Arch Virol (2001) 146: 2227–2238

Archives of Virology

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Printed in Austria

Entry of influenza virus into a glycosphingolipid-deficient mouse skin fibroblast cell line

Brief Report

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Accepted June 13, 2001

Summary. A glycosphingolipid (GSL)-deficient mouse skin fibroblast mutant cell line (GM95) was tested for its susceptibility to influenza virus infection and/or fusion. Octadecyl rhodamine labeled influenza virus fused at 37 °C and low pH with GM95 cells at similar rates and extents as with the parental cell lines which did bear glycosphingolipids. Influenza virus infected the GM95 cells at the same level as the parental cell lines. The infection and fusion was blocked when the cell lines were pre-treated with neuramindase. We conclude that influenza virus uses mainly sialoglycoproteins and that gangliosides are not essential for influenza virus fusion and infection.

*

Abbreviations

D-0	Dulbecco's modified Eagles medium with 4.5 g glucose per liter, without L-glutamine
D-I	Dulbecco's modified Eagle's medium with 4.5 g/l D-glucose, 4 mM L-glutamine, Penicillin G 100 u/ml, Streptomycin 100 µg/ml, 10% heat inactivated fetal bovine Serum, without Na Pyruvate.
D-II	Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 1.0 mM sodium pyruvate, Penicillin G100 u/ml, Streptomycin, 100 µg/ml, 10% HI fetal bovine serum,
PBS	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 pH 7.4
TLC	Thin Layer Chromatography
GSL	Glycosphingolipids
R18	Octadecyl rhodamine
R18-fdq	Octadecyl rhodamine fluorescence dequenching

Cell dissociation buffer	PBS-based non-enzymatic buffer
influenza HA	influenza hemagglutinin
FBS	fetal bovine serum
PPMP	d,1-threo-(1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol)
Nase	neuraminidase
GlcCer	glucocerebroside
GM_3	monosialoganglioside

Influenza virus enters cells by receptor-mediated endocytosis followed by low pH-triggered fusion between viral and endosomal membranes [14, 17]. N-acetyl neuraminic acid (sialic acid), the terminal component of oligosaccharide chains on cell-surface glycoproteins and acidic glycosphingolipids (also termed as gangliosides) is known to be an essential component of cellular receptors for influenza viruses [22, 28, 33, 34]. The role of target membrane sialic acid is based on the following observations: (i) pretreatment of target cells with the enzyme neuraminidase, which cleaves sialic acids from oligosaccharide chains of glycosphingolipids (GSL) and glycoproteins, inhibits influenza virus infection and fusion [2, 29, 30] (ii) addition of sialic acids to desialylated proteins on the target cells via sialyl transferase recovers fusion activity [5], (iii) addition of gangliosides to neuraminidase-treated erythrocytes recovers fusion [29], (iv) liposomes containing structurally specific gangliosides serve as suitable targets for low-pH triggered influenza virus fusion [9, 12, 26] and (v) biophysical studies show that sialic acid and its analogs bind with influenza hemagglutinin via specific interactions [23, 32].

Binding of various influenza virus strains is determined by oligosaccharide chains of sialoglycosphingolipids [28]. However, a direct contribution of gangliosides (sialic acids and their lipid counter parts) in influenza HA-mediated fusion and/or conformational changes preceding fusion remains to be investigated. Previous reports show that the efficiency of influenza virus fusion is significantly higher when a sialoglycoprotein, rather than a sialoglycolipid incorporated into liposomes [12]. Studies from this laboratory have shown that a glycoprotein complex in the erythrocyte membrane is preferred site of influenza fusion based on photolabeling of HA2 [18].

Although presence of sialoglycoproteins in the target membrane may lead to efficient entry, the question remains whether the presence of sialoglycosphingolipids in biological membranes is essential for influenza-HA mediated fusion. To resolve this issue, we have examined the susceptibility a GSL-deficient mouse melanoma skin fibroblast cell line, GM95 [11], to influenza virus X:31 ((A/Aichi/68, H3N2) fusion and infection. GM95 cells are derived from GSL^+ MEB4 cells (a mutant of B 16 cell line) and have a primary defect in the enzyme ceramide glucosyl transferase (EC 2.4.1.80) which is required for the synthesis of glucosyl ceramide, the precursor for over 300 different GSL species in animal cells.

GM95 and MEB4 cells were obtained from Riken Cell bank (Tsukuba Science City, Japan), and were cultured in DMEM supplemented with 10% serum + antibiotics (D-I)[1] [11]. B16-F1 and B16-F10 cells (ATCC, Manassas, VA) were maintained in DMEM supplemented with antibiotics and 10% serum (D-II)[1]. Since GSL-deficient GM95 are derived from GSL^{+}MEB4 cell line which is a clone of mouse melanoma skin fibroblast B16 cells, we have employed MEB4 an B16 cells clone F1 (B16F1) and clone F10 (B16F10) with low and high metastatic potential respectively as controls in our study. The source, propagation, and purification of influenza virus strains used in this study are as described previously [20]. The absence of GSLs in GM95 cells was re-confirmed by routine lipid analysis of the cells [10]. Total cellular lipids were extracted essentially as described [3]. Small aliquots of lipid extracts were separated on duplicate Silica Gel thin layer chromatography (TLC) – see Fig. 1 – and the presence of GSLs was identified by staining with Bial's reagent [13] which reacts with all types of GSLs (Fig. 1A). The development of purple-colored product was used to distinguish GSL from other lipids, which showed yellow/brown spots in the mixture. To further differentiate GSL-positive spots from phospholipids which are bulk constituents of the cell membrane, a second TLC plate was sprayed with Molybdenum blue spray [6] which reacts with the phosphate group of phospholipids to produce a distinct blue color (Fig. 1B). It is clear from the TLC analysis that GM95 cells do not express any GSLs (Fig. 1A) as reported earlier [7]. MEB4 cells showed the presence of glucosylceramide (GlcCer) and GM3 as indicated by arrow, consistent with previously published observations [7]. Depletion of GSL from the PPMP treated cells (see below) was assessed by TLC analysis of the GSLs in the total lipid extracts (Fig. 1C). Quantitation of GlcCer and GM3 before and after PPMP treatment was done as described [10]. GSL levels were significantly reduced (50–70%) under the experimental conditions used. B16-F1 and B16-F10 cells also showed similar GSL reduction following PPMP treatment (data not shown).

We tested susceptibility of the GM95 and parental cell lines to influenza virus infection by the ability of cell surface expressed influenza-HA to bind with calcein-labeled human erythrocytes [21]. The cells were plated on 24-well clusters overnight, incubated with an appropriate dilution of influenza X:31 virus and the infection was continued for 20–24 h at 37 °C. At the end of incubations, the medium was removed, the cells were washed with PBS and incubated with 1 ml calcein-labeled erythrocytes (see legend to Fig. 2) at room temperature for 20–30 min. Unbound erythrocytes were removed by washing with PBS. Attachment of erythrocytes to influenza-infected cells was examined by fluorescence microscopy (Fig. 2). Phase and fluorescence images were acquired using a cooled CCD camera attached to Olympus microscope using an 20 X-0.4NA objective and Metamorph software (Universal Imaging, Inc.) [10]. It is evident from the micrographs that calcein-labeled erythrocytes bound to influenza X:31 virus-infected GM95 cells. The results were reproducible from at least three independent experiments. Uninfected cells did not show any erythrocyte binding under similar conditions (data not shown). GM95 cells were infected by influenza virus with similar efficiency to that of the parent MEB4 and B16 cells when

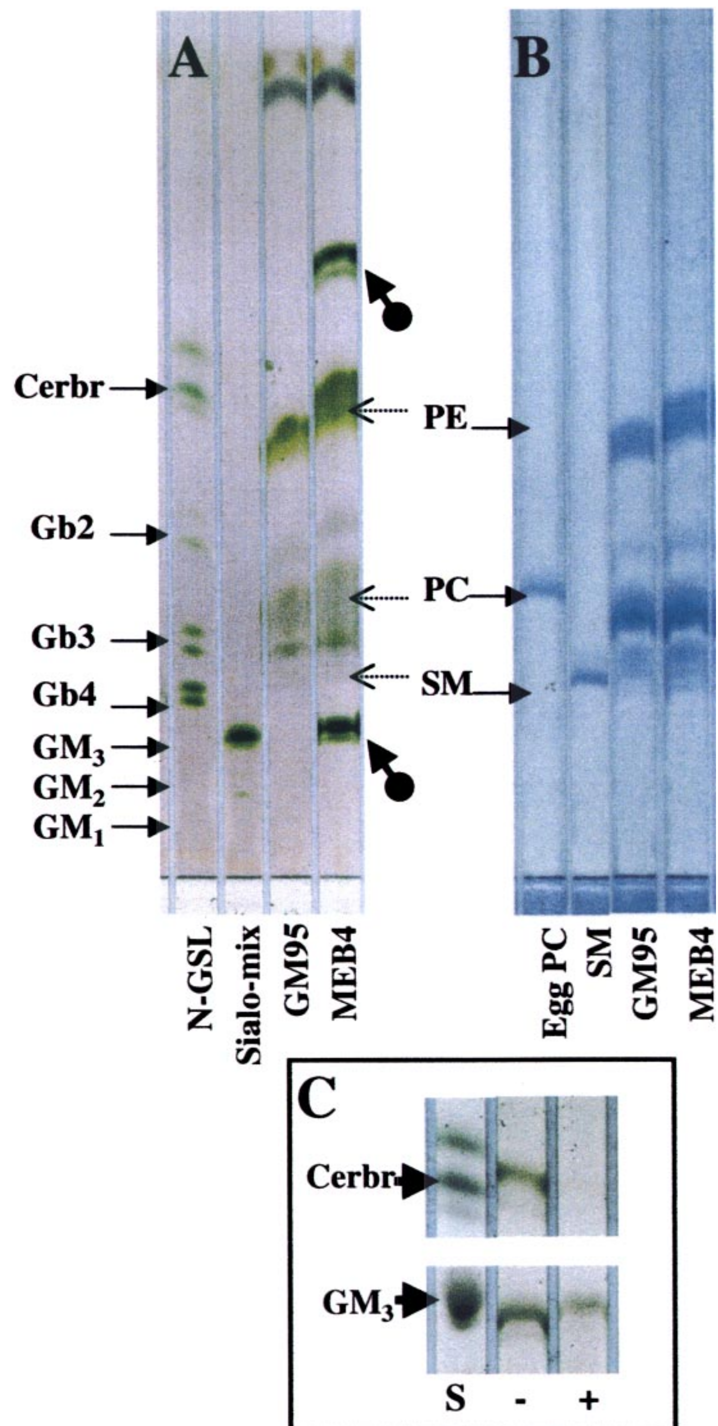

Fig. 1. GSL analysis of GM95 cells: 5 $\times$ 10^6 cells were harvested with trypsin/EDTA, and the total lipids were extracted following the published procedure [3] and solvents were removed. Dried lipids were re-solubilised in 100 μl chloroform:methanol (2:1, v/v). We used 50 μl (approx. 2.5. $\times$ 10^6 cells) and 15 μl samples (approx 7.5 $\times$ 10^5 cells) for

influenza virus was used at very low MOI (data not shown). GM95 cells were also readily infected by two other influenza sub-types, A/PR/8/34 (H1N1), and A/Japan/305/57 (H2N2) (data not shown).

Next, we monitored fusion of intact influenza virus directly with the plasma membrane of GM95 cells using the well-established octadecyl rhodamine (R18) fluorescence dequenching (R18-fdq) assay [8]. Purified X31 influenza virus, labeled with R18 [20], was incubated with the cells for 20 min at 20–25 °C to allow virus association to the cells, and unbound virus was removed by centrifugation. Small aliquots of virus-cell complexes were added to a stirred cuvette at 37 °C containing 2 ml buffer at preadjusted pH in a SLM8000 spectrofluorimeter (SLM Instruments, Urbana, IL), and fluorescence increases were recorded with a 1 sec-time resolution. Figure 3A shows a rapid increase in fluorescence at pH 5.0 and 37 °C as a result of fusion of the virus with the different cell lines. There was a slight decrease in fluorescence when incubations were done at pH 6.7 and 7.0, presumably due to slow sedimentation of virus-cell aggregates. However, at pH 7.0 and 37 °C fluorescence increased after a delay of about 100 sec which may be attributed to viral fusion in the low-pH compartment of endosome [4]. Given the greater increase in R18-fdq at pH 7 and 37 °C in the case of B16F10 cells, it appears that these are the most actively endocytosing cells. Lack of fusion at pH 6.7 was presumably due to block in the endocytosis of X31 influenza virus by the cells as shown previously [4]. However, the kinetics and extent of fusion at pH 5 and 37 °C was similar for GSL-deficient GM95 cells and GSL^{+}MEB4 cells and B16 cells (Fig. 3).

Previous studies using liposomes as targets have shown that influenza-HA mediated fusion can occur with membranes devoid of sialic-acid containing receptors provided that the virus is bound to the target membrane via an alternative (surrogate) receptor [15]. To examine involvement of terminal sialic acids on the glycoproteins of the GM95 cells, we studied the ability of desialylated cells to promote influenza-HA mediated fusion and infection. Cells were treated with Nase (Sigma Chemical Co. Type V, from Clostridium perfringens) for 30 min

Fig. 1 (*continued*)
GSL and phospholipid analysis respectively. Samples were loaded on preadsorbent channeled Silica Gel TLC plate (Whatman Inc., NJ, LK6D, Silica gel 60A, 250 μm thickness) and separated using the solvent system $CHCl_3$:MeOH:H_2O (65:25:4, v/v). The plates were air-dried and sprayed with Orcinol ferric chloride (Bial's reagent, Sigma Chemical Co, cat# O-7875), heated for 10 min at 100 °C to develop purple spots identifying GSL (**A**) [13]; or sprayed with molybdenum blue spray [6], heated at 40–50 °C for 10 to 20 min to develop the blue spots characteristics of phospholipids (**B**). The GSL levels in MEB4 cells before (−) and after treatment (+) with PPMP are shown in **C**. S, GlcCer and GM3 standards as indicated (**C**). The TLC plates were scanned with Hewlett Packard ScanJet 4P scanner and processed using Paint Shop Pro software (Jasc Software Inc.). The following lipid standards (10–20 μg) were used (**A**, **B**): egg PC; sphingomyelin; N-GSL (neutral GSL qualmix (Matreya, Inc cat#1505) containing Cerebrosides (Cerbr), Lactosyl ceramide (Gb2), Ceramide trihexoside (Gb3) and globoside (Gb4)); Sialo-mix, monosialoganglioside mixture (Matreya Inc. cat#1508) containing GM1, GM2 and GM3

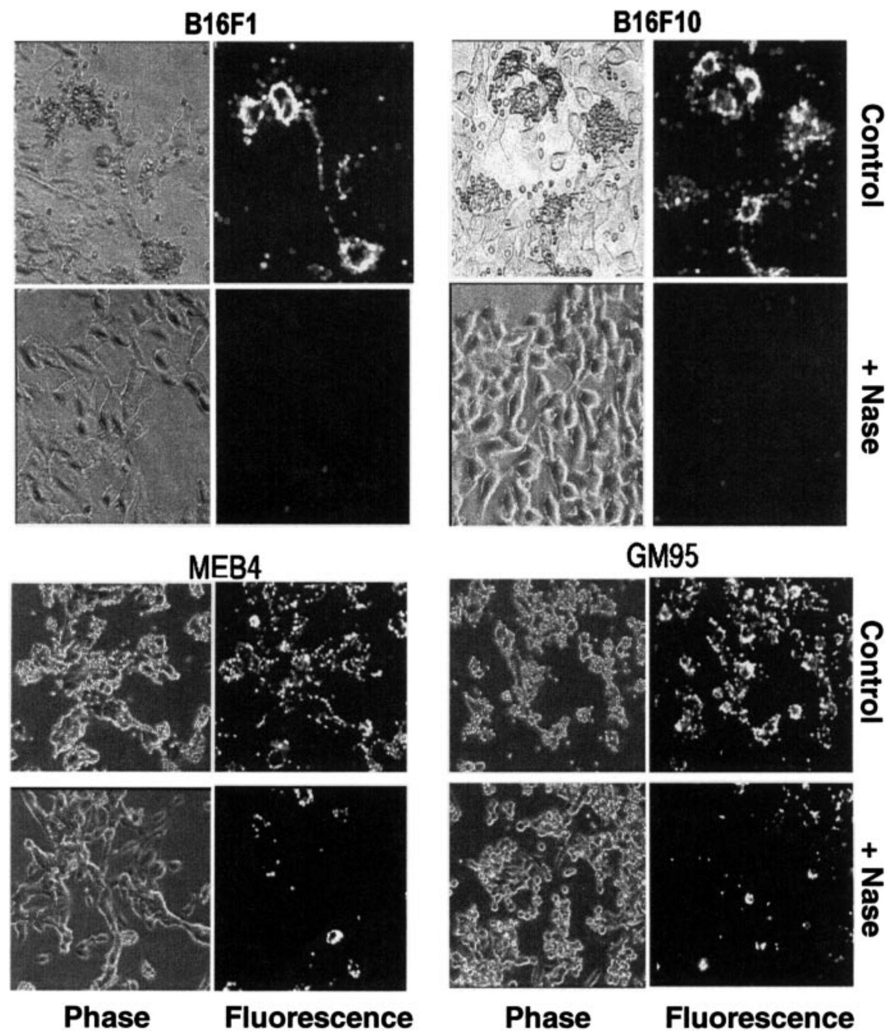

Fig. 2. Infection of cells by Influenza virus. Cells were plated on 24 well plates (5×10^4/well) one day prior to infection. To cleave sialic acids from the cell surface, cells were incubated with 0.5 ml D0 containing Nase (Sigma Chemical Co. Type V, from Clostridium perfringens, cat#N-2876, 5 units/ml) for 30 min at 37 °C. The enzyme was removed by washing with 0.5 ml D0 (×2). Control cells were incubated under exact conditions without the enzyme. X:31 influenza virus was added to the cells in a total volume of 0.25 ml for 30 min/37 °C. Unbound virus was replaced with fresh culture medium (0.5 ml) and infection was continued for 16–20 h at 37 °C. At the end of incubations, 0.5 ml suspension of calcein-labeled human erythrocytes in D0 (approx. 10^7 RBC) was added per well and the samples were incubated at room temperature for 15–20 min. Unbound RBC were removed by gentle washing and images were captured as described in the text. Labeling of human erythrocytes with calcein-AM (Molecular Probes, cat#C-3100) was done as follows: A solution of calcein-AM (50 μg solubilized in 10 μl DMSO and further diluted with 40 μl PBS) was incubated with 1 ml suspension of washed erythrocytes (10% = 10^9/ml in PBS) at 37 °C for 45–60 min with occasional stirring. At the end of incubation, the cells were pelleted by centrifugation and washed with PBS (10 ml × 3). Calcein-labeled cells were resuspended in 10 ml PBS @ 10^8/ml = 1% RBC suspension). Calcein-loaded erythrocytes were further diluted in DMEM without serum (D0) @ 2×10^7/ml = 0.2% RBC just before binding to influenza infected cells

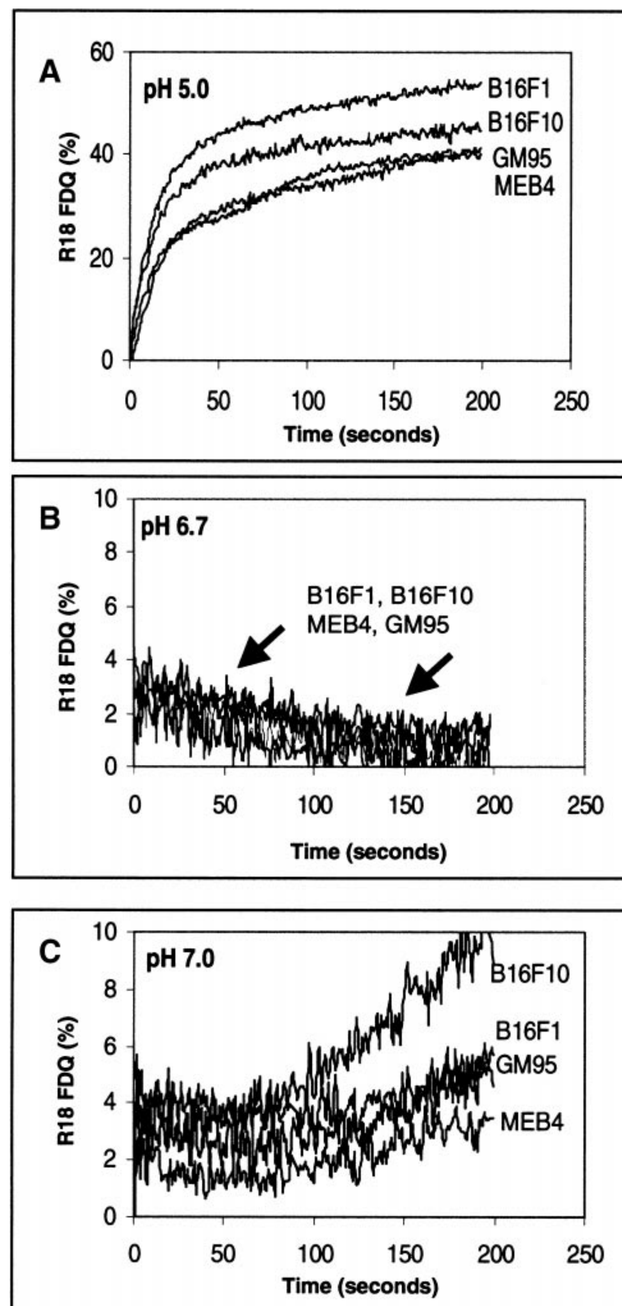

Fig. 3. Kinetics of influenza virus X31 fusion with cells: R18-labeled X:31 influenza virus (100 μg) was added to 1 ml cell suspension in PBS (5×10^6). Virus was allowed to bind to the cells at room temperature for 20–30 min and 10 ml PBS was added. Unbound virus was removed by centrifugation and the pellet was resuspended in 0.5 ml PBS. 100 μl of virus-cell complexes was added to a cuvette containing prewarmed buffer at 37 °C at **A** pH 5.0, **B** pH 6.7, **C** pH 7.0. Kinetics of fusion monitored using SLM spectrofluorimeter and percent FDQ was calculated as follows: 100 ×(F-Fo/Ft-Fo), where Fo, F, Ft are the fluorescence values at zero, given time and in fluorescence in the presence of tritonX100 respectively [20]

at 37 °C either on adherent cells for infection assays (see legend to Fig. 2) or in suspension for subsequent fusion kinetics (see legend to Fig. 3A). At the end of incubations, the enzyme was removed and desialylated cells were tested for infection or fusion with influenza X:31 virus. We found that desialylation of cells resulted in inhibition of influenza infection in all the cell types tested (Fig. 2). Control cells (not treated with Nase) on the other hand were readily infected with influenza X:31 as judged by the binding of calcein-labeled erythrocytes (Fig. 2) [21]. Low levels of fluorescent erythrocytes bound to MEB4 and GM95 cells following the treatment with neuramindase were close to the background as compared to the erythrocyte binding in untreated cells (Fig. 2). We made similar observations when desialylated cells were tested for the low-pH triggered

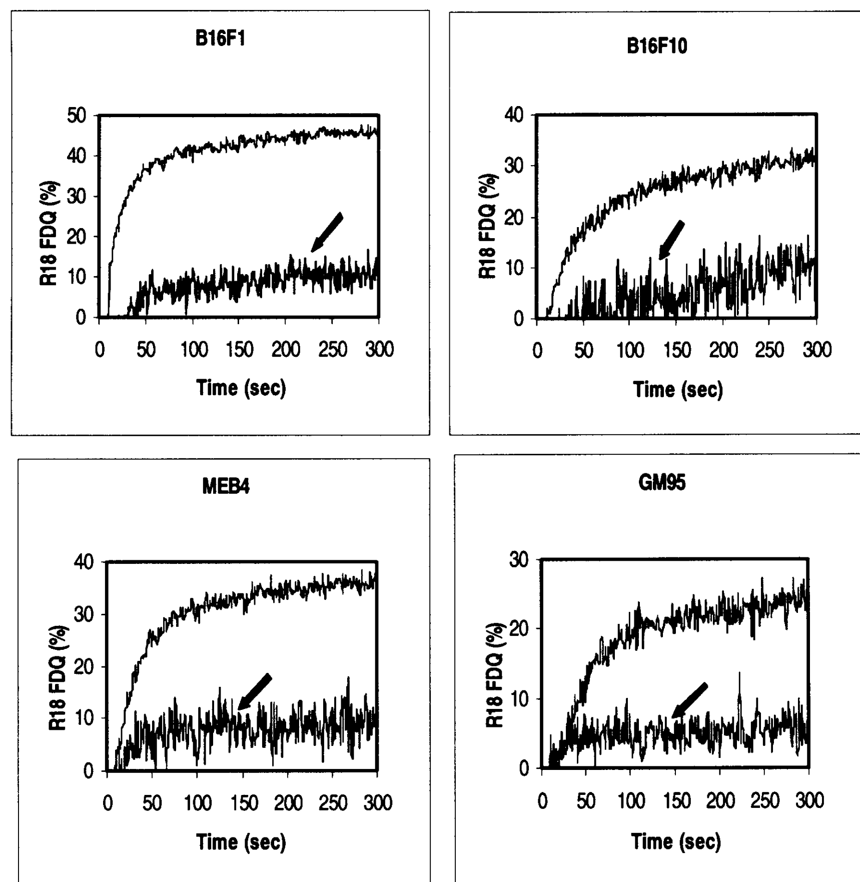

Fig. 4. Inhibition of Influenza virus fusion by pre-treatment of cells with neuraminidase: Cells (5×10^6) were harvested using cell dissociation buffer, washed, resuspended in 0.5 ml D-0 and incubated with Nase (0.5 ml, 5 units/ml) for 30 min at 37 °C as described in legend to Fig. 2. At the end of incubation, 5 ml D-0 was added and cells were pelleted by centrifugation. Cell pellets were washed with 5 ml D-0 and the cells were resuspended in PBS (5×10^6/ml). Desialylated cells were tested for fusion with R18-labeled X:31 at pH 5.0 and 37 °C as described in legend to Fig. 3. Arrows indicate fdq when Nase-treated cells were used as targets. The results were reproducible from at least three independent experiments

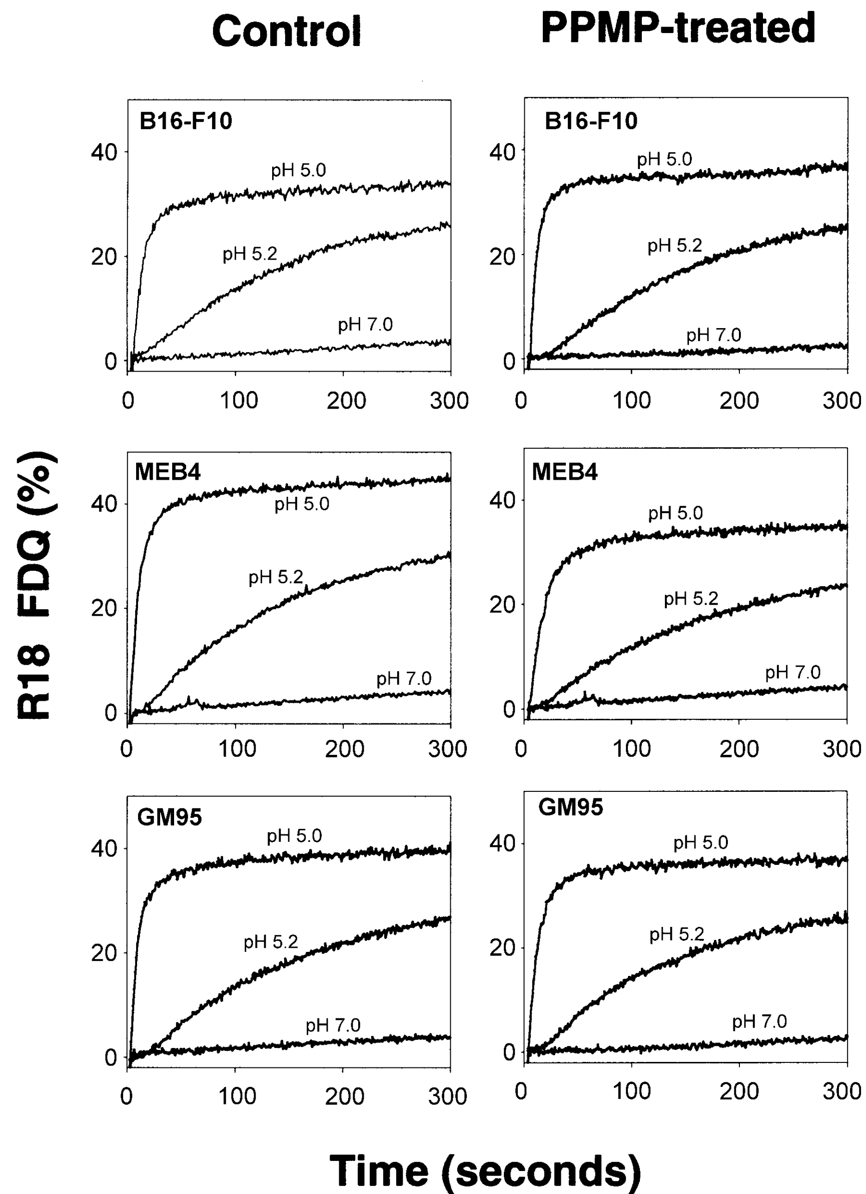

Fig. 5. Effect of GSL depletion from target cells on influenza virus fusion: Glycosphingolipids were depleted from the surface of B16 and MEB4 cells by incubation with an inhibitor of GSL biosynthesis PPMP. Cells were cultivated in T75 flask (3×10^6 per flask, in duplicate) in the absence or presence of 10 μM PPMP (Matreya Inc., cat# 1720) in culture medium (15 ml/flask) for 48 hours at 37 °C. At the end of incubations, cells were either harvested with cell-dissociation buffer for fusion assay or lifted using trypsin-EDTA and proceeded for extraction of total lipid as described. Fusion with R18-X:31 influenza virus was monitored as in Fig. 3. (GSL analysis of PPMP treated cells is shown in Fig. 1C). The results were reproducible from two independent experiments

fusion at the plasma membrane with R18-labeled X31 influenza virus (Fig. 4). Fusion was significantly reduced at pH 5.0, 37 °C (>80%, indicated by arrow) in all the cell lines tested following pretreatment with Nase. Taken together these findings confirm that influenza virus utilizes sialic acid residues on the surface of GSL-deficient GM95 cells for it's entry and membrane fusion.

Although sialoglycoproteins are required for entry of influenza virus into GM95 cell, the question remains whether sialo-GSL play an additional role in the parental cell lines. To explore the role of the sialo-GSL we treated MEB4 and B16 cells with a GSL synthesis inhibitor PPMP, which is known to down-modulate GSL but not glycoprotein synthesis [1]. Reduction in GSL levels was confirmed by TLC analysis of the lipid extracts. We observed a significant decrease in GlcCer and GM3, the two GSLs present in MEB4 cells when cells were incubated with 10 μM PPMP for 48 hours (Fig. 1C) [7]. A similar reduction in GSL levels was also observed for B16 cells following PPMP treatment (data not shown). However, GSL-depleted cells did not show any reduction in fusion with R18X31 influenza virus at various pH's tested (Fig. 5). These observations demonstrate that the presence of gangliosides in the biological membrane is not essential for influenza virus fusion.

It has been shown in model systems that influenza virus binds more tightly to sialoglycoproteins than to sialo-GSL [12, 26]. Presumably the close proximity of sialic acid residues on GSL to the membrane surface may make them less accessible for binding to the influenza virus. Despite the fact that gangliosides can serve as receptors for influenza virus fusion in model systems (liposomes, erythrocytes) [12, 25, 27, 31], we show here for the first time that influenza virus requires sialo-glycoproteins rather than sialo-GSL to infect animal cells. The notion that sialoglycoproteins serve as the receptors for influenza virus in animal cells makes sense given the fact that entry of influenza virus in animal cells occurs via clathrin coated pits [14]. In contrast to sialo-glycoproteins, sialo-GSL are localized on the cell surface in non-clathrin coated membrane microinvaginations called the membrane rafts or caveolae [16, 19]. The GSL utilize a unique, pH-independent pathway to reach compartments of the Golgi net work, whereas a variety of sialoglycoproteins utilize the clathrin coated pit pathway which involve low pH endocytic sorting compartments [24]. Therefore influenza virus entry via GSL may result in a non-infectious route, whereas sialo-glycoprotein receptor-mediated entry results in productive infection in vivo.

Acknowledgements

Members of the Blumenthal lab are appreciated for their insightful comments. We are grateful to Dr. Y. Hirabayashi for making the GM95 and MEB4 cell lines available to us.

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Received April 23, 2001