilized on microtitre plates was measured as follows. Serially diluted solutions of GLs in methanol were placed in 96-well polystyrene plates and solvents were evaporated to dryness. Unreactive sites were saturated with PBS–BSA at 4 °C overnight. Biotinylated HCMV in PBS–BSA was added and incubated for 1 h at 4 °C. After washing with buffer A, a solution of avidin–biotin complex conjugated with horseradish peroxidase (Vector) was added and incubation was continued for another 1 h at 4 °C. After washing, the substrate solution (ABTS) (Bio-Rad) was added to each well and the absorbance at 415 nm was measured. Absorbance values were corrected by subtraction of values obtained from wells without antigens.

For pretreatment with HNK-1 antibody, SGGLs (100 pmol) immobilized on polystyrene microtitre plates were preincubated with 1 or 0·1 μg/ml HNK-1 antibody or 1 μg/ml control IgM at 4 °C for 1 h. Wells were washed with PBS containing 1% gelatin, followed by the solid-phase binding assay as described above. For a competitive assay,

biotinylated HCMV was preincubated with the indicated amounts of SGPG or GA1 at 4 °C for 1 h and the binding to 100 pmole SGPG by the pretreated biotinylated HCMV was measured by the solid-phase binding assay described above.

■ **Detection of HCMV immediate-early (IE) gene products.** HCMV was preincubated with GLs (final concentration 20 or 100 μM) for 30 min at 37 °C and then used to inoculate cell monolayers at an m.o.i. of 0·1 in 6-well plates for 90 min at 37 °C. After washing the cells with MEM, the monolayers were additionally incubated in MEM with 2% FBS for 20 h. The infected cells were washed with PBS and lysed with sample buffer for SDS–PAGE (1% SDS, 0·1 M 2-mercaptoethanol, 0·1 M Tris–HCl pH 6·8). Lysates were subjected to SDS–PAGE and Western blotting, followed by blocking with 1% BSA for 2 h at 37 °C. The membrane was then incubated for 1 h with each antibody, firstly with monoclonal antibody MAB810 specific for the HCMV IE gene product (Chemicon) and secondly with peroxidase-conjugated goat anti-mouse Ig antibody. 4-Chloronaphthol was used for the visualization of the antibody-reactive spots.

■ **Plaque inhibition assay.** HCMV was preincubated with various concentrations of GLs (0·01–10 μM) for 30 min at 37 °C and used to inoculate cell monolayers at an m.o.i. of 0·003 in 6-well tissue culture plates for 90 min. The monolayers were washed with PBS and overlaid with 0·6% agarose in MEM containing 2% FBS. Thirteen days after infection the monolayers were fixed with formalin. Plaques were stained with methylene blue and counted. Inhibition by GLs was expressed as a percentage of plaques formed by the GL-pretreated virus to those formed by virus preincubated with PBS alone. About 70 plaques were counted.

For pretreatment with the HNK-1 monoclonal antibody, cell monolayers were incubated first with various dilutions of HNK-1 antibodies or with control IgM in PBS for 1 h at 37 °C. After washing with PBS, the cells were infected with about 70 p.f.u. of AD169 per well for 90 min, followed by plaque assay. Inhibition was expressed as a percentage of plaques formed in cultures after preincubation of cells with either HNK-1 or control IgM to those formed after preincubation with PBS alone.

Results

Binding of HCMV to SGGLs and the related GLs on TLC plates

Our previous report showed that the sera of HCMV-infected patients contained elevated titres of antibodies against SGGLs, which also bound to other sulfated GLs, including CSE, with low affinity (Ogawa-Goto *et al.*, 1994). We analysed the binding reactivity of HCMV particles to these sulfated GLs by a TLC-overlay assay using ^{35}S-labelled virus. As shown in Fig. 1 (B), HCMV bound to SGPG, SGLPG and CSE, but it did not bind to GPG, a de-sulfated derivative of SGPG. The binding of HCMV to SGLPG was much stronger than that to SGPG and CSE. SGLPG gave a positive result in the TLC-overlay assay with only 40 pmoles (Fig. 1 B, lane 2). By contrast, 100 pmol SGPG or CSE gave negative results (lane 2). This unique reactivity of HCMV was very similar to that of the anti-SGGL antibodies detected in the sera of HCMV-infected patients mentioned above (Ogawa-Goto *et al.*, 1994).

We subjected various GLs to TLC-overlay assays to study the detailed features of carbohydrate structure required for

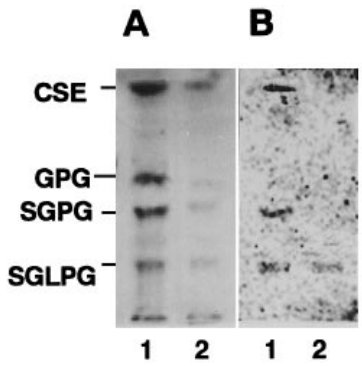

Fig. 1. Binding of HCMV to sulfated GLs separated by TLC. (A) Chemical staining with orcinol spray; (B) stained with ^{35}S-labelled HCMV as described in Methods. Lane 1, 400 pmol CSE, GPG and SGPG and 200 pmole SGLPG; lane 2, 100 pmol CSE, GPG and SGPG and 40 pmol SGLPG. HCMV bound to SGLPG, bound less strongly to SGPG and CSE, but did not bind to GPG.

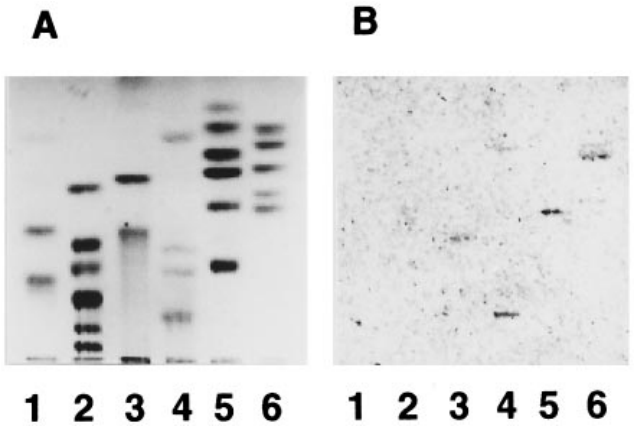

Fig. 2. Binding of HCMV to various GLs separated by TLC. (A) Chemical staining with orcinol spray; (B) stained with ^{35}S-labelled HCMV as described in Methods. Lanes: 1, gangliosides LM1, sialyl-nLc$_6$Cer and cholesterol-3-*O*-sulfate (from bottom to top); 2, gangliosides GT1b, GD1b, GD1a, GD3, GM1 and GM3 (from bottom to top); 3, nLc$_6$Cer (lower) and nLc$_4$Cer (upper); 4, SGLPG, SGPG, GPG and CSE (from bottom to top); 5, sialyl-Le^x, GA1, Gb$_4$Cer, Gb$_3$Cer, LacCer and GalCer (from bottom to top); 6, SB1a, SB2, SM2, SM3 and CSE (from bottom to top). Out of 24 different kinds of GLs and one sulfated lipid, ^{35}S-labelled HCMV bound specifically to certain GLs including SGLPG. Compared with SGLPG, the virus bound less strongly to other 3-*O*-sulfated GLs, which included CSE, SM3 and SB2 in lane 6, and faintly to a nLc$_6$Cer in lane 3, but did not bind to its sialylated derivative sialyl-nLc$_6$Cer in lane 1. GA1 also gave a positive result in lane 5. The results are summarized in Table 1.

binding of HCMV (Fig. 2). Out of 24 GLs and one sulfated lipid, ^{35}S-labelled HCMV bound to certain GLs including SGLPG (Fig. 2 B). In addition to SGLPG (lane 4), the virus bound less strongly to other 3-*O*-sulfated GLs, which included CSE, SM3 and SB2, but it did not bind to SB1a and SM2 (lane 6). Of seven neutral GLs, HCMV bound strongly to GA1, which is a 'ganglio-series' GL (lane 5). It also bound weakly to nLc$_6$Cer, but not to nLc$_4$Cer (lane 3), both of which belong to

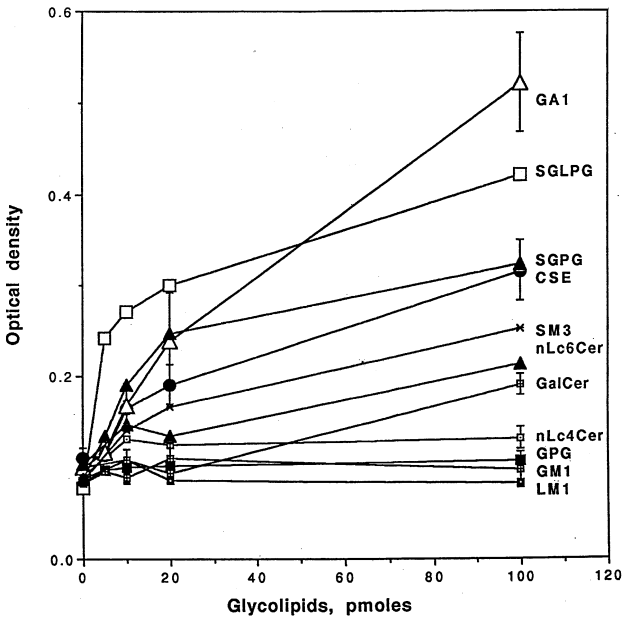

Fig. 3. Binding of HCMV to various GLs estimated by a solid-phase binding assay. GLs were immobilized on polystyrene microtitre plates at the indicated concentrations and the binding of biotinylated HCMV was measured as described in Methods. Results are means of triplicate measurements and bars represent the standard deviations, except SGLPG, SM3 and nLc_6Cer, which are means of duplicate measurements.

'neolacto-series' GLs with two units and one unit of lactosamine structures (-3Galβ1–4GlcNAc1-), respectively. All gangliosides tested gave negative results, including GM1, GD1a, GD1b, GT1b, GM3, GD3 (lane 2), LM1, sialyl-nLc_6Cer (lane 1) and sialyl-Le^x (lane 5). Cholesterol sulfate, which is a sulfated lipid without a carbohydrate moiety, gave a negative result (lane 1). Since HCMV did not bind to cholesterol sulfate, SM2 or SB1a, it appears that a sulfate residue with a specific carbohydrate structure was necessary for the binding of HCMV.

Binding of HCMV in solid-phase binding assays

By using a solid-phase binding assay we quantitatively assessed HCMV binding to the GLs, which gave positive results in the TLC-overlay assays. Fig. 3 shows the binding pattern of biotinylated HCMV to various GLs, which is in good agreement with the results of the TLC-overlay assay described above. Strong binding to SGLPG and GA1, and moderate binding to SGPG, CSE and nLc_6Cer was observed in this assay. Table 1 summarizes the sugar structures to which HCMV bound. The results suggest that 3-*O*-sulfated saccharide and repeating units of lactosamine (-3Galβ1–4GlcNAc1-)$_2$ are important structures for binding of HCMV.

The binding of HCMV to SGGLs was carbohydrate-specific and differed from that to GA1

Preincubation of immobilized SGGLs with HNK-1 antibody resulted in reduced binding of HCMV, but not with anti-GA1 antibody or control IgM (Fig. 4 A), suggesting that the binding of HCMV to SGGLs was carbohydrate-specific. A competition assay revealed that preincubation of HCMV with GA1 had no effect on the binding to SGGL (Fig. 4 B). It seems probable that SGGL and GA1 were recognized independently.

Effects of pretreatment of HCMV with GLs on expression of the IE gene

To elucidate the role of the binding of HCMV to SGGLs and other related GLs *in vitro*, we preincubated the virus with these GLs prior to inoculation, and studied expression of the IE gene by Western blotting (Fig. 5). SGLPG and SGPG had inhibitory effects on expression of the IE gene at 20 μM and CSE had an effect at 100 μM. GA1 showed only a marginal inhibitory effect at 100μM. Other GLs tested had no obvious effects in this assay.

Effects of pretreatment of HCMV with GLs on plaque formation

We assessed the inhibitory effects of SGGLs and other related GLs on HCMV infection by a plaque inhibition assay. Preincubation of HCMV with SGGLs efficiently inhibited the plaque-forming activity of HCMV in MRC-5 cells (Fig. 6). SGLPG had a fairly strong inhibitory effect: preincubation of HCMV with 1 μM SGLPG resulted in about 10% of the plaque-forming activity of the control. SGPG or CSE led to about 40–50% of the plaque-forming activity of the control at 10 μM, indicating less potent inhibitory effects than SGLPG (Fig. 6). nLc_6Cer had a weak effect, while nLc_4Cer had no obvious effect. While HCMV clearly bound to GA1 in the binding assays, GA1 showed only a marginal inhibitory effect on HCMV plaque formation (Fig. 6). Preincubation of HCMV with other GLs including LM1, GM3, GPG, GalCer and LacCer (data not shown) gave negative results. The inhibitory effects of the GLs on plaque formation by HCMV were positively correlated with their abilities to bind to HCMV as described above, except in the case of GA1 (Table 1). Cell monolayers that had been treated with 1 μM SGLPG or 10 μM SGPG or CSE prior to HCMV inoculation formed about 88–95% of plaques formed in the control (data not shown), indicating that these sulfated GLs barely affected the susceptibility of MRC-5 cells to HCMV infection.

The HNK-1 antibody partially inhibited plaque formation by HCMV

The 3-*O*-sulfated glucuronyl lactosamine structure (HSO_3-3GlcAβ1–3Galβ1–4GlcNAc-) in SGGLs is a specific carbohydrate structure recognized by the monoclonal antibody HNK-1. We tested the HNK-1 antibody for its ability to inhibit plaque formation by HCMV. Cell monolayers were incubated with purified HNK-1 antibody and washed prior to HCMV

Table 1. Structures of glycosphingolipids to which HCMV bound and their inhibitory effects on the infection

Type of GL	Name	Name in non-abbreviated form	Structure	Binding to HCMV*	Inhibition of IE synthesis†	Inhibition of plaque formation
Sulfated GL	SGPG	Sulfated glucuronylparagloboside	HSO_3-3GlcAβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1′Cer	++	++	+
	SGLPG	Sulfated glucuronyl lactosaminylparagloboside	HSO_3-3GlcAβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1′Cer	+++	++	+++
	CSE	Cerebroside sulfate ester	HSO_3-3Gaβ1–1′Cer	++	+	+
	SM3		HSO_3-3Galβ1–4Glcβ1–1′Cer	+		
	SM2		HSO_3-3GalNAcβ1–4Galβ1–4Glcβ1–1′Cer HSO_3	–		
	SB2		HSO_3-3GalNAcβ1–4Galβ1–4Glcβ1–1′Cer HSO_3	±		
	SB1a		HSO_3-3Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–1′Cer HSO_3	–		
Neutral GL	GalCer	Galactosylceramide	Galβ1–1′Cer	±	–	–
	GlcCer	Glucosylceramide	Glcβ1–1′Cer	–		
	LacCer	Lactosylceramide	Galβ1–4Glcβ1–1′Cer	–	–	–
	Gb_3Cer	Globotriaosylceramide	Galα1–4Galβ1–4Glcβ1–1′Cer	–		
	Gb_4Cer	Globotetraosylceramide	GalNAcβ1–3Galα1–4Galβ1–4Glcβ1–1′Cer	–		
	nLc_4Cer (PG)	Neolactotetraosylceramide, paragloboside	Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1′Cer	–	–	–
	nLc_6Cer (LPG)	Neolactohexaosylceramide, lactosaminylparagloboside	Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1′Cer	+	±	±
	Gg_4Cer (GA1)	Gangliotetraosylceramide, asialo GM1	Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–1′Cer	+++	±	±
Gangliosides	GM1		Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–1′Cer Neu5Ac	–	–	–
	GD1a		Neu5Acα2–3Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–1′Cer Neu5Ac	–		
	GD1b		Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–1′Cer $(Neu5Ac)_2$	–		
	GT1b		Neu5Acα2–3Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–1′Cer $(Neu5Ac)_2$	–		
	GD3		Neu5Acα2–8Neu5Acα2–3Galβ1–4Glcβ1–1′Cer	–	–	
	LM1	Sialylparagloboside	Neu5Acα2–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1′Cer	–	–	–
	Sialyl-nLc_6Cer		Neu5Acα2–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1′Cer	–		
Others	GPG	Glucuronylparagloboside	GlcAβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1′Cer	–	–	–
	Cholesterol-3-sulfate				–	–

* Binding of HCMV as estimated by a TLC-overlay assay and a solid-phase binding assay.
† Inhibition of synthesis of IE gene product as estimated by Western blotting. ++ or + represents clear inhibition at 100 μM or 20 μM, respectively, ± represents a marginal inhibition at 100 μM.

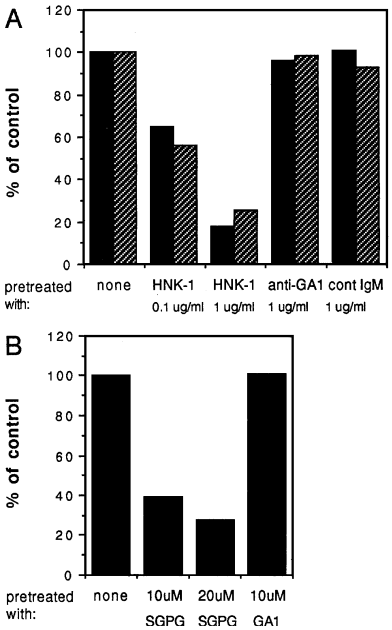

Fig. 4. Effects of HNK-1 monoclonal antibody or GA1 on the HCMV binding to SGGLs. (A) SGGLs (100 pmol) immobilized on polystyrene microtitre plates were preincubated with 1 or 0·1 μg/ml HNK-1 antibody or 1 μg/ml control IgM at 4 °C for 1 h. Wells were washed with PBS containing 1% gelatin and then the solid-phase binding assay was performed as described in Methods. Black bars, SGPG; hatched bars, SGLPG. Pretreatment with HNK-1 antibody led to decreased binding. Control IgM did not inhibit binding. Results are means of duplicate measurements. (B) After preincubation of biotinylated HCMV with indicated amounts of SGPG or GA1, the HCMV binding to SGPG was measured by a solid-phase binding assay as described in Methods. Preincubation of HCMV with GA1 did not have a distinct effect on the HCMV binding to SGPG. Results are means of duplicate measurements.

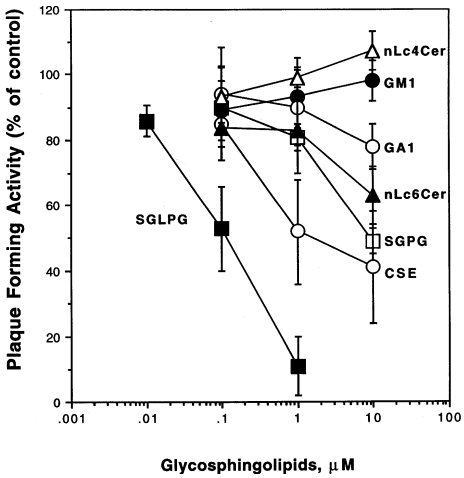

Fig. 6. Effects of pretreatment of HCMV with GLs on plaque formation. HCMV was pretreated with various concentrations of GLs and used to inoculate MRC-5 monolayer cells. The residual virus was analysed by the plaque formation assays as described in Methods. Results are means of three experiments and bars represent the standard deviations. The inhibitory effects of the GLs on plaque formation by HCMV were very well correlated with their abilities to bind to HCMV, except in the case of GA1.

inoculation. This treatment resulted in about a 40% decrease in plaque formation by HCMV (Fig. 7).

Discussion

SGGLs belong to sulfated GLs and share a unique epitope, HNK-1. The sugar structures of two major SGGLs were characterized originally from human peripheral nerves (Chou *et al.*, 1986; Ariga *et al.*, 1987); SGPG contains 3-sulfated glucuronic acid and 1 unit of lactosamine, (-3Galβ1–4GlcNAc-)$_1$, while SGLPG has 3-sulfated glucuronic acid and 2 units of lactosamine, (-3Galβ1–4GlcNAc-)$_2$. In the present paper we first demonstrate that HCMV shows a binding reactivity to SGGLs, especially to SGLPG. The binding of HCMV to SGGLs was carbohydrate-specific and the 3-sulfated glucuronic acid on the lactosamine structure was important for binding. We also demonstrated the inhibitory effects of SGGLs both on the expression of the IE gene and on plaque formation by HCMV, suggesting that the binding of HCMV to the sulfated carbohydrate epitope in SGGL plays an important role in the initial process of infection. It is of particular interest that HCMV bound more strongly to SGLPG than to SGPG and also that SGLPG had the stronger inhibitory effect on HCMV infection than SGPG. The repeating lactosamine structure (-3Galβ1–4GlcNAc1-)$_2$ of SGLPG, in addition to the 3-sulfated glucuronyl moiety, may be important for recognition by the virus. The importance of a repeating lactosamine structure is also suggested by the preferential binding of HCMV to nLc$_6$Cer rather than to nLc$_4$Cer. Studies on the potential ability

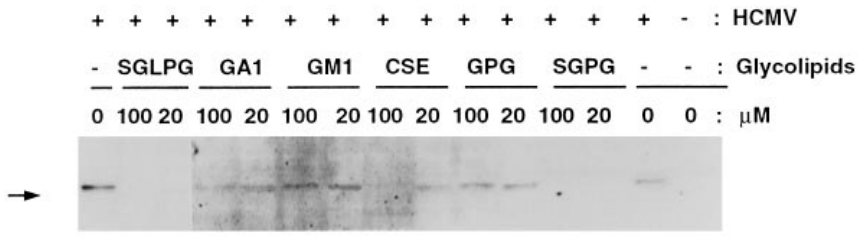

Fig. 5. Effects of pretreatment of HCMV with GLs on expression of the IE gene. HCMV was pretreated with 20 μM or 100 μM GLs and used to inoculate MRC-5 monolayer cells. At 20 h post-infection, cells were harvested and subjected to Western blotting as described in Methods.

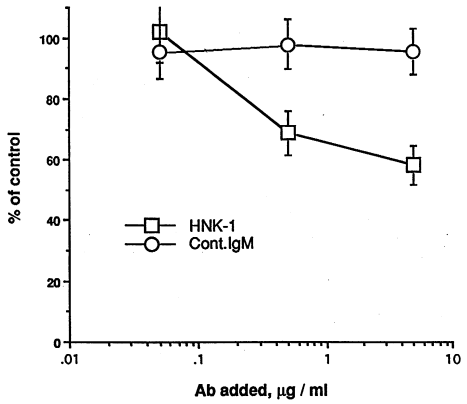

Fig. 7. Inhibitory effects of HNK-1 monoclonal antibody on HCMV plaque formation. Cells were pretreated with the HNK-1 monoclonal antibody or control IgM in PBS for 1 h at 37 °C. After washing with MEM, the cells were inoculated with HCMV and the plaque formation assay was carried out as described in Methods. Results are means of three experiments and bars represent the standard deviations.

of SGLPG or related compounds to serve as anti-HCMV agents might be useful.

The binding reactivity of HCMV to sulfated GLs demonstrated in the present study was very similar to those of anti-SGGL antibodies in the sera of HCMV-infected patients (Ogawa-Goto *et al.*, 1994). The virus bound to several 3-*O*-sulfated GLs that have been shown to be bound by the antibodies. Moreover, HCMV exhibited a higher reactivity to SGLPG than to SGPG, as in the case of the antibodies against SGGLs. Interestingly, a very analogous phenomenon has been demonstrated with respect to *Mycoplasma pneumoniae* infection, where patients develop high titre auto-antibodies (cold agglutinins) against branched poly-*N*-acetyllactosamine and its sialylated sequences. The latter has been shown to be a host cell receptor of the mycoplasma (Loomes *et al.*, 1984; Roelcke *et al.*, 1991). The mechanism by which these auto-antibodies arise in *M. pneumoniae* infection is unknown. It has been suggested that the infective agent or its adhesins serve as immunological adjuvants for the host cell carbohydrate antigens (Feizi & Loveless, 1996). We raise the possibility that the anti-SGGL antibodies in the sera of patients might have been anti-idiotypic antibodies. In other words, HCMV seems to bind to a specific carbohydrate moiety on the host cell surface via an association with a certain envelope glycoprotein, and antibodies against the envelope glycoprotein are produced. Then, anti-idiotypic antibodies are produced against these antibodies. The anti-idiotypic antibodies then recognize the specific carbohydrate moiety on the host cells. This hypothesis led us to the present finding that HCMV binds to SGGLs. To verify this hypothesis, however, further studies will be necessary, including extended and detailed analyses of antibody in human and experimental infection systems, if possible.

Besides the sulfated GLs, HCMV clearly bound to GA1 in our *in vitro* binding assays, but GA1 had only a marginal inhibitory effect on the infection under the conditions we used. The reason for the low inhibitory effect of GA1 on HCMV infection is not clear. One possibility is that HCMV may bind to GA1 less efficiently in the micellar solution because of the different oligosaccharide conformation from the solid-phase assay. Further experiments using GA1-containing liposome should be done to evaluate the biological significance of the binding. The inhibitory effects of GLs on the HCMV infection were mostly positively correlated with the results from binding assays. Gangliosides, which are sialic acid-containing GLs, neither bound to HCMV nor inhibited infection. It was reported that sialyllactose (Neu5Acα2–3Galβ1–4Glc) had a weak inhibitory activity on HCMV infection (Lobert *et al.*, 1995). The reason for this discrepancy is unclear, but it might be due to the difference in sugar derivatives, namely, an oligosaccharide and a GL. On the other hand, partial inhibitory activity of *N*-acetyllactosamine was also reported (Lobert *et al.*, 1995), which is consistent with our results.

The HNK-1 epitope is present both in GLs and in glycoproteins (Jungalwala, 1994; Schachner, 1989). A number of glycoproteins known as neural adhesion molecules have this epitope. They include myelin-associated glycoprotein (MAG), neural cell adhesion molecule (N-CAM) and PO glycoprotein (Schachner, 1989). Certain proteoglycans also carry this epitope on their core proteins (Margolis *et al.*, 1987; Krueger *et al.*, 1992). Therefore, it is not clear which type of glycoconjugate carrying the HNK-1 epitope is utilized by HCMV as an attachment molecule on the surface of the host cell. While HNK-1 antigen was detected on MRC-5 cells, preliminary study showed that the MRC-5 cells contained no detectable amounts of SGGLs in a TLC-immunostaining study (unpublished observation). Other types of glycoconjugate carrying the HNK-1 epitope rather than SGGLs seem to be involved in the initial process of HCMV infection at least in the human lung fibroblasts. We are now searching for such cell surface molecules on HCMV permissive cells. Our preliminary results from Western blotting analysis indicate the presence of such glycoproteins in MRC-5 cells (unpublished observation).

Heparan sulfate proteoglycans on the cell surface have been shown to serve as the initial attachment molecules of HCMV, as is the case for other herpesviruses including herpes simplex virus (WuDunn & Spear, 1989; Lycke *et al.*, 1991). The involvement of envelope glycoproteins gB and gCII has been reported for this process of HCMV (Kari & Gehrz, 1992; Compton *et al.*, 1993). Further studies are now required to identify the viral envelope glycoproteins which are responsible for binding to the HNK-1 epitope. Heparan sulfate chains are heterogeneous, varying in sites of *N*-acetylation, *N*-sulfation, *O*-sulfation and the epimerization of glucuronic acid to iduronic acid (Yanagishita & Hascall, 1992). Unique sugar sequences in the highly sulfated domains of heparan sulfate have been shown to be involved in specific interactions with some growth factors (Turnball *et al.*, 1992; Ishihara *et al.*, 1994). A pentasaccharide sequence including a novel 3-*O*-sulfate group has been reported to be responsible for the high-affinity

binding of heparin to antithrombin III (Oscarsson *et al*., 1989). It has been reported that a 3-*O*-sulfated glucuronic acid-containing oligosaccharide has been detected in human fibroblasts after addition of a sugar primer specific for synthesis of glycosaminoglycan chains (Shibata *et al*., 1995), suggesting that human fibroblasts can potentially synthesize a novel 3-*O*-sulfated glucuronic acid in their glycosaminoglycans. A unique sugar sequence might be present in the heparan sulfate of the host cell, which is closely related to and cross-reacts with the HNK-1 epitope. Therefore, the HNK-1 or closely related epitope can exist in the heparan sulfate chains of human fibroblasts, and could possibly be involved in the initial process of HCMV infection. An extensive study aiming at the identification of such molecules should result in a better understanding of the multistep process of HCMV infection.

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