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Influenza virus utilizes *N*-linked sialoglycans as receptors in A549 cells

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ABSTRACT

Influenza viruses (IFVs) recognize sialoglycans expressed on the host cell surface. To understand the mechanisms underlying tissue and host tropisms of IFV, it is essential to elucidate the molecular interaction of the virus with the host sialoglycan receptor. We established and applied a new monoclonal antibody, clone HYB4, which specifically recognizes the Neu5Acα2-3 determinant at the non-reducing terminal Gal residue of both glycoproteins and gangliosides to investigate the biochemical properties of IFV receptors in A549 cells. HYB4 significantly blocked virus binding to A549 cells in a dose-dependent manner. Virus overlay assay indicated that several glycoproteins with molecular masses of 80–120 kDa of A549 cells were commonly recognized by different subtypes of IFV, such as H1N1 and H3N2. H1N1 virus binding to the glycoproteins was diminished by pretreatment with either sialidase or PNGase *F*. On TLC-immunostaining experiments with HYB4, GM_3 ganglioside was only detected in A549 cells. Interestingly, this antibody bound to GM_3 gangliosides on TLC and plastic surfaces, but not on lipid bilayers. In comparison with the recognition of *Maackia amurensis* lectins, HYB4 exclusively recognized Neu5Acα2-3Galβ1-4GlcNAc residues expressed on glycoproteins. These results strongly suggest that *N*-linked sialoglycans with the Neu5Acα2-3 determinant on several glycoproteins are receptors for influenza virus in A549 cells.

1. Introduction

Many proteins and lipids on the cell surface membrane and secreted proteins are modified by oligosaccharide chains, which are structurally diverse. Specific glycan structures are intimately involved in not only biological phenomena but also pathogenesis *in vivo*. Sialoglycans such as glycoproteins and glycolipids that contain a carbohydrate residue termed sialic acid (Sia) on the cell surface are known to be involved in the progression of cancer and other diseases [1–3].

Previous studies indicated that sialoglycans account for receptor recognition by pathogenic microorganisms [4,5]. The sialoglycans are thought to function as host-specific receptors in host tropism of influenza virus (IFV). At the initial stage of IFV infection, the interaction between the viral spike protein termed hemagglutinin (HA) and specific sialoglycans on the host cell membrane triggers endocytosis and membrane fusion for virus entry [6,7]. Sialic acid linkages to non-reducing terminal Gal are related with IFV receptor recognition. Human and avian IFVs bind to sialoglycans with non-reducing terminal Siaα2-6Gal and Siaα2-3Gal residues with high affinity, respectively [8,9]. In recent years, several lines of evidence suggested that the internal carbohydrate structures of sialoglycans may also affect the receptor recognition of IFVs [10,11]. Both binding preference and affinity of IFVs with characteristic sialoglycans contribute to interspecies transmission between humans and other animals, such as chickens and pigs [12].

Investigation of effective protection strategies based on the host factors involved in IFV infection will lead to improved control of this disease. Comprehensive research regarding the host factors involved in IFV infection, such as determination of the nature and tissue distribution of the receptor molecules, including sialoglycans and specific proteins and lipid aglycons, is expected to contribute to the establishment and development of preventive strategies for control of IFV epidemics, such as the development of novel anti-influenza drugs with new mechanisms of action.

Reaction probes specific for sialoglycans are effective tools for IFV receptor research. Plant lectins have been widely used for the detection of sialoglycans. *Maackia amurensis* hemagglutinin (MAH) and mitogen (MAM), and *Sambucus sieboldiana* and *Sambucus nigra* agglutinins (SSA and SNA, respectively) have been used for the detection of Siaα2-3 and Siaα2-6 residues by biochemical and fluoromicroscopic analyses. MAM, which promotes lymphocyte proliferation activity *in vitro*, and MAH, which induces

Abbreviations: IFV, influenza virus; HA, hemagglutinin; Sia, sialic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; SPR, surface plasmon resonance; TLC, thin-layer chromatography; MAH, *Maackia amurensis* hemagglutinin; MAM, *Maackia amurensis* mitogen; SSA, *Sambucus sieboldiana* agglutinin.

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hemagglutination activity of erythrocytes, showed distinct carbohydrate recognition specificity as follows [13–15]. MAM binds to Siaα2-3Galβ1-4GlcNAc and SO_4^{3-}-3Galβ1-4GlcNAc residues with relatively high affinity, and Galβ1-4GlcNAc and SO_4^{3-}-3Gal residues with low affinity [16–21]. MAH shows preference for Siaα2-3Galβ1-3GalNAc over SO_4^{3-}-3Galβ1-3(±Siaα2-6)GalNAc and SO_4^{3-}-3Gal residues [19,22–25]. Previously researchers have designated MAM with different abbreviations, including MAL, MAL-I, MAA-1, and MAA [23,24,26,27]. MAH has also been referred to as MAL-II, MAA-2, and MAA [21,28,29]. This diversity of naming of the two lectins, MAH and MAM, has resulted in confusion in glycobiological research. In addition, these two lectins showed distinct glycan recognition, resulting in difficulties in appropriate evaluation and comparison in previous studies.

In the present study, we established and applied a new monoclonal antibody, clone HYB4, against Neu5Acα2-3Galβ1-4GlcNAc residues for IFV receptor research. To resolve the technical limitation of the current probes used for detection of sialoglycans, we characterized the biochemical properties of the antibody and evaluated its benefits as a reaction probe for sialoglycans. Using HYB4, we concluded that *N*-linked sialoglycoproteins with Neu5Acα2-3Galβ1-4GlcNAc residues predominantly act as IFV receptors on A549 cells.

2. Materials and methods

2.1. Cell culture and virus propagation

A549 cells were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). CHO-K1 and a mutant cell line, Lec2, were cultured at 37 °C under 5% CO_2 in alpha minimal essential medium (αMEM) supplemented with 10% FBS. Human and avian influenza virus strains used in this study were propagated and purified as described previously [30]. Aliquots of the virus were stored at −80 °C before use.

2.2. Cell-surface virus binding assay

Binding of viruses to cultured cells was performed as described previously [31]. Briefly, cells were seeded onto 96-well plates and cultured at 37 °C in appropriate medium supplemented with 10% FBS. After the cells were fixed at room temperature for 20 min with phosphate-buffered saline (PBS) containing 2% paraformaldehyde, virus was inoculated onto the cells at the indicated titer for 16 h at 4 °C. In the antibody inhibition experiments, virus (hemaggulutination unit, HAU = 2^8) was premixed on ice with antibodies at the indicated concentrations. The virus–antibody premixtures were inoculated immediately. After washing thoroughly, sialidase activity associated with the bound viruses were determined by the incubation with PBS containing 40 μM 2-(4-methylumbelliferyl)-α-d-*N*-acetylneuraminic acid as substrate. The fluorescence intensity was measured with excitation and emission wavelengths of 355 and 460 nm, respectively. The virus binding activity was determined from the quantity of virus antigens associated with the cell surface.

2.3. Preparation and lysis of membrane-bound proteins of A549 cells

Protein preparations described below were carried out at 4 °C or on ice. A549 cells (approximately 2–3 × 10^7 cells) were harvested and washed thoroughly with ice-cold PBS. For preparation of membrane-bound proteins, the cells were suspended in sonication buffer containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), and protease inhibitor cocktail (P8465; Sigma–Aldrich, St. Louis, MO), and pulse-disrupted using an ultrasonicator. The solution was centrifuged, and the sedimented membrane-bound proteins were lysed with 100 μl of lysis buffer containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 1% SDS, and protease inhibitor cocktail. The solution was again pulse-disrupted using an ultrasonicator and centrifuged. The supernatant was heat-treated under authentic reducing conditions, and resolved on 10% separation gels by SDS–PAGE [32]. The proteins resolved on the gel were blotted onto a PVDF membranes, followed by detection with HYB4, lectins, and IFVs.

2.4. Virus overlay assay

Virus overlay assay was carried out for detection of glycoproteins on PVDF membranes with IFVs. The blotted PVDF membranes were blocked at room temperature for 1 h with solution A [PBS containing 1% ovalbumin and 1% polyvinyl pyrrolidone (PVP)]. The membranes were washed 3 times for 5 min each time with ice-cold PBS and then incubated at 4 °C overnight in solution A containing biotinylated influenza virus (2^7 HAU). After washing 3 times for 5 min with ice-cold PBS, the membranes were then reacted at 4 °C for 2 h with alkaline phosphatase (ALP)-conjugated streptavidin in solution B (PBS containing 3% PVP). After washing 3times for 5 min with PBS, the membranes were visualized using Western blue reagent (Promega, Madison, WI).

2.5. Preparation of acidic lipid fractions

A549 cells (approximately 10^8 cells) were harvested and washed thoroughly with ice-cold PBS. Whole lipids were extracted with 30 ml of a mixture of chloroform/methanol/water (5:5:1, by vol.). The extracts were evaporated and subjected to DEAE-Sephadex A-25 column chromatography as described previously [33]. The acidic lipid fraction was prepared by elution with chloroform/methanol/0.8 M sodium acetate (30:60:8, by vol.). The fraction was dissolved in 0.2 ml of chloroform/methanol (1:1, by vol.). The lipids were then subjected to characterization of antibody binding properties by the TLC-immunostaining method.

2.6. Surface plasmon resonance analysis

All analyses of interactions between GM_3 ganglioside and antibodies, HYB4, or D4 were performed at 25 °C on a BIAcore 2000 (BIAcore AB, Uppsala, Sweden) using a sensor chip L1 [34]. For capture of lipid bilayer vesicles on the sensor chip, 0.5 mM total lipids containing 1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine (POPC) and cholesterol with or without 2.5% relative molar ratio of GM_3 ganglioside with respect to phospholipid were mixed and evaporated to form a lipid film. The lipid film was suspended in HEPES-buffered saline (pH 7.4) and sonicated briefly. The solution containing POPC, and cholesterol with or without GM_3 ganglioside were subjected to LiposoFast-basic (Avestin, Inc., Ottawa, Canada) using a membrane filter with a pore size of 50 nm according to the manufacturer's instructions [35]. The obtained solution was injected at a flow rate of 5 μl/min until around 7000 RUs as immobilized ligands. To stabilize captured lipid bilayers, 50 mM sodium hydroxide and 10 mM glycine buffer (pH 1.5) were successively injected. The lipid solution without GM_3 ganglioside was immobilized as a reference surface. Aliquots of 50 μl of antibody at concentrations of 50–400 nM were injected at a flow rate of 20 μl/min onto the surfaces of the sensor chip and the dissociation phase was monitored for 250 s postinjection. The sensor chip was regenerated by successive injections of 50 mM sodium hydroxide and 10 mM glycine–HCl (pH 1.5). Response curves were subtracted from the background signals generated from reference surfaces. Kinetic parameters were obtained by global fitting of the sensorgrams to a 1:1 (Langmuir) binding model using BIAevaluation 3.1 software. Two independent experiments were performed for each antibody.