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Gangliosides are not essential for influenza virus infection

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Abstract Sialic acid is known to be an essential part of influenza virus receptors, but the specific identity of the receptor molecules on target cells is still not defined. In particular, the relative roles played by cellular sialylglycoproteins and gangliosides in virus entry into target cells remain unclear. To test whether gangliosides are essential for virus infection, we used the GM-95 mutant cell line of mouse B16 melanoma which lacks synthesis of major glycosphingolipids including gangliosides. We found that GM-95 cells grown in serum-containing medium harboured substantial amounts of ganglioside receptors for influenza virus due to incorporation of serum gangliosides. To obtain ganglioside-free cells, we adapted GM-95 cells to growth in defined serum-free (sf) medium. Ganglioside-free GM-95-sf cells could be infected by avian and human influenza A viruses and produced infectious virus progeny demonstrating that gangliosides were neither absolutely necessary for the early nor for the late stages of the infection. However, sensitivity of the GM-95-sf cells to the viruses was 2–4 times lower than that of the ganglioside-containing parent cell line. Further studies are needed to specify whether this effect was due to the lack of gangliosides, neutral glycosphingolipids, or other effects.

Keywords Influenza virus · Glycosphingolipid-deficient cells · TLC overlay assay · GM-95 · MEB-4 · Serum-free medium

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Abbreviations

BSA	bovine serum albumin
Dk/98	A/Mallard duck/Alberta/279/98 (H3N8)
DMEM	Dulbecco's modified Eagle medium
FCS	fetal calf serum
GM-95-sf	GM-95 cells adapted to serum-free medium
GD1a	disialoganglioside Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer
GM3	monosialoganglioside Neu5Acα2-3Galβ1-4Glcβ1-1Cer
GT1b	trisialoganglioside Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glcβ1-1Cer
GalCer	galactosylceramide
GlcCer	glucosylceramide
HRP	horseradish peroxidase
LacCer	lactosylceramide
NP	nucleoprotein of influenza virus
MDCK	Madine-Darby canine kidney cells

PBS phosphate-buffered saline
TLC thin layer chromatography

Introduction

Infection by influenza viruses is initiated by the binding of virus particles to terminal sialic acid moieties of cell-surface molecules called viral receptors (reviewed in references [1–3]). Sialic acids are represented on the cell surface as components of oligosaccharide chains of glycoproteins and gangliosides (sialic acid-containing glycosphingolipids) [4–6]. Influenza viruses bind to both glycoproteins and gangliosides in various in vitro assays (reviewed in references [1,2,7]); however, the roles played by these two structurally distinct types of molecules in the early stages of virus infection following attachment are still not clear.

It is generally believed that large glycoproteins better exposed on the cell surface are more likely to serve for the initial virus attachment, whereas subsequent binding to gangliosides could bring the viral and cell membranes into closer proximity and, thus, facilitate the membrane fusion required for the entry of the viral genome into the cell (for a discussion, see [1,2]). Previous studies suggested that gangliosides can mediate virus entry into cells even in the absence of glycoprotein receptors, as exogenous gangliosides restored susceptibility to influenza A and C virus infection of cells that have been rendered resistant by destroying endogenous receptors with bacterial sialidases [8–10].

Several lines of evidence indirectly suggested that binding to cellular gangliosides may be essential for the infection. For example, clinical isolates of human influenza A and B viruses replicate efficiently in MDCK cells but not in the allantoic cavity of embryonated chicken eggs, and adaptation of human viruses to eggs always results in a selection of receptor-binding mutants with amino acid substitutions in the HA protein (reviewed by Robertson [11]). To evaluate the mechanisms by which these substitutions enable growth of human virus in eggs, Gambaryan *et al.* [12] studied the binding of MDCK- and egg-grown human influenza virus pairs to preparations of cellular membranes, gangliosides, and sialylglycoproteins. In marked contrast with non-egg-adapted viruses, all egg-adapted variants bound to gangliosides of the egg allantoic membrane. The authors concluded that growth of human influenza viruses in eggs is restricted because of their inefficient binding to cellular receptors and that gangliosides can play an important role in virus binding and/or penetration.

As another example, a common property of influenza viruses in wild aquatic birds distinguishing these viruses from most human viruses was strong avian virus binding to Neu5Acα2-3Gal-terminated gangliosides with relatively short sugar cores, such as GM3, GD1a, and 3′ sialylparaglo-boside [13–15]. A strict conservation of this property among avian viruses raised the hypothesis that binding to gangliosides could be essential for virus replication in the intestine [13,15], the primary target tissue of influenza viruses in aquatic birds [16].

Ablan and colleagues have recently addressed the role of gangliosides as biological receptors for influenza virus by using the ganglioside-deficient cell line GM-95 [17]. This cell line was selected from the parent MEB-4 cells of B16 mouse melanoma using antibodies against GM3 [18]. Unlike the MEB-4 cells, the GM-95 cells lack activity of ceramide glucosyltransferase (EC 2.4.1.80), and do not synthesize glucosylceramide (GlcCer), a precursor of all major glycosphingolipids, including gangliosides. Octadecyl rhodamine labeled human influenza virus fused with GM-95 cells at similar rates and extents as with the parental MEB-4 cells, the virus also infected the GM-95 cells at the same level as MEB-4 cells [17]. The authors concluded that influenza virus uses mainly sialoglycoproteins and that gangliosides are not essential for influenza virus fusion and infection. These important observations were compromised, however, by the fact that the cells used in these experiments were cultivated in serum-containing medium and could incorporate gangliosides from the serum [19,20]. The present study was initiated to address this issue and to give a formal answer to a long-standing question of whether binding to gangliosides is essential for influenza virus infection. We confirmed the hypothesis that GM-95 cells grown in serum-containing medium harbour substantial amounts of ganglioside receptors for influenza virus. To overcome this problem, we adapted GM-95 cells to growth in serum-free medium and demonstrated that ganglioside-free cells are sensitive to infection with human and avian influenza viruses.

Materials and methods

Viruses

Influenza viruses A/Mallard duck/Alberta/279/98 (H3N8), (Dk/98), A/Los Angeles/2/87 (H3N2), and A/WSN/33 (H1N1) were propagated in embryonated chicken eggs.

Virus labelled with horseradish peroxidase (HRP)

Dk/98 virus was clarified by low-speed centrifugation, pelleted by high-speed centrifugation through a 25% sucrose cushion and resuspended in PBS. Thirty μl of aqueous solution containing 0.3 mg HRP (Sigma) and 1 μmole sodium periodate was incubated for 20 min at room temperature. Periodate-oxidized HRP was desalted by using 1 ml Sephadex G-25 column equilibrated with water and immediately added to the solution containing 1.5 mg of the purified

virus in 0.8 ml PBS. We next added 0.2 ml of 0.2 M carbonate buffer (pH 9.3) and incubated the mixture for 3 h on ice followed by addition of 0.5 mg sodium borohydride. To remove non-conjugated HRP, the labelled virus was pelleted through 25% sucrose. The labelled virus was suspended in 0.1 ml of 0.1 M Tris-HCl buffer (pH 7.3), diluted with an equal volume of glycerol, and stored at −20°C. Assay of the viral hemagglutination titers before and after labelling revealed no changes the virus capacity to agglutinate chicken red blood cells.

Cells

MEB-4 cells of mouse B16 melanoma and GM-95 cells, a mutant cell line of MEB-4 lacking glycosphingolipids have been described previously [18]. MEB-4, GM-95 and Madin Darby canine kidney (MDCK) cells were propagated in Dulbecco's modified Eagle medium (Cambrex) supplemented with 10% fetal calf serum (FCS). DMEM-F12 medium (Cambrex) supplemented with sodium selenite, insulin, transferrin, and ethanolamine (SITE liquid media supplement, Sigma) was used to grow GM-95 cells under serum-free conditions. For the adaptation, the cells were passaged 5 times in DMEM-F12-SITE medium that was supplemented with gradually decreasing concentrations of FCS (from 10 to 1%). After 5 more passages without FCS, the cells were frozen in aliquots. These cells were designated GM-95-sf ("serum-free") and they were used for the experiments at passage levels from 3 to 15.

Isolation and analysis of lipids

Confluent monolayers of cells were washed 3 times with phosphate-buffered saline (PBS) followed by scraping the cells into PBS, two additional washings with PBS, and lyophilization. Total cellular lipids were extracted with 20 volumes of chloroform/methanol (2:1) and separated by thin layer chromatography (TLC) on plastic-backed Polygram Sil G TLC plates (Macherey-Nagel GmbH, Germany) in chlorofrom/methanol/0.25% KCl (60:35:8) as a solvent system. Reference neutral glycosphingolipids and gangliosides were obtained from Sigma. Glycolipids were detected with orcinol-ferric chloride solution (Bial's reagent, Aldrich). Alternatively, we detected the virus-binding activity in lipid extracts by overlaying separated lipids directly on TLC plates with HRP-labelled influenza virus [14,21] as outlined below.

Virus overlay assay

The TLC plate with separated lipids was blocked with 1% solution of bovine serum albumin (BSA) (Sigma) in PBS (PBS-BSA) for 1 h at room temperature. After 3 washings with PBS, the plate was overlaid with HRP-labelled virus diluted to a hemagglutinating titer of about 100. The solution was prepared in PBS-BSA supplemented with 1 μM of sialidase inhibitor 2,3-didehydro-2,4-dideoxy-4-guanidino-*N*-acetyl-D-neuraminic acid (zanamivir), kindly provided by R. Bethel, Glaxo Wellcome Co, UK. After 1 h incubation at the room temperature, the plate was washed 5 times with PBS, and the virus attached to the lipid bands was detected using precipitate-forming peroxidase substrate (True Blue, KPL). To test whether the virus binding in this assay was sialic acid-dependent, a replicate plate was treated with 50 mU/ml of *V. Cholerae* neuraminidase (Sigma) added to the blocking solution.

Virus infection

Cells grown in 96-well plates (Costar) were washed 3 times with DMEM and inoculated with 0.1 ml of 10-fold dilutions of the viruses in serum- and supplement-free basal medium (4 to 6 replicates per dilution). Eight to ten h post-inoculation, the cells were fixed with 80% acetone for 10 min at −20°. Fixed cultures were immunostained for the expression of influenza virus nucleoprotein (NP) by incubating for 1 h with anti-NP monoclonal antibodies (kindly provided by A. Klimov, Centers for Disease Control, USA) followed by 1 h of incubation with HRP-labelled anti-mouse antibodies (Sigma) and 30 min of incubation with aminoethylcarbazole substrate (Sigma). Ten-percent horse serum plus 0.05% Tween 80 in PBS was used for the preparation of working dilutions of immuno-reagents. To express the level of infection in quantitative terms, we counted infected cells under the microscope. The average number of infected cells per well were calculated for four to six replicate wells infected with the virus dilution that produced from 10 to 100 infected cells per well.

Results and discussion

GM-95 cells incorporate gangliosides from serum-containing cultivation medium

To test whether GM-95 cells grown in FCS-containing medium were free from gangliosides, we compared total lipids extracted from these cells with those extracted from MEB-4 and MDCK cells. The lipids were first analyzed by thin-layer chromatography with chemical detection. In addition, the presence of virus-binding activity in lipid extracts was assayed by overlaying separated lipids directly on TLC plates with peroxidase-labelled avian influenza virus. As reported previously [17,18], two major purple glycolipid bands were identified in the MEB-4 lipid sample by orcinol staining that corresponded to GlcCer and GM3, with neither glycolipid being detected by this method in GM-95 cells

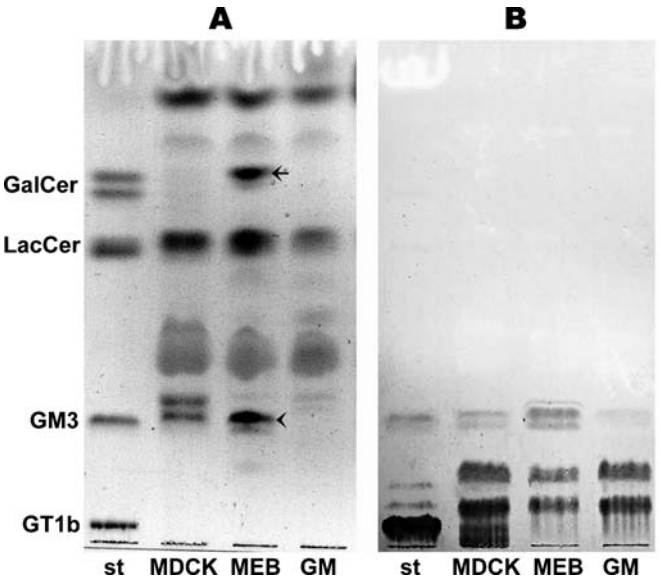

Fig. 1 Orcinol stain (A) and virus overlay assay (B) of lipids separated by TLC. Lanes: standard lipid mixture containing 1 μg of each GM3, GT1b, GalCer, and LacCer (st); total lipids extracted from 10^5 MDCK, MEB-4 and GM-95 cells (MDCK, MEB, and GM, respectively). All cells were grown in DMEM-FCS. Arrow and arrowhead in the MEB lane depict GlcCer and GM3, respectively

(Figure 1A). This finding confirmed that GM-95 cells did not synthesize GlcCer, a precursor of all glycosphingolipids [18]. Remarkably, the virus overlay assay detected several lipids with virus-binding activity in each cell line including GM-95 (Figure 1B). As there was no virus binding to any of the abundant lipid bands between GM3 and the front and as the binding was abolished by treatment of the plate before the assay with *V. Cholerae* sialidase (not shown), we concluded that all positive bands in the Figure 1B were gangliosides. The patterns of the virus binding differed between lipid extracts from MDCK, MEB-4, and GM-95 cells, however, all three samples contained comparable amounts of two ganglioside bands that migrated between GT1b and GM3. Although these gangliosides went undetected by orcinol staining, they displayed substantial virus-binding activity. In fact, due to the presence of these gangliosides in GM-95 cells, the virus binding to lipid extracts from these cells was only 2 to 3 times lower than its binding to extracts from the same amounts of MDCK and MEB-4 cells (Figure 1B). These results were confirmed in replicate experiment using lipids isolated from another lot of serum-grown MEB-4 and GM-95 cells (data not shown).

To test whether cultivation in serum-containing medium was responsible for the presence of gangliosides in GM-95 cells, we decided to exclude FCS from the medium. After testing of several different serum-free media with no success, we finally managed to adapt the cells to growth in the DMEM-F12 medium supplemented with selenite, insulin, transferrin and ethanolamine (SITE). No virus-binding material could be detected in the lipid fraction of GM-95-sf cells that were adapted to and serially passaged in this medium (Figure 2A). However, after a single passage in the FCS-supplemented medium, the lipid extracts from GM-95-sf cells contained gangliosides with a TLC-overlay profile indistinguishable from that of the lipid extracts from original FCS (Figure 2B). These data proved our initial hypothesis that GM-95 cells grown in the presence of FCS accumulate exogenous gangliosides thus compromising the use of these cells in the studies on the role of gangliosides in the virus infection.

GM-95-sf cells support replication of influenza viruses

Having confirmed that GM-95-sf cells grown in serum-free medium contain no gangliosides, we compared these cells with the parent MEB-4 cells and with MDCK cells for their sensitivity to influenza viruses. Both human and avian in-

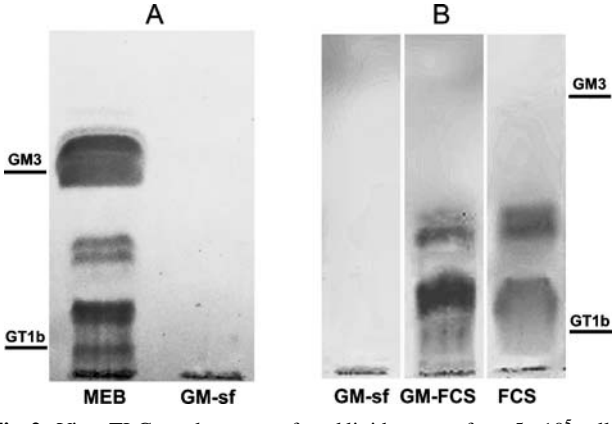

Fig. 2 Virus TLC overlay assay of total lipid extracts from 5×10^5 cells. (A) MEB-4 cells grown in FCS-containing medium (MEB) and GM-95-sf cells grown in serum free medium (GM-sf). (B) GM-95-sf cells were plated in either serum-free medium described in the Materials and Methods or the same medium supplemented with 10% FCS. After 3 days of growth in these media total cellular lipids were extracted and tested in the virus overlay assay (lanes GM-sf and GM-FCS, respectively). Lane FCS, total lipids extracted from 30 μl of fetal calf serum. Horizontal bars mark positions of ganglioside standards (GM3 and GT1b)

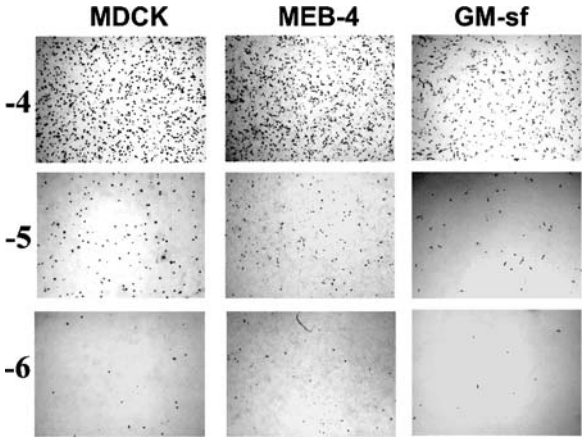

Fig. 3 Microscopic views of MDCK, MEB-4, and GM-95-sf cells infected with the same doses of human influenza virus A/Los Angeles/2/87 (H3N2). We inoculated the cultures in parallel with the 10^{-4}, 10^{-5} and 10^{-6} dilutions of the virus stock, fixed the cultures 8 h post infection, and immuno-stained them for the viral NP

fluenza virus strains were tested to account for the known differences between viruses from these hosts in binding to gangliosides [7,13–15]. We inoculated cultures with the viruses, stopped infection by fixing the cells after one cycle of virus replication, and immuno-stained the cells for the expression of viral nucleoprotein (Figure 3). To compare sensitivity of different cell lines to virus infection in quantitative terms, we counted numbers of cells infected in each culture by the same virus dose. In these experiments, GM-95-sf cells were readily infected by both human and avian viruses, although their sensitivity to infection was somewhat lower than that of the parent MEB-4 cells. Namely, in replicate experiments performed on different days with different lots of cultures, the avian and human virus infected 2 to 4 times less cells in GM-95-sf cultures than in MEB-4 cultures (see Figure 4 for the results of arepresentative experiment). This finding suggested that an absence of either gangliosides, or neutral glycosphingolipids, or both lipid components in GM-95 cells slightly reduce virus entry into these cells. Alternatively, a lower sensitivity to infection could be a result of mutations acquired during the selection of the original GM-95 cells, this explanation would be consistent with the slower growth rate of GM-95 cells as compared to MEB-4 cells [18]. Another potential explanation for the observed differences might be that GM-95-sf cells were grown at sub-optimal conditions of serum-free medium. Whatever is the case, our data on susceptibility of ganglioside-free GM-95-sf cells to influenza viruses for the first time unambiguously demonstrate that gangliosides are dispensable for the early studies of infection and that cellular sialylglycoproteins alone can mediate virus entry into cells. This conclusion is further emphasised by the recent data on the essential role of N-linked glycosylated glycoproteins for the influenza virus entry into CHO cells [22].

Glycosphingolipid- and cholesterol-enriched raft microdomains of the cellular membrane are believed to function as the site of budding of several enveloped viruses, including influenza virus (reviewed in references [23,24]). To test whether a lack of glycosphingolipid synthesis in GM-95 cells could prevent assembly and budding of infectious virus progeny, we studied multi-cycle virus infection in these cells. Because newly formed influenza virus particles remain non-infectious until the HA is proteolytically cleaved [25,26], addition of trypsin to the culture medium is routinely used to provide for multi-cycle infection in the laboratory cell lines, such as MDCK and VERO. Unfortunately, GM-95 cells as well as their parent MEB-4 cells detached from the plastic support in the presence of required amounts of trypsin (from 0.25 to 1 μg/ml). To deal with this problem, we utilized A/WSN/33 (H1N1), the mouse-adapted influenza virus strain which

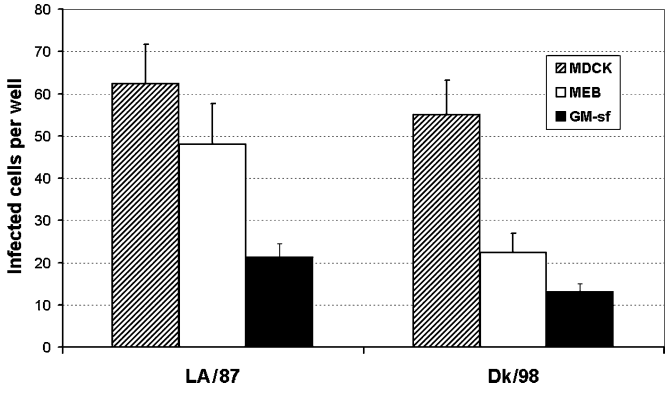

Fig. 4 Numbers of cells infected by identical influenza virus doses in MDCK (dashed bars), MEB-4 (open bars), and GM-95-sf (closed bars) cultures. The cultures were inoculated with either 10^{-6} dilution of A/Los Angeles/2/87 (LA/87) or 10^{-5} dilution of A/Mallard duck/Alberta/279/98 (Dk/98) to achieve the multiplicity of infection of about 0.005 infectious virus particles per cell. The cultures were fixed 8 hrs after infection, immuno-stained for the viral NP, and infected cells were counted under the microscope. Mean values of 6 replicate wells are presented. The patterns of susceptibility observed in this experiment were reproducible in replicate experiments performed on different days.

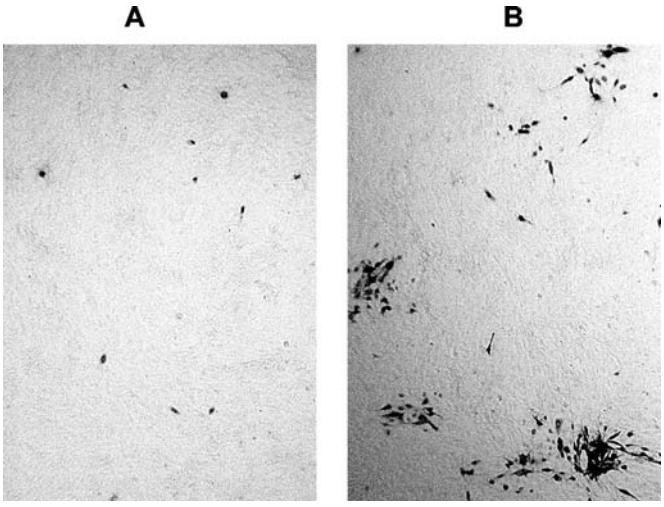

Fig. 5 Multi-cycle infection in GM-95-sf cells. Cultures were inoculated with 10^{-5} dilution of A/WSN/33 (H1N1) in the presence of 5 μg/ml plasminogen (B) or without addition of plasminogen (A). The cells were fixed and immuno-stained for NP 18 h post infection.

is known to be proteolytically activated by plasminogen [27,28]. Eighteen h after infection of GM-95-sf cells with this virus in the presence of plasminogen, we observed prominent foci of infected cells indicative of the efficient formation of viable virus progeny (Figure 5). Thus, neither gangliosides, nor neutral glycosphingolipids are essential at the late stages of virus infection. This finding does not contradict the theory about influenza virus budding from rafts, since the biochemical analysis of detergent-resistant membranes from GM-95 and MEB-4 cells suggested that glycosphingolipids are not crucial for the raft's formation and do not play major role in determining their properties [29,30].

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