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Binding of influenza A virus to monosialoganglioside (GM_3) reconstituted in glucosylceramide and sphingomyelin membranes

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Abstract

The binding of influenza A virus to GM_3-containing monolayers at an air/water interface was quantitatively investigated by use of a quartz crystal microbalance (QCM). A QCM was horizontally attached to the monolayer from the air phase and the binding behavior of influenza virus was followed by the frequency changes of the QCM. GM_3 was reconstituted in the momolayer of sphingomyelin (SM) or glucosylceramide (GlcCer). When the mole fraction of GM_3 was below 30 mol%, the binding rate of the influenza A virus to the GM_3/GlcCer membrane was significantly faster than that to GM_3/SM membranes. When the mole fraction of GM_3 in SM was below 20 mol%, specific binding of influenza virus was not observed at all. Binding of the virus to the GM_3/GlcCer mixed membrane was inhibited by the addition of sialyllactose (Neu5Acα2-3Galβ1-4Glc). The virus binding was also visually observed by scanning electron microscopy. Viruses selectively bound to GM_3/GlcCer (20:80, by mol%) membrane, but not to GM_3/SM (20:80, by mol%) membrane. Furthermore, it was suggested that specific binding of influenza virus to the GM_3/GlcCer membrane induced the changes in morphology of virus. It was clearly demonstrated that binding of influenza virus to GM_3 was influenced by matrix lipids surrounding GM_3.

Keywords: Ganglioside; Sphingomyelin; Glucosylceramide; Quartz crystal microbalance; Air/water interface monolayer; Influenza A virus

1. Introduction

Gangliosides are known as receptors for influenza viruses. Specificities of gangliosides to influenza viruses have been investigated by ganglioside-coated erythrocyte/virus-binding assay or thin-layer chromatography/virus-binding assay [1]. Many epitopes of gangliosides against viruses were determined by these methods. For example, influenza A virus [A/PR/8/34(H1N1)] can bind to GM_3, sialyl Lewis^X, GM_{1b}, but can not bind to their derivatives and other gangliosides [1]. Gangliosides usually exist surrounded by various kinds of lipids such as a phosphatidylcholine or a neutral glycolipid in plasma membrane. Recognition of ganglioside is expected to be modulated by such a matrix lipid. However, we could not know the influence of membrane composi-

Abbreviations: GM_3, *N*-acetylneuraminylgalactosylglucosylceramide; SM, sphingomyelin; GlcCer, glucosylceramide; LacCer, lactosylceramide; DSPC, distearoylphosphatidylcholine; Neu5Ac, *N*-acetylneuraminic acid; Gal, galactose; Glc, glucose; WGA, wheat germ agglutinin; QCM, a quartz crystal microbalance; SEM, scanning electron microscopy.

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tion on the receptor functions of gangliosides by the previous methods.

Liposomal membranes and monolayers have been used as a biomembrane model. In ganglioside-lecithin liposome, production of complex phase behavior with increasing mole fraction of ganglioside has been observed [2,3]. On the other hand, the ganglioside-containing air/water interface monolayer is very stable and gives a simple membrane structure at every mole fraction of GM_3 [4–6]. The well-defined monolayer membranes are considered to be the more suitable models to study the influence of mole fraction of ganglioside and lipid composition on the virus binding to GM_3-containing mixed membrane.

Studies in binding of proteins to a lipid monolayer have been carried out by surface tension measurement [7–9], surface plasmon resonance [10], fluorescence-labeling technique [11–13] and radio-labeling technique [8,14], and so forth. In the previous papers [4,5,15,16], we reported a new method to detect directly the binding of lectins to a specific glycolipid monolayer at the air/water interface monolayer on which a quartz-crystal microbalance (QCM) was attached horizontally in the air phase. Binding amounts and initial binding rate of sialic acid-specific lectin (wheat germ agglutinin, WGA) to a GM_3 reconstituted in distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), glucosylceramide (GlcCer), and lactosylceramide (LacCer) was obtained from frequency decrease (mass increase) of the QCM [4,5]. These values for the GM_3/GlcCer and the GM_3/GlcCer mixed membranes were found to be significantly higher than those for the GM_3/SM and the GM_3/DSPC mixed membranes. These results suggest that the recognition of sialic acid on GM_3 was regulated by the surrounding matrix lipids.

In this study, binding of the influenza A virus to GM_3 reconstituted in SM and GlcCer monolayers is investigated by using QCM (Fig. 1) and scanning electron microscopy. The binding behavior of virus to GM_3-containing membranes was found to be influenced by the mole fraction of ganglioside and by the membrane composition.

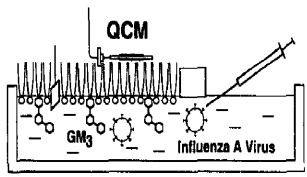

Fig. 1. An experimental setup of a QCM attached horizontally on a ganglioside-containing monolayer.

2. Materials and methods

2.1. Materials

GM_3, GlcCer, and sialyllactose (Neu5Acα2-3Galβ1-4Glc) were obtained from Snow Brand Milk Products, Japan, and SM and lactose (Galβ1-4Glc) were purchased from Sigma, USA. Those were used without further purification. Influenza A virus [A/PR/8/34(H1N1)] was kindly given by Prof. K. Nagata (Department of Biomolecular Engineering, Tokyo Institute of Technology).

2.2. Lipid monolayer

A mixed solvent of chloroform and methanol (4:1, v/v) containing sphingolipids was spread on the 10 mM Tris-HCl buffer (pH 7.6) in a Teflon-coated trough with a microcomputer-controlled Teflon barrier (USI, Fukuoka, Japan). The solutions of the sphingolipids were used within 2 days after the preparation. It was confirmed from π–A isotherms that GM_3 mixed homogeneously with matrix lipids and did not show phase separation in matrix membranes. Since molecular areas of monolayer at the surface pressure of 30 mN m^{-1} were constant for several hours, the stability of monolayer was evidenced.

The membrane composition after spreading on the air/water interface was investigated by X-ray photoelectron spectroscopy (XPS). A lipid monolayer was transferred onto a glass plate (4.5 × 18 mm) by lifting at a surface pressure of 30 mN m^{-1}. XPS measurements were carried out by a ESCA 850M (Shimadzu, Tokyo, Japan) which is connected with a analyzer ESPAC-1000 (Shimadzu, Tokyo, Japan). For example, when the GM_3/SM (40:60, by mole before spreading) mixed membrane was investigated by XPS, the found mole fraction of GM_3 was 43 mol%, which was calculated from the peak area due to N_{1s}(choline)

and N_{1s}(amide). This indicates that the gangliosides are incorporated totally into the monolayer.

2.3. The quartz crystal microbalance (QCM)

Detection of virus binding to monolayers was carried out by a QCM technique which is basically the same method as previously reported [4,5,15,16]. A QCM plate was attached horizontally on the mixed monolayer in the air phase at a surface pressure of 30 mN m^{-1}. The frequency decrease of the QCM (mass increase) responding to the addition of influenza viruses ($1.5 \cdot 10^7$ ml^{-1}) in the subphase of 10 mM Tris-HCl buffer (pH 7.4) was followed with time. Experiments were carried out at 4°C to decrease the activity of the viral neuraminidase. The QCM employed is commercially available 9 MHz, AT-cut quartz (diameter: 9 mm) deposited with gold electrode on both sides (diameter: 4.5 mm). The QCM was connected to a handmade oscillator designed to drive the quartz at its resonance frequency at the air/water interface. The frequency changes were followed by a universal frequency counter model SC 7201 (Iwatsu, Tokyo) attached to the microcomputer system model PC 9801 (NEC, Tokyo). The following Sauerbrey's equation was obtained for the AT-cut shear mode QCM [17]:

$$\Delta F = \frac{-2F_o^2}{A\sqrt{\rho_q \mu_q}} \Delta m \qquad (1)$$

where ΔF is the measured frequency shift (Hz), F_o the parent frequency of QCM ($9 \cdot 10^6$ Hz), Δm the mass change (g), A the electrode area (0.16 cm^2), ρ_q the density of quartz (2.65 g cm^{-3}), and μ_q the shear modulus ($2.95 \cdot 10^{11}$ dyne cm^{-2}). Calibration showed that a frequency decrease of 2 Hz corresponded to a mass increase of 1 ng on the QCM electrode at the air/water interface, when the adsorption of protein was measured in an aqueous solution.

2.4. Scanning electron microscopy

A lipid monolayer was transferred onto a plastic plate (diameter: 13.5 mm, cell disk, Sumitomo Bakelite, Tokyo) by a horizontal lowering method at a surface pressure of 30 mN m^{-1}. The monolayer-coated plastic plate was moved to a plastic petri dish (diameter: 18 mm) filled with 10 mM Tris buffer (pH 7.6) without contacting it to air. Influenza virus ($1.5 \cdot 10^7$ ml^{-1}) in 10 mM Tris buffer was added to the petri dish. After soaking at 4°C for 12 h, the plate was rinsed three times in cold 10 mM phosphate buffer (pH 7.2). The viruses adhered to the lipid monolayer were fixed with 1% glutaraldehyde at 4°C for 2 h, followed by 1% OsO_4 for 1 h at 4°C. Dehydration was carried out by 50, 70, 80, 90, 95, and 99.5% ethanol, and *t*-butanol. The samples were lyophilized in *t*-butanol at −10°C (ES-2030, Hitachi, Tokyo). Au-Pd was deposited at the thickness of 10 nm (E-1020, Hitachi). The samples were examined in a Hitachi electron microscope (S-2380N).

3. Results and discussion

3.1. Detection for the virus bindings to GM_3-containing membranes by a QCM

When the experiments for virus binding to GM_3-containing monolayer were carried out at 37°C, no virus binding was observed. Suzuki et al. [1] have observed a similar phenomenon by means of erythrocyte agglutination and thin-layer chromatography. They have explained that such a phenomenon is caused by desialization of GM_3 catalyzed by membrane sialidase of the influenza virus. Since membrane sialidase is inactive at low temperature, experiments for influenza virus binding experiments are usually carried out at 4°C.

Fig. 2 shows typical time-courses of frequency changes of the QCM attached to monolayer responding to the addition of influenza viruses ($1.5 \cdot 10^7$ ml^{-1}) into the subphase at 4°C. Binding rates of virus to simple SM and GlcCer were very slow as shown in Fig. 2. The GM_3/GlcCer (20:80, mol%) mixed membrane showed large frequency decrease (mass increase) with time. On the other hand, the GM_3/SM (20:80, mol%) mixed membrane showed small frequency changes, which are almost equal to those of the SM and GlcCer matrix membranes. Significant differences in virus binding between the GM_3/GlcCer and GM_3/SM membranes were observed, even if the mole fraction of GM_3 was the same.

The mass of the influenza A virus (diameter: 100 nm) may be assumed to be $7.4 \cdot 10^{-7}$ ng per one

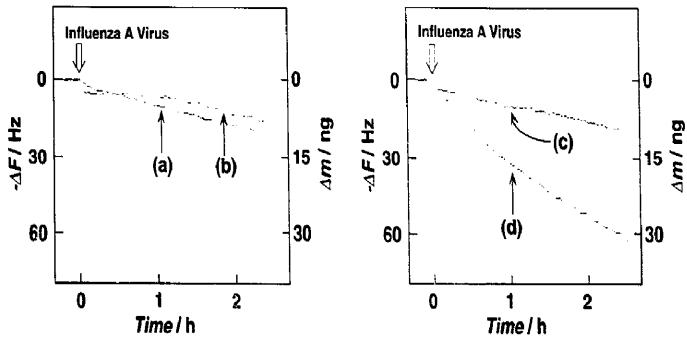

Fig. 2. Time-courses of frequency change ($-\Delta F$) and mass change (Δm) of the QCM on (a) the SM membrane, (b) the GM_3/SM (20:80, by mol%) mixed membrane, (c) the GlcCer membrane, and (d) the GM_3/GlcCer (20:80, by mol%) mixed membranes, responding to the addition of the influenza viruses ($1.5 \cdot 10^7$ ml^{-1}) in aqueous solution (10 mM Tris-HCl buffer, pH 7.6 at 4°C).

virus from the number of nucleic acids, proteins and lipids per one virus [18]. When the virus covers the membrane surface as a monolayer, a frequency decrease ($-\Delta F$) of 2400 Hz and a mass increase (Δm) of 1200 ng are expected. Frequencies of QCM gradually decreased with time responding to the addition of viruses for about one day, and reached equilibrium ($-\Delta F \approx 1000$ Hz; $\Delta m \approx 500$ ng) after two or three days. This binding amount of virus means that the virus can cover about 40% of the overall surface area at the equilibrium.

Binding rates (V_{bind}) of virus at several mole fractions of GM_3 in the monolayer were shown in Fig. 3. Significant differences between the GM_3/SM and GM_3/GlcCer membrane were observed at mole fractions of GM_3 below 20 mol%. The V_{bind} for GM_3/SM membrane raised at mole fractions of GM_3 above 30 mol%. However, virus binding to GM_3/GlcCer membranes occurred even at low mole fractions of GM_3. Influences of ganglioside mole fraction and membrane composition on virus binding were clearly demonstrated by the present experiment using monolayers. Interaction of the influenza virus with GM_3 was found to be modulated by the matrix lipid.

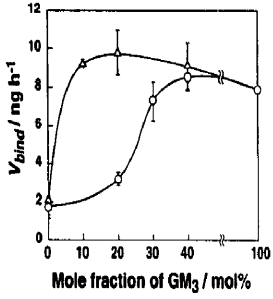

Fig. 3. Binding rates (V_{ads}) of the influenza viruses to GM_3 reconstituted in the GlcCer matrix (△) and the SM matrix (○) as a function of the mole fraction of GM_3. The data represent the means ± S.D. for three or four separate experiments.

3.2. Inhibition of virus binding by sialyllactose

Sialyllactose is expected to inhibit the virus binding to the GM_3/GlcCer mixed membrane. Sialyllactose corresponds to hydrophilic oligosaccharide in GM_3. Table 1 shows the effects of sialyllactose and lactose on V_{bind}. The presence of sialyllactose resulted in the decrease of V_{bind}, depending on the

Table 1
Binding rate (V_{bind}) of the influenza virus to the GM_3/GlcCer (20:80, mol%) mixed monolayer in the presence of sialyllactose or lactose at 4°C ($n = 3$)

Additive	Concentration (mM)	V_{bind} (/ng h^{-1})
Sialyllactose	0	10 ± 1.3
	1	6.2 ± 1.4
	2	2.0 ± 0.8
Lactose	2	11 ± 1.5

concentration of sialyllactose. In the presence of 2 mM sialyllactose, V_{bind} was decreased to 2 ng h^{-1}, which was almost the same as nonspecific binding to the SM and the GlcCer membranes (2.5 ng h^{-1}, see Fig. 2). Specific binding was completely inhibited by the addition of sialyllactose. On the other hand, 2 mM lactose had no influence on the V_{bind} values. Sialyllactose was found to be a better substrate for hemagglutinin of influenza A virus than lactose. To our knowledge, this is the first evidence that naturally occurring sialyllactose can inhibit the binding of the influenza A virus to GM_3.

3.3. Observation of viruses bound to monolayers by scanning electron microscopy (SEM)

In order to confirm that the frequency changes of QCM correspond to the virus binding, the influenza viruses bound to the GM_3-containing membranes were directly observed by SEM. Typical electron micrographs of viruses bound to lipid monolayers are shown in Fig. 4. It was clearly observed that the influenza viruses bound in a large amount to the GM_3/GlcCer (20:80, by mole) mixed membrane, compared with the SM, the GlcCer membranes and the GM_3/SM (20:80, by mole) mixed membranes. Viruses selectively bound to GM_3 reconstituted in the GlcCer matrix, but not to GM_3 in the SM matrix. The shape of viruses on the GM_3/GlcCer mixed membrane seems to be flat, and was different from the shape of the viruses bound to other membranes. It is considered that multivalent binding between GM_3 and the viral receptor caused strong interaction.

The number of viruses bound to SM, GlcCer, and GM_3/SM membranes was one or two on an average in one observed region (2 μm × 1.5 μm), when the magnification was ×60 000. On the other hand, larger number of viruses on GM_3/GlcCer membrane were

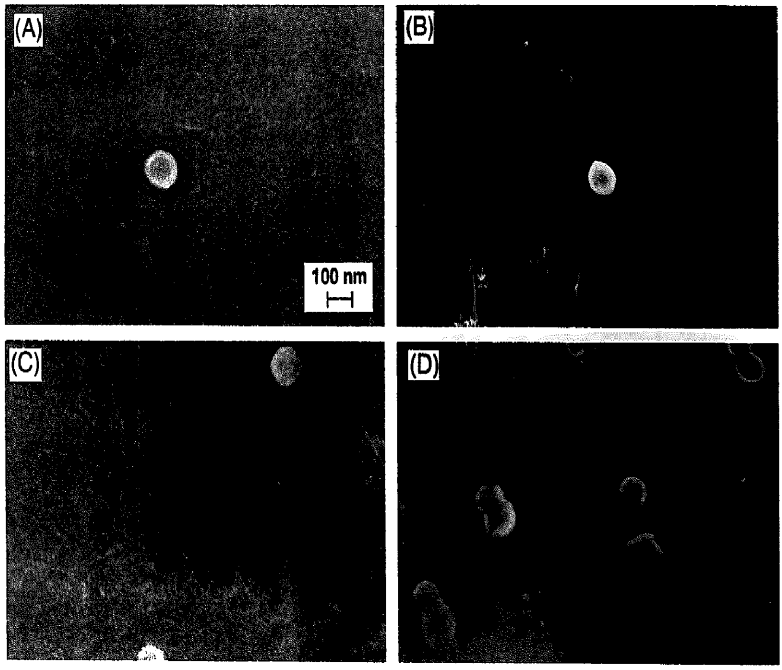

Fig. 4. Scanning electron micrographs of influenza A virus bound to (A) the SM membrane,(B) the GM_3/SM (20:80, mol%) mixed membrane, (C) the GlcCer membrane, and (D) the GM_3/GlcCer (20:80, mol%) mixed membranes.

observed at all parts of the membrane. At the lower magnification, however, the viruses on GM3/GlcCer membrane were not detected because the contrast became weak due to the flatness of the adhered viruses.

The binding amounts of viruses obtained by SEM were compared with the results of the QCM experiments. When the GM_3/GlcCer-coated plate was soaked into virus solution for 12 h, the expected binding amount of virus to the membrane was calculated to be about 130 ng from the QCM experiments ($V_{bind} = 11$ ng h^{-1}). When the viruses cover the membrane surface as a monolayer, the mass increase is theoretically calculated to be 1200 ng. Thus, the viruses are estimated to cover about 10% of the overall surface area. Also in the electron micrograph of Fig. 4D, the adhered viruses seem to occupy about 10% of the surface area. The observations by SEM were well consistent with the results of the QCM experiments.

3.4. Receptor function of GM_3 in lipid membranes

Through the present experiments, it is apparent that the recognition of GM_3 against the influenza A virus was altered by the lipid composition. Though GM_3 reconstituted in the GlcCer matrix membrane acts as receptor for the influenza A virus, recognition of GM_3 in the SM matrix membrane was cryptic when the mole fraction of GM_3 was below 30 mol%. The binding specificity of influenza A virus (H1N1 type) to GM_3 has been clearly demonstrated by TLC/virus binding assay [1,19]. However, our present study indicated that the binding behavior of influenza virus to GM_3 was depend on membrane composition and on mole fraction of GM_3 in membrane.

It has been reported that anti-GM_3 antibody can not react with a certain cell despite the cell expressing GM_3 [20–23]. Especially, Inokuchi et al. [23] have published interesting results. They have found that anti-GM_3 antibody hardly reacts with B16 melanoma cells that were treated with the blocker of glycosphingolipid synthesis and showed large loss of GlcCer and LacCer and no detectable loss of GM_3. However, since there may be a lot of unidentified factors possible to regulate the GM_3 recognition on the cell membrane, it is very difficult to discuss the reason for hidden recognition of GM_3. To study the recognition mechanism of ganglioside on the cell surface, artificial lipid membranes could be a convenient and useful model. Our investigations by ganglioside-containing monolayers strongly suggest that interaction of GM_3 with other matrix lipid is an important factor to regulate the antigenicity of GM_3 in the cell membrane. Radin [24] also described that glycosphingolipids are normally localized in the plasma membrane in the form of aggregates. If GM_3 and GlcCer form the aggregate together in the plasma membrane, GM_3 would be expected to show high recognition. When the concentration of GlcCer decreased, GM_3 may dissociate from the glycosphingolipids aggregate and associate with phosphatidylcholine [24]. Such a location of GM_3 may result in the disappearance of GM_3 recognition.

3.5. Possible mechanisms for GM_3 recognition regulated by matrix lipids

Regulation of GM_3 recognition by matrix lipids may be caused by membrane fluidity, phase separation, cluster formation or lipid–lipid interaction. In the present system, the phase-transition temperatures of the lipids employed in this study are higher than measurement temperature [25]. Phase separation was not detected from π–A isotherms of monolayer membranes [4]. Therefore the influence of membrane fluidity and phase separation may be neglected. Formation of a ganglioside cluster in lipid membranes has been demonstrated by Rock et al. [26]. They described that a glycosphingolipid is dispersed in a cluster of several molecules when present at a low mole fraction in phospholipid liposome. Formation of sugar cluster may lead to the high recognition of a sugar-binding protein since the multiantennary oligosaccharide chain often shows high recognition [27,28]. If the GM_3 cluster in the GlcCer matrix is greater than that in SM, recognition of GM_3 in the GlcCer matrix may be higher than in the SM matrix. Another possible factor is lipid–lipid interaction. Maggio et al. [6] have investigated the feature of ganglioside-containing monolayers from π–A isotherms [5]. They have demonstrated that di- and tri-sialoganglioside showed interactions with a phosphatidylcholine characterized by a decrease in mean molecular area and average surface potential per molecule. The interac-

tion was explained as the result of electrostatic interaction between anionic carboxylic acid and the positive charge of choline. In our study, however, significant differences in the changes of molecular area between the GM_3/GlcCer and the GM_3/SM mixed membranes were not observed when the mole fraction of GM_3 was less than 20 mol% (data not shown). Changes in molecular area of GM_3, that have smaller oligosaccharide headgroups than di- and tri-sialoganglioside, may not be detected by π–A isotherms. Though evidence of GM_3–SM interaction was not obtained by the preliminary experiments of the π–A isotherm, electrostatic interactions between GM_3 and SM would be expected. Low recognition of GM_3 in the SM matrix may be caused by such a lipid–lipid interaction. In the case of the GM_3/GlcCer mixed monolayer, recognition of GM_3 may not be restricted since electrostatic interaction between GM_3 and GlcCer is neglected. Though we can not discuss in detail how recognition of GM_3 is regulated by surrounding lipids, it is expected that the effects of GlcCer and SM on the recognizability of GM_3 were probably caused by the orientation of the oligosaccharides of the GM_3 molecules in the mixed membrane. This will be evidenced by further investigations such as STM, nuclear magnetic resonance or X-ray diffraction.

In conclusion, the present study demonstrates that a QCM technique combined with lipid monolayer will be a useful assay system for the detection of virus binding to lipid membranes. Furthermore, it was suggested that the investigations on the recognition of ganglioside in highly organized lipid membrane give a very important knowledge of the function of ganglioside in the cell membrane.

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