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Represented on a Solid-Phase Assay Surface

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Polyglycosylceramides (PGCs), complex glycolipids containing up to 50 or more sugar residues, are recognized as the minor components of the cell-surface membranes, but a knowledge on their tissue distribution, structure, and function is limited. In this study, the binding of influenza viruses to preparations of PGCs was investigated using a TLC overlay assay and a microwell adsorption assay. The ability of PGCs to bind influenza virus was dependent on the source from which they were derived. Preparations of PGCs from human erythrocytes were found to support binding of A and B influenza virus strains at a much lower concentration than sialyl-6-paragloboside and to be somewhat better receptors for these viruses compared to the sialylglycoprotein fetuin. A high virus-binding activity of PGCs suggests that these species could potentially serve as biologically important cell-surface receptors for influenza viruses. © 1996 Academic Press, Inc.

Influenza virus infection is initiated by attachment of the virus to host cells due to specific interactions of the viral hemagglutinin with sialic acid-terminated oligosaccharide chains expressed on cell-surface glycoproteins and/or gangliosides [see (*1–3*) for a review]. In spite of the early identification of sialic acid as an essential part of influenza virus cellular receptors, the biologically relevant receptor species on target cells have not been defined. Gangliosides, glycosphingolipids bearing terminal sialic acid residues (*4*), are regarded as possible candidates since they were shown to support virus binding in several model assay systems (*5–7*) and to stimulate experimental viral infection in tissue culture cells and in mice (*8, 9*).

Influenza virus binding has been shown to depend on the type of sialic acid, the sialyl-Gal linkage, and the carbohydrate core structure of gangliosides [see (*10*) for a review]. In a recent study (*7*) on influenza virus binding to gangliosides from human granulocytes using a TLC overlay assay, three minor nonidentified most polar gangliosides displayed stronger binding compared to ganglioside species with shorter oligosaccharide chains (3–7 sugars). This finding prompted us to investigate adhesion of influenza viruses to polyglycosylceramides (PGCs), glycosphingolipid species containing up to 50 or more sugars (*11–13*).

As a first step, binding of influenza virus to PGC preparations was evaluated using a TLC overlay assay (*14*). The PGCs were prepared in the Department of Medical Biochemistry, Göteborg University, Sweden, as described in (*13*). As shown in Fig. 1, influenza virus bound strongly to PGC fractions isolated from human erythrocytes and human leukocytes. Both preparations contained mixtures of highly polar components generally migrating slower than brain gangliosides, with the leukocyte material being more enriched in faster migrating species. The fastest migrating bands of the leukocyte fraction overlapped with the GD1a region of bovine brain gangliosides, as judged from several chromatographic runs. However, it was very difficult to draw conclusions concerning the complexity and structure of these species because the chromatographic system was not suitable for separation of less complex glycolipids. PGCs from erythrocytes contained on average 25 sugar residues per ceramide with 1 sialic acid residue per about 14 glycosyl units, and the trailing appearance of this fraction on TLC plates is due to the complexity and microheterogeneity of the sugar chains. The presence of *N*-acetylneuraminic acid in erythrocyte and leukocyte preparations and the glycosphingolipid nature of the fractions were confirmed, respectively, by electron impact ionization mass spectrometry after permethylation and by endoglycoceramidase treatment (not shown) [for a detailed description of

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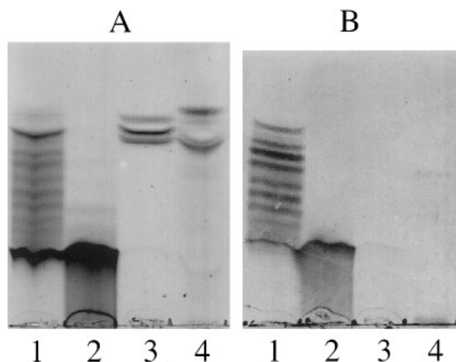

FIG. 1. Binding of influenza virus to complex glycosphingolipids on TLC plates. (A) Glycolipids visualized by 4-methoxybenzaldehyde stain for carbohydrates. (B) Glycolipids visualized by binding of the influenza virus labeled with horseradish peroxidase (HRP). Lane 1, PGCs from human leukocytes, 10 mg; lane 2, PGCs from human erythrocytes, 10 mg; lane 3, bovine brain gangliosides (a mixture of GM1, GD1a, GD1b, and GT1b; Calbiochem, USA), 6 mg; and lane 4, gangliosides from human erythrocyte membranes recovered from the upper phase after Folch's partition (*18*), 30 mg. The plates (silica gel 60 on aluminum; Merck, Germany) were developed in chloroform/methanol/water, 50:55:19 (*17*). The TLC overlay assay was performed as described in (*14*). A/Chile/1/83 (H1N1) influenza virus propagated in 10-day-old embryonated hen eggs and purified by sucrose density gradient centrifugation was labeled with HRP as described before for the preparation of fetuin–HRP conjugates (*15*), with the labeled virus being separated from unconjugated HRP by a sucrose density gradient centrifugation. The conjugation with HRP did not alter the ability of the virus to bind to sialylglycoproteins and to gangliosides compared to the initial virus preparation, as judged from their comparison in the microwell adsorption assay (data not shown). Five milliliters of the solution of labeled virus in PBS–BSA (20 mg/ml of virus protein) was applied to the TLC plate for 2 hr at 4°. After five washings with PBS, the virus binding was visualized using 3,3-diaminobenzidine as a substrate.

PGCs from human erythrocytes see (*13*)]. The binding of virus to erythrocyte and leukocyte PGCs was to the entire bands of the chromatogram, although some components seemed to be more active than others (compare lanes 1A and 1B, Fig. 1). Mild acid treatment of the PGCs abolished the binding, confirming the role of sialic acid in virus–PGC interaction (not shown). There was no binding of the influenza virus to brain gangliosides (lane 3) nor to the upper phase ganglioside fraction isolated from human erythrocytes according to the conventional procedure (*16*) and Folch's partition (*18*) (lane 4). We do not know at present if the complexity of the molecules is of importance for the binding, but it seems likely that long and branched multivalent carbohydrate chains provide better conditions for interaction with the virus than shorter simple chains.

PGCs isolated from rabbit intestine and from dog intestine and complex gangliosides from bovine erythrocytes (*19*) were also tested in the TLC overlay assay and no virus was bound under identical assay conditions (data not shown) despite the fact that they all, the same as human PGCs, had type 2 (neolacto) carbohydrate chains and contained sialic acid in comparable or higher amounts than PGCs from human erythrocytes. As detailed structural data on PGCs from different hosts are not available, we cannot rationalize for possible constraints for virus binding to rabbit, dog, and bovine preparations.

The preparation of PGCs from human erythrocytes was further tested for its ability to support the binding of different influenza virus strains in a microwell adsorption assay (*15, 20*). Figure 2 represents the data on binding of human H1, H3, and type B influenza virus isolates to PGCs, as well as to fetuin and to sialyl-α6-paragloboside (Neu5Acα6Galβ4GlcNAcβ3Galβ4Glcβ1Cer; 6-SPG). 6-SPG was isolated from the acid glycosphingolipid fraction of human meconium by chromatography on DEAE-Sepharose followed by HPLC on a silicic acid column (*21*). The structure of this glycosphingolipid was confirmed by negative-ion FAB mass spectrometry and proton NMR spectroscopy (not shown). In Fig. 2, amounts of the compounds added per well of the microplate are expressed in respect to sialic acid, to facilitate a comparison of their virus-binding activity. As shown in Fig. 2, at least an order of magnitude lower concentration of sialic acid residues of PGCs added per well is required for the same virus binding compared to 6-SPG. PGCs are also two to five times more potent receptors than fetuin. Although the values of the compound concentration in Fig. 2 may not represent the real amounts adsorbed in the wells due to a possible desorption during the washing and blocking steps, we believe that the conclusion about the high virus binding activity of PGCs is correct. Indeed, less than 50% of the fetuin is desorbed under the assay conditions as was estimated in the separate experiments with the labeled fetuin preparation (data not shown). Even with an assumption that no PGCs is desorbed at all, the binding curves still indicate the same or a better binding to PGCs compared to fetuin. In fact, substantial desorption of PGCs could be expected, as these compounds bear long highly soluble sugar chains anchored to the solid phase only via their ceramide portion. As to 6-SPG, its desorption should be lower than that of PGCs due to its poorer hydrophilicity. It can be suspected, therefore, that the binding potency of PGCs in respect to that of fetuin and to 6-SPG could be underestimated in our assay.

6-SPG is one of the most active receptor analogs for influenza viruses among various ganglioside species tested previously (*10*), while fetuin displays a binding capacity which is comparable in this assay to that of various sialylglycoproteins (*15*). It can be concluded, therefore, that human erythrocyte PGCs exposed on a membrane-like assay surface are among the most potent known binders of the influenza viruses.

PGCs are minor cell components, and their isolation from biological materials is difficult and laborious. Our present knowledge on the PGC distribution is limited to only few cells and tissues, namely to human erythrocytes

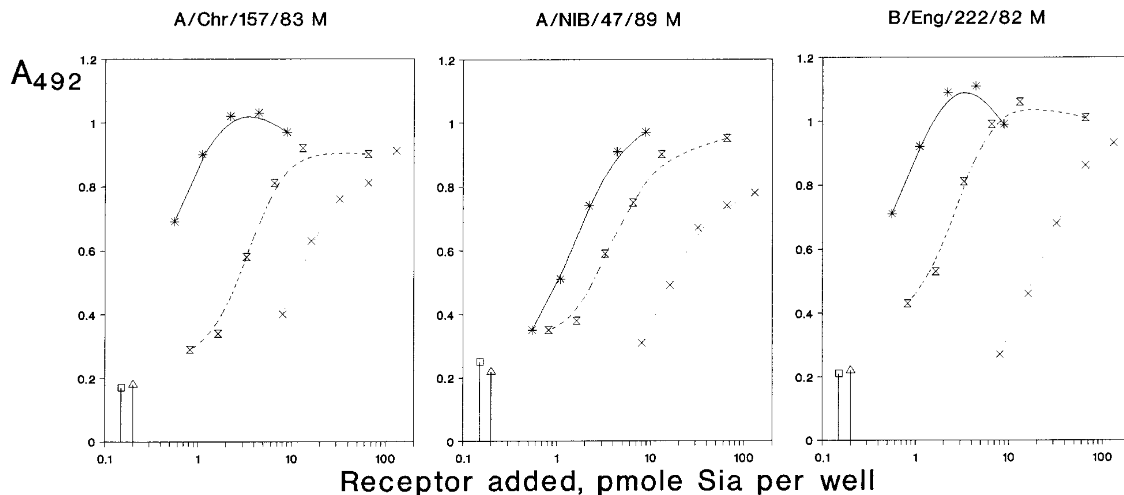

FIG. 2. Binding of influenza viruses to polyglycosylceramides (solid lines), fetuin (dashed lines), and sialyl-6-paragloboside (dotted lines) adsorbed in the wells of plastic microtiter plates. The human strains A/Chr/157/83M (H1N1), A/NIB/47/89M (H3N2), and B/Eng/222/82M isolated and propagated solely in MDCK cells (*22–24*) were used. For the binding assay, 0–20 ng PGCs from human erythrocytes (*13*) (0–8.8 pmol in respect to sialic acid) or 0–130 pmol 6-SPG from human granulocytes (*21*) dissolved in 50 ml of methanolic solution containing 500 pmol egg lecithin and 500 pmol cholesterol (auxiliary lipids used to provide for the natural membrane-like environment) was added to wells of polyvinylchloride microtiter plates (Cook, USA) prepared in triplicate. Fetuin (Sigma Chemical Co., USA) was added in 50-ml solutions in water (0–0.25 mg of the protein per well, which corresponded to 0–65 pmol of bound sialic acid residues (*25*)). For negative controls, the wells without coating (squares) and those coated with auxiliary lipids alone (triangles) were used. The plates were incubated at 37° for 4–5 hr for evaporation of methanol and water. One hundred microliters of 0.2% solution of BSA in phosphate-buffered saline (0.2% BSA) was added per well and incubated for 1 hr at 37°. The wells were emptied and 50 μl of virus suspensions in 0.2% BSA with a hemagglutinating titer of 32–128 was added. The plates were incubated at 4° for 2 hr for virus adsorption, rinsed three to four times with ice-cold washing solution (WS: 0.2× PBS–0.02% Tween 80), and further incubated with 50 μl/well of fetuin–horseradish peroxidase conjugate (*15*) in PBS–0.02% Tween 80 for 0.5 hr at 4°. Unbound conjugate was removed by six washings with WS, and the amount of bound conjugate was quantified using *o*-phenylenediamine as a substrate.

and leukocytes, rabbit erythrocytes, human placenta and pancreatic carcinoma cells, pig stomach mucosa, and rabbit and dog intestine (*11–13* and references therein; Miller-Podraza and Karlsson, in preparation); their presence in the cells permissive for influenza virus infection has not been studied. Although PGCs contain regular core poly-*N*-acetyllactosamine chains, the detailed structural elucidation of PGC molecules is complicated. The lack of definitive structural data hampers rationalization of a high virus-binding activity of PGCs from human erythrocytes and leukocytes. The effect may be due to a presentation of sialic acid moieties in a favorable context of penultimate sugar ring(s) (*26*), to the creation of high-affinity bivalent sialo-sugar determinants (*27*) on the neighboring branches of the PGC core, or to easy access of the virus to the sialo-sugar epitopes exposed on the distal end of long and flexible oligosaccharide sequences of PGCs. Whichever is the case, PGCs seem to be worthy of further studies for their relevance as biological receptors of influenza virus. Compared to sialylglycoproteins, they may create binding sites in closer proximity to the cell surface, a feature of possible importance for fusion of the viral envelope with the cellular membranes.

In addition to mediating infection of susceptible cells, viral binding to cell-surface sialoglycoconjugates may be important for antiviral immune response (*28*), activation of neutrophils (*29*), and pyrogenic activity of influenza viruses (*30*). In future studies on the molecular mechanisms of these effects a possible involvement of PGCs should, therefore, also be considered.

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