Sulphatide binds to human and animal influenza A viruses, and inhibits the viral infection

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We found, by using a virus overlay assay, that influenza A virus isolates bind to sulphatide ($\text{HSO}_3\text{-Gal}\beta 1 \rightarrow 1'\text{Cer}$), which has no sialic acid residue, and that the infection of Madin–Darby canine kidney cells with the human influenza virus A/Memphis/1/71 (H3N2) is inhibited by sulphatide. A/Memphis/1/71 (H3N2) causes obvious haemagglutination and low-pH haemolysis of asialoerythrocytes reconstituted with sulphatide. All influenza A virus isolates from the species of animals so far tested bound to

sulphatide. The sulphatide-binding specificity of the isolates was different from the viral sialyl-linkage specificity. Influenza A virus isolates also bound to galactosyl ceramide (GalCer; $\text{Gal}\beta 1 \rightarrow 1'\text{Cer}$), as well as sulphatide, in the virus overlay assays. In contrast, the influenza virus did not bind to *N*-deacyl, a derivative of sulphatide, glucosyl ceramide or the other neutral glycolipids tested. These results indicate that the linkage of galactose, or sulphated galactose, to ceramide is important for viral binding.

INTRODUCTION

Influenza A viruses infect many animal species including birds, pigs, horses and humans. The binding specificity of the influenza A virus for a host cell receptor is an important factor in the range of hosts and tissue tropism of the virus. The interaction between the influenza A virus and the host cells is mediated by a viral spike glycoprotein, a haemagglutinin that recognizes glycoproteins and glycolipids containing terminal sialic acid residues [1–5]. Recent studies have shown that cell-surface non-sialyl glycolipids might also play a critical role as receptors for several viruses [6–8], bacteria [9,10], and cell adhesion molecules [11]. Asialo GM1 (Gg_2Cer ; $\text{Gd}\beta 1 \rightarrow 3\text{GalNAc}\beta 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$) mediates the binding of rotavirus to polarized epithelial cells [6]. Galactosyl ceramide (GalCer; $\text{Gal}\beta 1 \rightarrow 1'\text{Cer}$) has been identified as a potential alternative receptor for HIV-1 [7]. In addition, human parvovirus B19 was shown to bind to blood-group P antigen [globoside (Gb_4Cer ; $\text{GalNAc}\beta 1 \rightarrow 3\text{Gal}\alpha 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$)] [8].

We show here that sulphatide ($\text{HSO}_3\text{-Gal}\beta 1 \rightarrow 1'\text{Cer}$) binds to influenza A viruses and inhibits viral infection.

EXPERIMENTAL

Glycolipids

All naturally occurring glycolipids were prepared as described previously [5,11–14]. Sulphatide, GalCer and $\text{IV}^3\text{-NeuAc}$, $\text{II}^3\text{NeuAc}\alpha\text{-Gg}_4\text{Cer}$ [GD1a; $\text{NeuAc}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 3\text{GalNAc}\beta 1 \rightarrow 4(\text{NeuAc}\alpha 2 \rightarrow 3)\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$] were prepared from bovine brain; glucosyl ceramide (GlcCer ; $\text{Glc}\beta 1$

$\rightarrow 1'\text{Cer}$), trihexosyl ceramide (Gb_3Cer ; $\text{Gal}\alpha 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$) and Gb_4Cer were prepared from pig erythrocytes; lactosyl ceramide (LacCer ; $\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$) was prepared from bovine buttermilk; $\text{II}^3\text{NeuGc}\alpha\text{-LacCer}$ ($\text{NeuGc}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$) was prepared from equine erythrocytes; and paragloboside (nLc_4Cer ; $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$) was prepared from $\text{IV}^3\text{NeuGc}\alpha\text{-nLc}_4\text{Cer}$ ($\text{NeuGc}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$) by sialidase treatment. Synthetic $\text{IV}^3\text{NeuAc}\alpha\text{-Lc}_4\text{Cer}$ ($\text{NeuAc}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$) and $\text{IV}^6\text{NeuAc}\alpha\text{-Lc}_4\text{Cer}$ ($\text{NeuAc}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$), containing the lacto-series type I chain, were prepared as described previously [15,16]. Lysosulphatide ($\text{HSO}_3\text{-Gal}\beta 1 \rightarrow 1'\text{LysoCer}$) was purchased from Sigma.

Influenza A viruses and cells

Influenza A virus isolates were grown in the allantoic sac of 10-day-old embryonated eggs and were purified by sucrose-density-gradient centrifugation. Viral haemagglutination units (HAU) were determined at 4 °C as described previously [2]. Madin–Darby canine kidney (MDCK) cell monolayers were propagated in Eagle's minimum essential medium (EMEM) containing 10 % fetal calf serum.

Anti-(influenza virus) antibodies

Rabbit anti-(influenza virus) antibodies were raised against whole A/PR/8/34 (H1N1), Eq/Miami/1/63 (H3N8), A/Aichi/2/68 (H3N2), A/Memphis/1/71 (H3N2) and A/Mal/Alb/7/87 (H8N4) viruses grown in eggs, as described previously [5]. Dr.

Abbreviations used: CPE, cytopathic effects; EMEM, Eagle's minimum essential medium; HAU, haemagglutination units; sulphatide, $\text{HSO}_3\text{-Gal}\beta 1 \rightarrow 1'\text{Cer}$; lysosulphatide, $\text{HSO}_3\text{-Gal}\beta 1 \rightarrow 1'\text{LysoCer}$; galactosyl ceramide (GalCer), $\text{Gal}\beta 1 \rightarrow 1'\text{Cer}$; glucosyl ceramide (GlcCer), $\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$; lactosyl ceramide (LacCer), $\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$; trihexosyl ceramide (Gb_3Cer), $\text{Gal}\alpha 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$; globoside (Gb_4Cer), $\text{GalNAc}\beta 1 \rightarrow 3\text{Gal}\alpha 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$; paragloboside (nLc_4Cer), $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$; $\text{II}^3\text{NeuGc}\alpha\text{-LacCer}$, $\text{NeuGc}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$; $\text{IV}^3\text{-NeuAc}$, $\text{II}^3\text{NeuAc}\alpha\text{-Gg}_4\text{Cer}$ (GD1a), $\text{NeuAc}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 3\text{GalNAc}\beta 1 \rightarrow 4(\text{NeuAc}\alpha 2 \rightarrow 3)\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$; $\text{IV}^6\text{NeuAc}\alpha\text{-Lc}_4\text{Cer}$, $\text{NeuAc}\alpha 2 \rightarrow 6\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$; $\text{IV}^3\text{NeuAc}\alpha\text{-Lc}_4\text{Cer}$, $\text{NeuAc}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1'\text{Cer}$; LDH, lactate dehydrogenase; MDCK, Madin–Darby canine kidney; TCID_{50} , 50 % tissue-culture infectious dose.

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Figure 1 Binding of influenza virus A/Memphis/1/71 (H3N2) to glycolipids

(A) Each glycolipid (1 nmol) was layered on to a silica gel plate (50 mm × 100 mm). The plate was incubated with 2^8 HAU of A/Memphis/1/71 (H3N2) at 4 °C for 12 h and then revealed by the immunostaining method described in the Experimental section. (B) Each glycolipid was revealed by using an orcinol/HCl reagent sprayed on to the plate. (C) A plate was incubated without the virus and served as the negative control. Lane 1, sulphatide; lane 2, lysosulphatide; lane 3, GalCer; lane 4, GlcCer; lane 5, LacCer; lane 6, Gb₃Cer; lane 7, Gb₄Cer; lane 8, nLc₄Cer; lane 9, II³NeuGcα-LacCer; lane 10, GD1a; lane 11, IV⁶NeuAcα-Lc₄Cer; lane 12, IV³NeuAcα-Lc₄Cer.

Robert G. Webster (St. Jude Children's Research Hospital) provided anti-HA monoclonal antibodies for the detection of the following viruses: A/Teal/Alb/69/87 (H1N4), A/Mal/Alb/25/85 (H4N6), A/Eq/Prag/56 (H7N7), A/Eq/Lon/1416/73 (H7N7), A/Eq/Font/1/79 (H3N8), or A/Eq/Tenn/5/86 (H3N8), and anti-NA monoclonal antibodies for the detection of A/Mal/24/92 (H11N9).

The virus overlay assay

Silica gel plastic plates (Polygram Sil G; Macherey-Nagel, Germany) were developed with glycolipids (1 nmol) by using a solvent containing chloroform, methanol and 12 mM aqueous MgCl₂ (5:4:1, by vol.). Immunochemical detection of virions on these thin-layer plates was performed as described previously [5,17]. Briefly, the chromatography plates were incubated with an influenza A viral suspension (2^8 HAU, 9 µg/ml viral protein) for 12 h at 4 °C. As a negative control some plates were incubated without virus. After being washed five times with PBS, pH 7.2, containing 131 mM NaCl, 14 mM Na₂HPO₄, 1.5 mM KH₂PO₄ and 2.7 mM KCl, the plates were incubated at 4 °C for 2 h with each anti-(influenza virus) antibody diluted 1:1000 with PBS containing 1% (w/v) egg albumin and 1% polyvinylpyrrolidone (solution A), and then they were reacted with the horseradish peroxidase-conjugated F(ab')₂ fragment of goat anti-(mouse IgG + IgM) (Jackson ImmunoResearch Laboratories) for the anti-(influenza virus) monoclonal antibodies, or horseradish peroxidase-conjugated Protein A (Organon Teknika N.V.-Cappel Products) for the rabbit anti-(influenza virus) antibodies, both diluted 1:1000 with solution A at 4 °C for 2 h. After this the plates were washed five times with PBS, and the viruses bound to the plates were revealed by incubation with an immunostaining reagent [18] containing *N,N*-diethylphenylenediamine monohydrochloride and 4-chloro-1-naphthol.

The binding reactivity of the viruses to each glycolipid was determined by scanning the stained chromatogram at 620 nm (reference 430 nm). As a control, the developed glycolipids were revealed by spraying the plate with orcinol/H₂SO₄ reagent. Virus overlay assays were performed in duplicate.

Neutralization of the influenza virus by glycolipids

MDCK cell monolayers were inoculated with EMEM containing 0.2% BSA and approx. 100 TCID₅₀ (50% tissue-culture infectious dose) of A/Memphis/1/71 in the presence of glycolipids (0.05–500 µg/ml) at 34.5 °C for 5 h. After removal of the

inoculum, the monolayers were washed three times with EMEM containing 0.2% BSA and incubated in 100 µl of the medium. The cell cultures were examined by light microscopy for the progression of viral-induced cytopathic effects (CPE) after incubation at 34.5 °C for 20 h. As a control, to examine viral-induced CPE, the cells were inoculated with the virus in the presence of the viral antibodies diluted 1:500 with EMEM. The activity of lactate dehydrogenase (LDH) released from MDCK cells was used as a measure for virus neutralization, and it was determined by a slightly modified colorimetric assay previously described [19,20]. The monolayers were inoculated with A/Memphis/1/71 (100 TCID₅₀) in the presence of increasing concentrations (0.05–500 µg/ml) of sulphatide, GD1a and II³NeuGcα-LacCer at 34.5 °C for 5 h. The inoculum was removed and the monolayers were washed three times with EMEM containing 0.2% BSA and incubated in 100 µl of the medium. After incubation at 34.5 °C for 20 h, LDH activity in the medium was determined as described previously [20]. The medium (12.5 µl) was diluted 1:4 with 100 mM Tris/HCl, pH 8.2, and mixed with 50 µl of a reagent containing 2 mM NAD⁺, 200 m-units/ml diaphorase, 190 mM lithium lactate, 0.78 mM Nitro Blue Tetrazolium in 100 mM Tris/HCl, pH 8.2. The mixture was incubated at 37 °C for 10 min and the reaction was stopped by the addition of 100 µl of 0.5 M HCl. Absorbance was measured at 550 nm (reference 630 nm). The assays were performed in duplicate.

Biological assay of the responsiveness of glycolipid-coated erythrocytes to influenza virus

To measure the biological responsiveness of glycolipids to the influenza virus, asialoerythrocytes were prepared by treating chicken erythrocytes with *Arthrobacter ureafaciens* sialidase and coating them with glycolipids (0.25–2 nmol per 10⁶ cells) at 37 °C for 30 min as described previously [2,3,21,22]. After being washed with cold PBS by centrifugation at 500 *g* for 10 min at 4 °C, 100 µl of 0.1% glycolipid-coated asialoerythrocytes (1 nmol per 10⁶ cells) were mixed with 10 µl of A/Memphis/1/71 (2^{12} HAU) in the same buffer, and shaken at 4 °C for 5 min. Cell agglutination was monitored by observation with a microscope. The cells incubated without any virus served as the negative control. To estimate virus-mediated haemolysis, 1.0 ml of 2% glycolipid-coated asialoerythrocytes was mixed with 100 µl of a PBS suspension containing A/Memphis/1/71 (2^{10} HAU), and this was kept at 4 °C for 10 min. After washing with cold saline solution (0.9% NaCl), the cells were suspended with 20 mM

acetate-buffered saline, pH 5.2, and virus-mediated haemolysis was determined by measuring the absorbance at 540 nm as described previously [2,3,21,22]. The percentage of lysed asialoerythrocytes is expressed relative to the number of intact cells. Haemolysis of cells incubated without any virus served as the negative control. The assays were performed in duplicate.

RESULTS AND DISCUSSION

Initially we examined the binding reaction between various glycolipids and the human influenza virus A/Memphis/1/71 (H3N2) by using virus overlay assays as described previously [5,23,24]. Virus bound to the plate was detected by immuno-

staining with specific rabbit anti-(influenza virus) antibodies. As shown in Figure 1, A/Memphis/1/71 (H3N2) bound most effectively to a lacto-series ganglioside, IV⁶NeuAc α -Lc₄Cer, which contains a terminal *N*-acetylneuraminic acid (NeuAc) α 2 $\rightarrow$ 6-Gal linkage, followed by IV³NeuAc α -Lc₄Cer, which contains a terminal NeuAc α 2 $\rightarrow$ 3Gal linkage. We found that the virus bound to sulphatide and GalCer as well as GD1a, but not to lysosulphatide, GlcCer, Gb₃Cer, nLc₄Cer, Gb₄Cer or II³NeuGc α -LacCer containing a terminal *N*-glycolylneuraminic acid. In addition the virus bound to LacCer; however, the binding reaction was feeble compared with sulphatide or GalCer.

To verify the ability of sulphatide to neutralize viral infectivity, monolayers of MDCK cells were inoculated with A/Memphis/1/71 (H3N2), which has similar binding specificity to some human strains for sialo-sugar chains [3,5,25], in the presence of increasing concentrations of sulphatide, GD1a and II³NeuGc α -LacCer at 34.5 °C for 5 h. The inoculum was removed and the monolayers were incubated in EMEM for 20 h. Neutralization of A/Memphis/1/71 by sulphatide was assessed by the reduced number of viral-induced CPE relative to the controls inoculated in the absence of the glycolipid. As shown in Figure 2 (upper panel), the appearance of viral-induced CPE in the cells was distinctly inhibited by sulphatide, relative to the control. Additionally, the appearance of viral-induced CPE was inhibited by GD1a as well as sulphatide, whereas II³NeuGc α -LacCer had no effect on viral-induced CPE (results not shown).

After inoculation in the presence of sulphatide, GD1a and II³NeuGc α -LacCer, LDH activity released from the cells was examined to estimate the cytotoxicity of the virus, as previously described [19]. The activity of the enzyme was markedly reduced after viral inoculation in the presence of either sulphatide or GD1a relative to II³NeuGc α -LacCer, which has no A/Memphis/1/71 (H3N2) binding ability in the virus overlay assay (Figure 2, lower panel). The dose of sulphatide used did not affect the stability of the cells or the LDH assay (results not shown). In contrast with sulphatide, GalCer, which has a binding potential similar to sulphatide, inhibits viral infection to a lesser degree (results not shown). This result might be related to the fact that GalCer, which is a neutral glycolipid consisting of a hydrophobic ceramide and a galactose residue, has a low solubility in aqueous solution compared with acidic glycolipids such as sulphatide or gangliosides.

We examined whether asialoerythrocytes coated with exogenous sulphatide, II³NeuGc α -LacCer or IV⁶NeuAc α -Lc₄Cer were biologically responsive to A/Memphis/1/71 (H3N2), as established by agglutination at 4 °C and haemolysis at low pH at 37 °C, as well as the responsiveness of asialoerythrocytes coated with gangliosides as described previously [2,3]. As shown in Figure 3, the asialoerythrocytes coated with sulphatide (Figure 3E) were agglutinated by A/Memphis/1/71 (H3N2) at 4 °C at pH 7.2, as were the asialoerythrocytes coated with IV⁶NeuAc α -Lc₄Cer (Figure 3C). Conversely, the asialoerythrocytes coated with II³NeuGc α -LacCer, which had no A/Memphis/1/71 (H3N2) binding ability in the virus overlay assay, did not react with the isolate (Figure 3D). In addition, the degree of asialoerythrocyte haemolysis at 37 °C at pH 5.2 increased in proportion to the sulphatide concentration as shown in Figure 4. It has been shown that human strains preferentially recognize the terminal NeuAc α 2 $\rightarrow$ 6Gal linkage of sialo-sugar chains, whereas avian and equine strains preferentially recognize terminal NeuAc α 2 $\rightarrow$ 3Gal sequences [26–28]. We examined the binding reactions between sulphatide and human, avian and equine influenza virus isolates by a virus overlay assay. The binding activities of sulphatide to human, equine and avian strains were compared with the activity of the gangliosides IV³NeuAc α -Lc₄Cer and

Figure 2 Neutralization of A/Memphis/1/71 (H3N2) infection by sulphatide

Upper panel: MDCK cells were inoculated with A/Memphis/1/71 (100 TCID₅₀) in the presence of 500 µg/ml sulphatide (a) or anti-influenza A virus antibodies (b). After removal of the inoculum and incubation in EMEM at 34.5 °C for 20 h, the cell cultures were examined under the light microscope for the progression of viral-induced CPE in the cells relative to the controls inoculated in the absence of the glycolipid (c); (d) mock-inoculated cells. Lower panel: MDCK cells were inoculated with A/Memphis/1/71 (100 TCID₅₀) in the presence of increasing concentrations (0.05–500 µg/ml) of sulphatide (○), GD1a (△) or II³NeuGc α -LacCer (●) at 34.5 °C for 5 h. Neutralization was assessed by the decrease in LDH activity released from the cells after incubation as described above. The results show means $\pm$ S.D. for duplicate experiments.

Figure 3 Agglutination of sulphatide-coated asialoerythrocytes by A/Memphis/1/71 (H3N2)

Sulphatide or gangliosides (1 nmol) were incorporated into chicken asialoerythrocytes (10^6 cells) by incubation at 37 °C for 30 min as described in the Experimental section. After being washed with cold PBS by centrifugation, a suspension of 0.1% glycolipid-coated asialoerythrocytes in PBS (100 μ l) was mixed with 10 μ l of A/Memphis/1/71 (2^{12} HAU) in the same buffer and shaken at 4 °C for 5 min (A–E). Cells incubated without any virus served as the negative control (F–J). Cell agglutination was observed by phase contrast microscopy. The agglutination of intact erythrocytes (A, F), asialoerythrocytes (B, G) and asialoerythrocytes coated with IV⁶NeuAc α -Lc₄Cer (C, H), II³NeuGc α -LacCer (D, I) or sulphatide (E, J).

Figure 4 Restoration of A/Memphis/1/71 (H3N2)-mediated haemolysis of asialoerythrocytes coated with sulphatide

Sulphatide, IV⁶NeuAc α -Lc₄Cer and II³NeuGc α -LacCer (0.25–2 nmol) were incorporated into chicken asialoerythrocytes (10^6 cells) as described in the Experimental section. Suspensions of 2% asialoerythrocytes coated with each glycolipid (1.0 ml) were mixed with 100 μ l of PBS suspension containing A/Memphis/1/71 (2^{10} HAU) and kept at 4 °C for 10 min. After being washed with cold saline, the cells were suspended with low-pH saline (pH 5.2) and virus-mediated haemolysis was determined by measuring the absorbance at 540 nm. Haemolysis of the cells incubated without virus served as the negative control. Haemolysis of the asialoerythrocytes coated with IV⁶NeuAc α -Lc₄Cer (Δ), II³NeuGc α -LacCer ($\bullet$) and sulphatide ($\circ$) is expressed as a percentage relative to untreated erythrocytes. The results show means $\pm$ S.D. for duplicate experiments.

IV⁶NeuAc α -Lc₄Cer, each of which contains a sialyl-sugar chain, which is preferentially recognized by the influenza virus. As shown in Table 1, all the isolates tested from human and animal species bound to sulphatide. The binding specificity of the isolates for sulphatide was different from their viral sialyl-linkage specificity.

In this study we have demonstrated that influenza A virus isolates bind to sulphatide, and also that asialoerythrocytes coated with sulphatide underwent virus-mediated agglutination and haemolysis. We found that GalCer also has influenza A virus

Table 1 Binding reactivity of human and animal strains of influenza virus to sulphatide and gangliosides

Each glycolipid (1 nmol) on a silica gel plate was examined for its ability to bind human and animal influenza virus strains by a virus overlay assay. The binding reactivity of the virus to the glycolipids was determined by scanning the positive spots with a dual-wavelength chromatoscanner. The percentage binding is expressed relative to the ganglioside (IV³NeuAc α -Lc₄Cer or IV⁶NeuAc α -Lc₄Cer) that is preferentially recognized by each strain. The results show the means $\pm$ S.D. for duplicate experiments.

Influenza virus	Relative binding activity (%)		
	IV ⁶ NeuAc-Lc ₄ Cer	IV ³ NeuAc-Lc ₄ Cer	Sulphatide
Human isolates			
A/PR/8/34 (H1N1)	18 $\pm$ 5	100 $\pm$ 3	22 $\pm$ 8
A/Aichi/2/68 (H3N2)	100 $\pm$ 7	21 $\pm$ 4	18 $\pm$ 3
A/Memphis/1/71 (H3N2)	100 $\pm$ 4	26 $\pm$ 2	20 $\pm$ 6
Avian isolates			
A/Mal/Alb/25/85 (H4N6)	28 $\pm$ 7	100 $\pm$ 2	26 $\pm$ 7
A/Teal/Alb/68/87 (H1N4)	64 $\pm$ 3	100 $\pm$ 6	28 $\pm$ 6
A/Mal/Alb/7/87 (H8N4)	2 $\pm$ 1	100 $\pm$ 8	18 $\pm$ 4
A/Mal/Alb/24/92 (H11N9)	9 $\pm$ 5	100 $\pm$ 3	8 $\pm$ 5
Equine isolates			
A/Eq/Prague/56 (H7N7)	2 $\pm$ 1	100 $\pm$ 4	4 $\pm$ 3
A/Eq/Miami/1/63 (H3N8)	13 $\pm$ 6	100 $\pm$ 7	26 $\pm$ 7
A/Eq/Lon/1416/73 (H7N7)	45 $\pm$ 7	100 $\pm$ 5	4 $\pm$ 2
A/Eq/Font/1/79 (H3N8)	14 $\pm$ 5	100 $\pm$ 8	20 $\pm$ 6
A/Eq/Tenn/5/86 (H3N8)	4 $\pm$ 3	100 $\pm$ 2	8 $\pm$ 3

binding activity, as shown by the virus overlay assay (Figure 1). GalCer, however, does not inhibit the virus to the same degree as sulphatide (results not shown). The solubility of GalCer in aqueous solution is lower than acidic glycolipids such as sulphatide (which consists of a hydrophobic ceramide and an ionic sulphogalactose residue) or gangliosides (which contain sialic acid), and neutral glycolipids (which contain long sugar

chains). Insoluble aggregates of GalCer in the medium do not react with the virus efficiently, and asialoerythrocytes are not coated with GalCer sufficiently because of its low solubility.

In contrast, *N*-deacyl, a sulphatide derivative (lysosulphatide), and GlcCer, containing glucose, an epimer of galactose at the C-4 position, have no virus-binding ability. Additionally, ceramides and their derivatives, such as *N*-stearoyl-D-sphingosine 1-sulphate, also lack the ability to bind the virus (results not shown). These results suggest that galactose or sulphated galactose linked to ceramide is important in viral binding. It has been reported that the main receptor for human immunodeficiency virus type 1 (HIV-1) is the CD4 molecule of lymphoid cells, whereas recombinant gp120 from HIV-1 specifically binds to galactosylceramide or molecules such as sulphatide that are derived from it [7]. In addition, galactosylceramide has been identified as a potential alternative receptor that enables HIV-1 to enter CD4-negative cells [29,30]. Conversely, sulphatide induced virus binding and virus-mediated fusion of chicken erythrocyte membranes from which the sialic acid had been removed by the receptor-destroying enzyme (sialidase). Our results indicate that sulphatide is a potential candidate for a secondary receptor of influenza A virus.

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