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Binding kinetics of influenza viruses to sialic acid-containing carbohydrates

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Abstract To elucidate the molecular mechanisms of transmission of influenza viruses between different host species, such as human and birds, binding properties of sialic acid-containing carbohydrates that are recognized by human and/or avian influenza viruses were characterized by the surface plasmon resonance (SPR) method. Differences in the binding of influenza viruses to three gangliosides were monitored in real-time and correlated with receptor specificity between avian and human viruses. SPR analysis with ganglioside-containing lipid bilayers demonstrated the recognition profile of influenza viruses to not only sialic acid linkages, but also core carbohydrate structures on the basis of equilibrated rate constants. Kinetic analysis showed different binding preferences to gangliosides between avian and human strains. An avian strain bound to Neu5Acα2-3nLc_4Cer with much slower dissociation rate than its sialyl-linkage analog, Neu5Acα2-6nLc_4Cer, on the lipid bilayer. In contrast, a human strain bound equally to both gangliosides. An avian strain, but not a human strain, also interacted with GM_3 carrying a shorter carbohydrate chain. Our findings demonstrated the remarkable distinction in the binding kinetics of sialic acid-containing carbohydrates between avian and human influenza viruses on the lipid bilayer.

Keywords Avian influenza virus · Sialic acid linkage · Lipid bilayer · Association rate constant · Dissociation rate constant · Surface plasmon resonance

Abbreviations

SPR	surface plasmon resonance
RU	resonance unit
POPC	1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine
HA	hemagglutinin

Introduction

Glycoconjugates act as receptors for various types of virulent agent involved in pathogenesis, such as bacteria and viruses [1, 2]. Some glycoconjugates containing sialic acids are dominant receptors for influenza viruses [2]. Influenza viruses infect host cells through binding of viral hemagglutinins (HAs) to sialoglycoproteins or sialoglycosphingolipids (gangliosides) as receptors on the host cell surface. The viral genome is replicated in the infected cells and viral proteins are synthesized by host translation systems. These materials are carried and assembled along vesicular trafficking systems in the cells. Finally, mature viral particles exit the host cells via the action of neuraminidase, a viral glycoprotein [2].

As aquatic birds, such as ducks and gulls, maintain all subtypes of HA (H1–H16) and neuraminidase (NA, N1–N9), influenza viruses found in humans and other animals appear to have originated in wild birds [3–6]. When avian

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and human viruses simultaneously infect an intermediate host, such as pigs, genetic recombination can occur between avian and human viruses. This can lead to the emergence of new viruses that may give rise to a worldwide flu pandemic. The 1957 (Asian flu) pandemic virus consisted of avian-derived HA (H2), NA (N2), and PB1 segment genes, and other human genes. In the 1968 (Hong Kong flu) pandemic viruses, HA (H3) and PB1 segments were derived from avian viruses in the human background [7, 8]. Another mutation mechanism is active in influenza virus. Antigenic drift of HA and NA gene segments results in production of one dominant circulating virus strain (annual epidemics), which escapes the host immune system and adapts to receptor specificity in a certain host [2, 9]. However, in 1997, purely avian H5N1 virus was found to have been transmitted directly into humans. Since 1997, other avian virus subtypes, H7N7 and H9N2, have emerged. Very recently, advanced techniques to determine gene sequences and artificially generate influenza viruses, so-called reverse genetics, demonstrated that the 1918 (Spanish flu) virus was also an avian precursor [10–12]. Interspecies transmission, especially both initial infection from avian to human and outbreaks of pandemic viruses in the human population, seems to be rare and restricted by limited adaptation of avian viruses to humans [13–15].

Sialic acid-containing carbohydrate molecules expressed on the host cell surface are possible candidates as critical determinants for both interspecies transmission and dominantly spread in a specific host. Several lines of evidence indicated that the influenza viruses recognize not only sialic acids in the receptors but also particular sugar chain structures, such as sialyllacto-series type I (Neu5Acα2-3/6Galβ1-3GlcNAcβ1-) and type II (Neu5Acα2-3/6Galβ1-4GlcNAcβ1-). There are differences among influenza viruses in the types (Neu5Ac or Neu5Gc) and linkages (α2-3 or α2-6) of sialic acid residues that they recognize [2, 16–20]. Human influenza A viruses, which have been isolated from humans for the last 30 years, preferentially recognize Neu5Acα2-6 residues. On the other hand, avian influenza A viruses predominantly bind to Neu5Acα2-3 residues. Recently, a histochemical study using lectins specific for sialic acid linkages demonstrated that characteristic distribution of sialoglycoconjugates is associated with viral transmission from avian to human. The expression profiles of Neu5Acα2-3 and Neu5Aα2-6-containing glycoconjugates are different among human respiratory organs [20]. However, the kinetic properties of receptor carbohydrates for avian and/or human influenza viruses have yet to be determined. To fully understand the molecular mechanisms on interspecies transmission of the viruses, elucidation of the binding kinetics of sialic acid-containing carbohydrates for avian and human influenza viruses is essential.

We previously isolated and characterized carbohydrate determinants that are strongly recognized by avian and human viruses, such as Neu5Acα2-3nLc$_4$ for avian viruses, and Neu5Acα2-6nLc$_4$ for human viruses [2, 21]. In this study, we investigated the binding kinetics of sialic acid-containing carbohydrates for both avian and human viruses using newly established surface plasmon resonance (SPR) assays.

Materials and methods

Materials

Cholesterol and 1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine (POPC) were purchased from Sigma-Aldrich. A sensor chip L1 was obtained from BIAcore AB. Authentic Neu5Acα2-3nLc$_4$Cer and Neu5Acα2-6nLc$_4$Cer gangliosides were purified from human meconium as described previously [22]. Neolactotetraosylceramide (nLc$_4$Cer) was prepared by hydrolysis of Neu5Acα2-3nLc$_4$Cer with *Althrobacter ureafaciens* sialidase. Ganglioside GM$_3$ was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). All other chemicals were of the highest quality commercially available.

Viruses

The human and avian influenza virus strains used in this study were propagated and purified as described previously [23].

Surface plasmon resonance analysis

All analyses of interactions between gangliosides and influenza viruses, human A/Aichi/2/6,8 (H3N2), or avian A/Duck/Hongkong/313/4/78 (H5N3) were performed at 25°C on a BIAcore 1000 (BIAcore AB, Uppsala, Sweden) using a sensor chip L1 [24]. For capture of lipid bilayer vesicles on the sensor chip, 0.5 mM of total lipids containing 2- oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (POPC) and cholesterol with or without 0.5% relative molar ratio of gangliosides with respect to phospholipid were mixed and evaporated. The dried mixture was re-dissolved in chloroform and evaporated again to form a lipid film. The lipid film was suspended in HEPES-buffered saline (pH 7.4) and sonicated briefly. Subsequently, liposomes containing POPC, cholesterol with or without gangliosides were prepared using LiposoFast-basic (Avestin) with a membrane filter with a pore size of 50 nm according to the manufacturer's instructions [25]. The liposomes were injected at a flow rate of 5 μl/min until around 8,000 RUs as immobilized ligands. To stabilize captured lipid bilayers,

50 mM sodium hydroxide and 10 mM glycine buffer (pH 1.5) were injected successively and a stable baseline was obtained. Liposomes without ganglioside were immobilized as a reference surface. Viruses as analytes were defined by hemagglutination titration, and total protein levels were quantified using a BCA protein assay kit (Pierce). The concentration of physical virus particles was calculated on the basis of an average figure of 2,883 molecules of matrix (M1) protein per virus particle [26–28]. The molecular weight of the virus was estimated as 2.5×10^8. Viruses were diluted in HEPES buffer supplemented with 2 μg/ml zanamivir to between 10^5–10^7 particles/l equivalent to 10^{-13}–10^{-11} M (approximately 10^8–10^{10} hemagglutinin spikes/l) [29]. Before injection, aliquots of virus solutions were filtrated using a filter with a pore size of 0.45 μm. Aliquots of 50 μl of virus were injected at a flow rate of 20 μl/min onto the surfaces of the sensor chip. 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. At least three independent experiments were performed for each influenza virus. Rate constants are described by the following set of equations, where A=influenza virus and B=receptor ganglioside. At t=0,

$$\mathrm{A} = \text{concentration}, \mathrm{B}[0] = \mathrm{Rmax}, \mathrm{AB}[0] = 0 \quad (1)$$

$$\mathrm{dB/dt} = -[k_{\mathrm{on}} \times \mathrm{A} \times \mathrm{B} - k_{\mathrm{off}} \times \mathrm{AB}] \quad (2)$$

$$\mathrm{dAB/dt} = [k_{\mathrm{on}} \times \mathrm{A} \times \mathrm{B} - k_{\mathrm{off}} \times \mathrm{AB}] \quad (3)$$

$$\text{Total response: AB} + \text{RI (residual bulk shift)} \quad (4)$$

Results

Immobilization of lipid bilayer containing ganglioside on the surface of L1 sensor chip

For quantitative assessment of virus binding to sialic acid-containing carbohydrate molecules, we utilized SPR to measure the interactions between virions and carbohydrates containing Neu5Acα2-3nLc$_4$ or Neu5Acα2-6nLc$_4$ residues. There are two types of sialic acid-containing glycoconjugates, such as sialoglycoproteins and gangliosides. As sialoglycoproteins carrying heterogeneous carbohydrate species on their molecules, they are not suitable materials to investigate kinetic interaction by SPR. According to our previous findings, gangliosides also showed receptor binding activity for avian and human influenza viruses [2]. Thus we used gangliosides to analyze the interaction between virions and gangliosides carrying Neu5Acα2-3nLc$_4$ or Neu5Acα2-6nLc$_4$ residues. As the ligands immobilized on the surface were gangliosides containing ceramide as a lipid portion, sensor chip L1 was chosen. The surface of L1 chip coated with a homogenous lipid bilayer containing phosphatidylcholine (POPC), cholesterol, and gangliosides was generated by lipophilic interaction of hydrophobic residues of dextran matrix and liposomes [30, 31]. Figure 1 shows the typical immobilization process of the lipid bilayer formed on the L1-sensor chip. To assess liposome binding by SPR, the sensor chip was cleaned with 20 mM CHAPS (Fig. 1, injection a), before the liposomes prepared by extrusion were injected at a concentration of 0.5 mM total lipids (Fig. 1, injection b). The surface was treated with 50 mM NaOH, followed by 10 mM glycine–HCl (pH 1.5) to remove excess liposomes (Fig. 1, injection c and d). The SPR signal was stable for at least 12 h and was not reduced by washing with 50 mM NaOH, followed by 10 mM glycine–HCl (pH 1.5) during multiple regeneration steps. As shown in Fig. 1, there were no significant differences in binding capacity when the lipid composition of liposomes was changed. Approximately 8,000 resonance units (RU) of lipids at the final signal were immobilized on the surface.

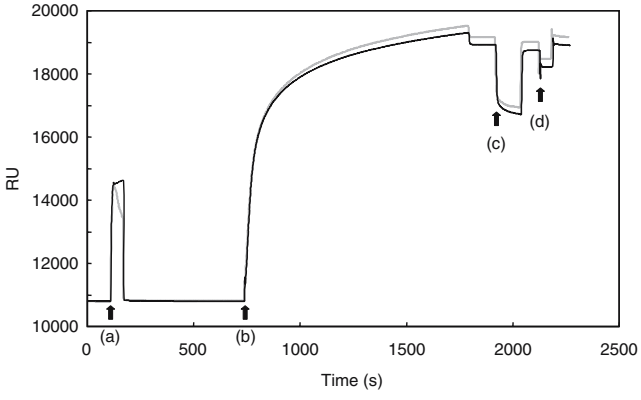

Fig. 1 Immobilization of liposomes for generation of a lipid bilayer on the L1 sensor chip surface. Preparation and immobilization of the liposomes are described in "Materials and methods". The liposomes contained POPC (350 μM) and cholesterol (150 μM) with (*black*) or without (*gray*) 0.5% Neu5Acα2-3nLc$_4$Cer (2.5 μM). The immobilization steps of liposomes are shown in sequential order: *(a)* injection of 20 mM CHAPS (20 μl/ml for 1 min); *(b)* injection of liposomes until immobilization reaches about 8,000 resonance units (5 μl/ml); *(c)* injection of 50 mM NaOH (5 μl/ml for 2 min); *(d)* injection of 10 mM glycine buffer (pH 1.5; 5 μl/ml for 1 min)

Binding kinetics of influenza virus

As shown in Fig. 2, Neu5Acα2-3nLc$_4$Cer-immobilized flow cell, but not the control flow cell (0.5% nLc$_4$Cer-containing liposome), showed specific binding of influenza virus, A/Aichi/2/68 (H3N2). The signals increased in a dose-dependent manner. Similar dose-dependent increases were observed with different influenza virus strains (data not shown). We also used 0.1% Neu5Acα2-3nLc$_4$Cer-containing liposome for immobilization on the surface. The binding signals were detected at a similar level to those in the case when 0.5% Neu5Acα2-3nLc$_4$Cer-containing liposome was used for immobilization. This suggests that the content of Neu5Acα2-3nLc$_4$Cer into lipid bilayer covered on the surface is saturated for the virus binding under the present conditions with 0.5% Neu5Acα2-3nLc$_4$Cer (data not shown).

To calculate and compare the association (k_{on}) and dissociation rate (k_{off}) constants between avian and human influenza virus strains, sensorgrams were obtained with different concentrations of the viruses and 0.5% Neu5Acα2-3nLc$_4$Cer or analogous ganglioside, Neu5Acα2-6nLc$_4$Cer (Fig. 3). We examined two influenza viruses, human A/Aichi/2/68 (H3N2) strain and avian A/Duck/HongKong/313/4/78 (H5N3) strain, independently over the three gangliosides, Neu5Acα2-3nLc$_4$Cer, Neu5Acα2-6nLc$_4$Cer, and GM$_3$. In the present study, we prepared liposomes containing very low concentration of gangliosides for immobilization onto the sensor chip. Trimers of HA are functionally expressed on virus particles and bromelain-released HA glycoprotein binds multivalently to a receptor sialo-molecule, fetuin [32]. However, since the virus, not a protein, serves as a ligand in this experiment, multiple ligand-binding to a single lipid is supposed to be improbable. Thus, a monomeric hemagglutinin (HA) of influenza virus is assumed to bind to a single sialic acid-containing carbohydrate molecule in the 1:1 model. Under our conditions, one influenza virus particle is supposed to bind to one ganglioside molecule immobilized on the surface. Therefore, the binding curves obtained from ganglioside-immobilized flow cells with both viruses were computationally fitted to the 1:1 (Langmuir) binding model using BIAevaluation software [32]. Global fitting of the data using the 1:1 model yielded a χ^2 value of <20, indicating that the fitting procedure by the Langmuir model described reasonable data. The relative association and dissociation rate constants for three independent experiments on each test solution are summarized in Table 1. As the concentration of both viruses increased, resonance signals over Neu5Acα2-3nLc$_4$Cer and Neu5Acα2-6nLc$_4$Cer gangliosides increased in a dose-dependent manner (Fig. 3a–d). Both viruses bound well to Neu5Acα2-3nLc$_4$Cer. A human strain, A/Aich/2/68, over Neu5Acα2-6nLc$_4$Cer also showed resonance signals equivalent to those with Neu5Acα2-3nLc$_4$Cer. On the other hand, higher concentrations of a duck strain, A/Duck/HongKong/313/4/78, were required for resonance signals over Neu5Acα2-6nLc$_4$Cer equal levels to those in the case of Neu5Acα2-3nLc$_4$Cer. Human virus showed similar k_{on} values between Neu5Acα2-3nLc$_4$Cer and Neu5Acα2-6nLc$_4$Cer gangliosides. The k_{off} value of a human strain to Neu5Acα2-3nLc$_4$Cer was three-fold higher than that to Neu5Acα2-6nLc$_4$Cer, indicating that the human virus detaches from Neu5Acα2-3nLc$_4$Cer more rapidly than from Neu5Acα2-6nLc$_4$Cer. In contrast, duck virus showed similar k_{on} values between Neu5Acα2-3nLc$_4$Cer and Neu5Acα2-6nLc$_4$Cer gangliosides. However, the dissociation rate constant to Neu5Acα2-3nLc$_4$Cer was 20-fold lower than that to Neu5Acα2-6nLc$_4$Cer. Thus, the duck virus bound on Neu5Acα2-3nLc$_4$Cer detaches from the surface much more slowly than that on Neu5Acα2-6nLc$_4$Cer under these conditions (Table 1). In the case of

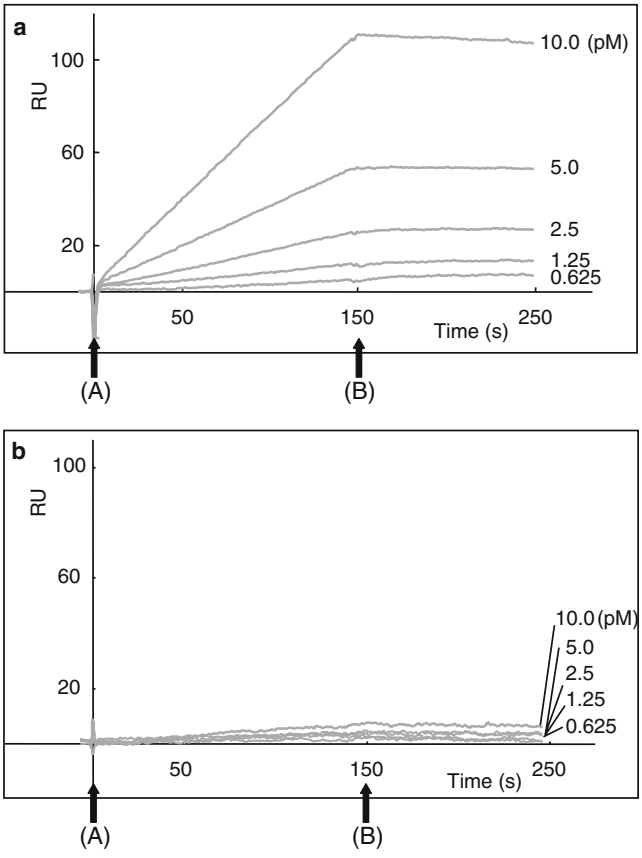

Fig. 2 Surface plasmon sensorgrams of different concentrations of influenza virus A/Aichi/2/68 binding to captured liposomes containing POPC and cholesterol with 0.5% Neu5Acα2-3nLc$_4$Cer (sensorgram **a**) or nLc$_4$Cer (sensorgram **b**). Aliquots of 50 μl of viruses at the indicated concentrations were injected, and HBS buffer was injected during the 100-s dissociation phase at a flow rate of 20 μl/min. Arrows indicate injections of influenza viruses at the indicated concentrations (*A*) and HBS buffer alone (*B*), respectively

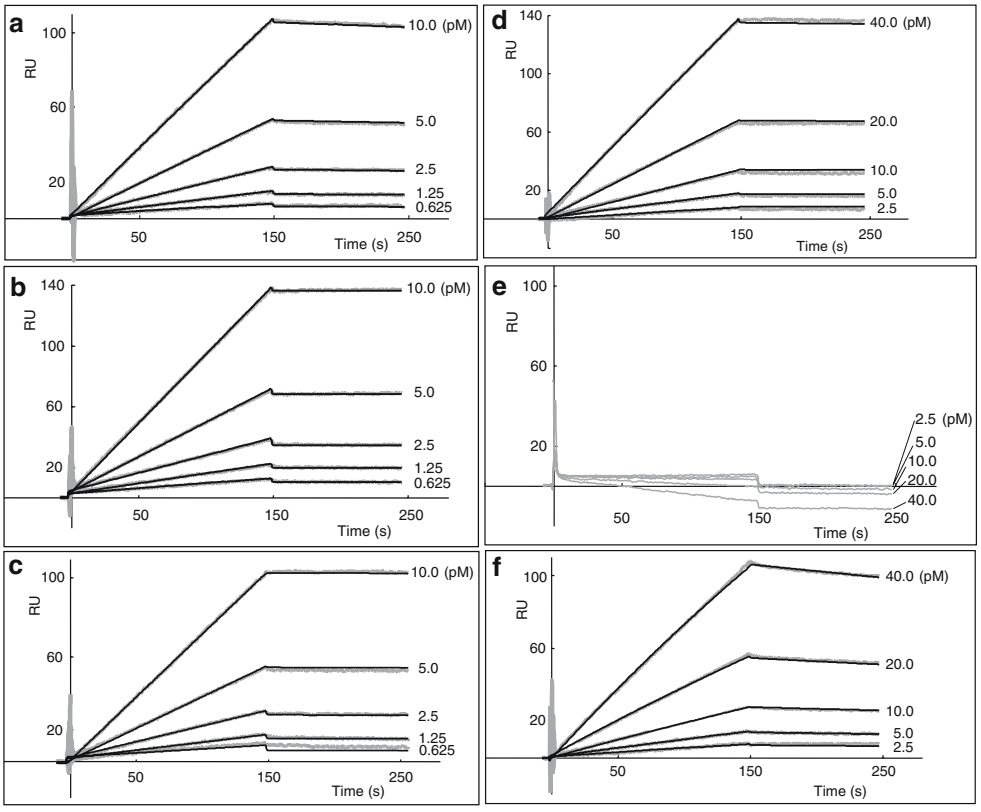

Fig. 3 Surface plasmon sensorgrams of influenza virus A/Aichi/2/68 and A/Duck/Hongkong/313/4/78 binding to captured liposomes containing POPC and cholesterol with three different gangliosides (0.5%). Aliquots of 50 μl of viruses were injected, and HBS buffer was injected during 100-s dissociation phase at a flow rate of 20 μl/min. Sensorgrams **a**, **c**, and **e** show A/Aichi/2/68 binding to L1 surface expressing Neu5Acα2-3nLc$_4$Cer, Neu5Acα2-6nLc$_4$Cer, and GM3, respectively. Sensorgrams **b**, **d**, and **f** show A/Duck/Hongkong/313/4/78 binding to L1 surface expressing Neu5Acα2-3nLc$_4$Cer, Neu5Acα2-6nLc$_4$Cer, and GM3, respectively. Actual responses in RU are shown as gray shaded lines. They were fitted to a monovalent analyte model and depicted as *solid lines*, as described in "Materials and methods". Data shown in this figure are representative of three independent experiments for each virus yielding similar results

the GM_3 monolayer on the sensor chip, the signal of human A/Aichi/2/68 strain was abolished completely. The avian strain, A/Duck/HongKong/313/4/78, still bound to GM_3 with much higher k_{off} value than Neu5Acα2-3nLc$_4$Cer although both gangliosides contain a terminal NeuAcα2-3Gal residue (Fig. 3e, f). This suggests that duck virus bound preferentially to Neu5Acα2-3nLc$_4$Cer with longer carbohydrate core structure, as compared to GM3 ganglioside.

Table 1 summarizes the equilibrium rate constants of both viruses over three independent ganglioside surfaces. The rate constant values indicate that there are significant differences in characteristics between the avian strain, A/Duck/HongKong/313/4/78, and the human A/Aichi/2/68 strain. First, the duck virus bound to Neu5Acα2-3nLc$_4$Cer with very slow dissociation rate. Second, the duck strain, but not the human strain, bound to GM3 with 20-fold faster k_{off} value than to Neu5Acα2-3nLc$_4$Cer.

Table 1 Summary of equilibrated rate constants of influenza viruses to gangliosides on lipid bilayer

Gangliosides	Viruses	k_{on} (1/Ms)	k_{off} (1/s)
Neu5Acα2-3nLc$_4$Cer	Aichi	1.61×10^{6}	3.15×10^{-4}
	Duck	1.85×10^{6}	0.75×10^{-5}
Neu5Acα2-6nLc$_4$Cer	Aichi	2.74×10^{6}	0.90×10^{-4}
	Duck	1.15×10^{6}	1.85×10^{-4}
GM_3	Aichi	NB	NB
	Duck	0.69×10^{7}	2.36×10^{-4}

Rates constants are calculated according to the 1:1 binding model. Values are shown as averages for three independent experiments yielding similar results.
Aichi A human strain, A/Aichi/2/68 (H3N2); *Duck* A/Duck/HongKong/313/4/78 (H5N3); *NB* Not bound

Taken together, SPR analysis with ganglioside-containing lipid bilayers effectively demonstrated the recognition profile of influenza viruses to not only sialic acid linkages but also core carbohydrate structures on the basis of equilibrated rate (k_{off} value).

Discussion

To assess the binding properties of endogenous carbohydrate molecules to influenza viruses, we utilized SPR analysis in this study. Recently, several studies indicated that SPR analysis could be useful to determine interaction of not only micro-organisms such as viruses and bacteria but also cells with their ligand molecules [33–37]. Hence, we applied this technology for analysis of the interaction between whole influenza viral particles and gangliosides, which were determined to be binding molecules by TLC. Critchley and Dimmock [35] measured the concentrations of physical virus particles and the molecular weights of viruses were determined. The binding of influenza viruses was analyzed over lipid bilayers containing the gangliosides constituted on L1-sensor chips [31].

The avian influenza virus strain, A/Duck/HongKong/313/4/78, bound to Neu5Acα2-3nLc$_4$Cer-containing surface with much slower dissociation rate than Neu5Acα2-6nLc$_4$Cer, suggesting that duck virus interacts with Neu5Acα2-3 residue with higher affinity as suggested previously [38, 39]. In contrast, human virus showed dual binding specificity for both Neu5Acα2-3nLc$_4$Cer and Neu5Acα2-6nLc$_4$Cer on lipid bilayers. Previous studies using cell-based and/or virus-overlay assays showed that human viruses bind preferentially to Neu5Acα2-6nLc$_4$Cer, but not Neu5Acα2-3nLc$_4$Cer. Some studies also demonstrated that human viruses interact with Neu5Acα2-6nLc$_4$Cer with higher affinity than with Neu5Acα2-3nLc$_4$Cer [38, 40, 41]. There are possible explanations for the discrepancies between our findings and these previous results. Previous cell-based assays were performed using sialidases and linkage-specific sialyltransferases to generate homogenous sialic acid residues with binding activity to influenza viruses. However, such assays may be affected by uncontrolled enzyme reactions and may sometimes cause false interactions either positively or negatively. The others primarily employed synthetic analogs of receptor carbohydrates or non-endogenous carbohydrates, such as serum glycoproteins, as competitive inhibitors for affinity measurement of viruses. These studies did not directly indicate binding properties between endogenous receptor carbohydrates and viruses. Our assay with SPR technology allowed real-time detection of the interaction of viral particles with receptor gangliosides exposed on lipid bilayers and direct determination of binding properties, such as binding kinetics (k_{on} and k_{off}). Indeed, this system revealed a unique binding character in that avian virus hardly detaches from lipid bilayers containing Neu5Acα2-3nLc$_4$Cer. There are additional advantages of our SPR assay. Our method is capable of detecting very small amounts of viruses. As virus particles can be used directly for the measurement, it is not necessary to generate recombinant proteins. Thus, our method is much more effective with regard to cost, time, and effort than those reported by other methods [32, 39]. Finally, SPR instruments are closed and so the system can provide a high level of biosafety.

We also observed differences in binding specificity, in that avian virus bound to GM3 ganglioside that contains Neu5Acα2-3 linkage attached to a shorter carbohydrate chain (Galβ1-4Glc) than nLc$_4$Cer. A previous study also suggested that avian-type viruses bind preferentially to Neu5Acα2-3 linkage attached to short carbohydrate chains using synthetic carbohydrates [39]. Although the duck virus seems to have lower affinity with GM3 ganglioside than with Neu5Acα2-3nLc$_4$Cer, a specific interaction was observed between GM3 ganglioside and avian virus, but not the human virus. This characteristic binding may help us judge transmission and expansion of avian influenza viruses circulating in wild birds to the human population. Further studies using SPR are required to establish an effective and exhaustive assay to understand the binding preferences of a variety of avian viruses.

Previously, using SPR technology, the binding of influenza virus hemagglutinin (BHA) rosettes, which were prepared by cleavage with bromelain, was determined to a sialic acid-containing glycoprotein, fetuin, immobilized on a sensor chip [32]. When a soluble protein, BHA rosette was injected over fetuin-immobilized surfaces, specific interaction was observed. However, k_{on} value of BHA over fetuin is about 1000-fold lower than that of virus particle over ganglioside on lipid bilayer in this study. There are three possible explanations. First, gangliosides into lipid bilayer on L1-sensor chip may give clustering effect of sugar chains as described previously [35, 42], resulting in enhancement of interaction between virus particles and gangliosides. Second, multivalency of HA trimers on viral envelope may allow high affinity interaction through avidity effects. Third, fetuin contains three complex N-glycosylated sugar chains with both Neu5Acα2-3 and Neu5Acα2-6 residues [43]. The structures of oligosaccharides are heterogeneous and complicated. Heterogeneity of carbohydrate structures might affect binding affinity of influenza viruses.

In conclusion, we determined unique binding properties of sialic acid-containing carbohydrates by SPR techniques. Differences in the binding of influenza viruses to three gangliosides can be monitored in real-time and correlated with receptor specificity between avian and human viruses. In-depth binding studies to the receptor carbohydrates by the SPR technique will be helpful to survey the emergence of new pandemics or epidemics.

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Abbreviations Glycolipids and carbohydrates are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Lipids* (1977) 12:455–468). Gangliosides are abbreviated according to Svennerholm (*J. Lipid Res.* (1964) 5:145–155).

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