Glycoconjugate Journal 16, 223–227 (1999)

Binding affinity of GM3 lactone for influenza virus

Toshinori Sato[1]*, Megumi Ishii, Fuyuka Ohtake, Kyosuke Nagata, Takashi Terabayashi[2]
Yasuhiro Kawanishi[3], and Yoshio Okahata[1]*

[1]*Department of Biomolecular Engineering, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226-8501*
[2]*Department of Physics, Kitasato University, 1-15-1 Kitasato, Sagamihara 228-8555*
[3]*Ogata Institute for Medical and Chemical Research, 2-10-14 Higashikanda, Chiyoda-ku 101, Japan*

We investigated the interaction of GM3 lactone with influenza virus. The specific bindings of influenza virus and its hemagglutinin to GM3 lactone-containing mixed monolayers were studied by using a quartz-crystal microbalance. It has been known that gangliosides as receptors for influenza virus are also substrates for virus neuraminidase. GM3 lactone, however, was found to bind to influenza virus hemagglutinin, but not to be substrate for virus neuraminidase.

Keywords: **GM3, GM3 lactone, influenza virus, hemagglutinin, air-water interface monolayer, quartz-crystal microbalance**

Introduction

Ganglioside GM3 is known as an antigen on melanoma cells [1], a receptor for influenza virus [2], and a cell adhesion molecule [3]. When subjected to mild acidic condition, gangliosides form an internal ester, so called "lactone", between a carboxyl group of sialic acid and a hydroxyl group of neighboring saccharide [4]. Figure 1 showed the chemical structures of GM3 and GM3 lactone. Ganglioside lactones have been detected in tumor cells and normal brain [5]. An IgM monoclonal antibody established after immunization with B16 melanoma showed stronger affinity with GM3 lactone than with GM3 [6]. Therefore, ganglioside lactone is considered to be real immunogen on B16 melanoma cells.

In our previous paper, GM3 lactone showed higher binding affinity with a sialic acid-binding protein (wheat germ agglutinin, WGA) than the parent GM3 did [7]. We had also an interest in the binding affinity of GM3 lactone for influenza virus. Influenza virus is known to have membrane proteins such as hemagglutinin and neuraminidase [8]. Hemagglutinin selectively binds to sialylgalactose groups conjugated with lipids. Such the sialylglycolipids are also substrates for neuraminidase. Exploration of glycolipids that are not hydrolyzed by neuraminidase should be important to know the mechanisms for the transfection of influenza virus to host cells, and to develop inhibitors for influenza virus. In this report, we describe that GM3 lactone has a potential to bind to influenza virus without being hydrolyzed by neuraminidase.

*To whom correspondence should be addressed. Tel:+81-45-924-5782. Fax:+81-45-924-5836. E-mail: tsato@bio.titech.ac.jp

Materials and methods

GM3 and GlcCer were obtained from Snow Brand Milk Products Co., Ltd., Japan. Preparation of GM3 lactone was described in our previous paper [7]. Formation of lacton bond was observed by CD spectrometry [9]. The purity was confirmed by TLC [7]. The degree of lactonization was 90%. Major component was Neu5Acl→2 in 90%, and the rest was Neu5Acl→4 [6,10].

Influenza virus (A/PR/8/34(H1N1)) was grown in allantoic sacs of 10-day-old embryonated eggs for 48h at 35.5 °C. Virions were purified as described in the previous paper [11]. Influenza virions were deactivated by the irradiation with the two 15W-UV lights for 3 min. Plaques were not generated with such the UV-treated virions. Membrane proteins extracted from influenza virus (A/PR/8/34(H1N1)) with ether were kindly gifted by Mr. Yujirou Suzuki (The Kitasato Institute). Hemagglutinin content was determined by TLC to be 50%. Neuraminidase activity per protein was determined by using p-nitrophenyl *N*-acetylneuraminic acid, and found to be 1/500 compared with intact virus.

Hydrolysis of GM3 and GM3 lactone by influenza virus was carried out as follows. Gangliosides (10 nmol) were reacted with 1.5×10^8 influenza virus in 15 μl of 10 mM Tris-HCl buffer (pH 7.6) or 10 mM sodium acetate buffer (pH 5.4) at 37 °C and 4 °C for 1 h. After the virus neuraminidase was deactivated at 99 °C for 1 min, the virions

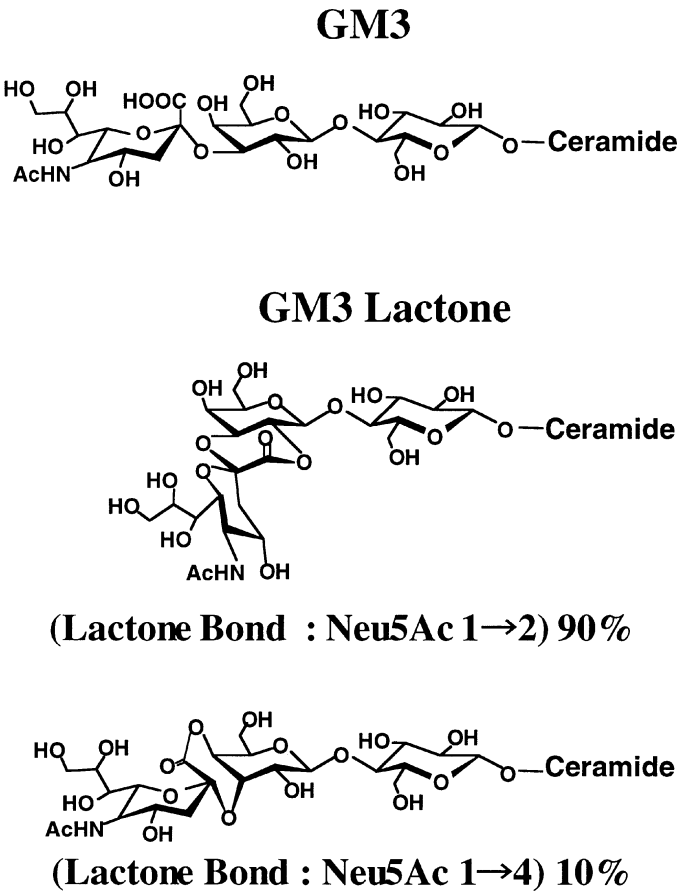

Figure 1. Chemical structures of GM3 and GM3 lactones.

were removed by centrifugation (138.000G, 45 min). The supernatants were dried by a centrifugal vaporizer. The products were dissolved with chloroform : methanol : 0.2%$CaCl_2$ aqueous solution = 5 : 5 : 1), and applied on a thin layer chromatography. Quantification of sialic acid was carried out by staining with orcinol sulfate. The degree of hydrolysis of GM3 was determined from the amount of sialic acid released from GM3.

Preparations of GM3 lactone-containing monolayers and quantitative analyses for the binding of influenza virus to the monolayers were done according to the previous paper [7,12–14]. A mixed solvent of chloroform and methanol (4: 1, v/v) containing GM3 or GM3 lactone, was spread on an aqueous solution (10 mM phosphate buffer, pH 7.2) in a Teflon-coated trough with a microcomputer-controlled Teflon barrier (USI, Fukuoka, Japan). A QCM plate was attached horizontally on the mixed monolayer. A notional illustration for an experimental apparatus is shown in Figure 2. The QCM employed is 9 MHz AT-cut quartz. Calibration showed that a frequency decrease of 1 Hz corresponded to a mass increase of 0.5 ng on the QCM electrode.

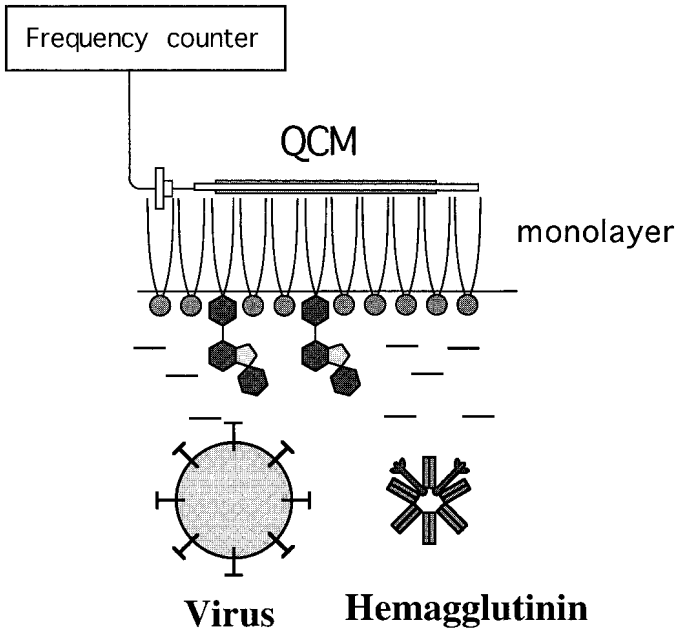

Figure 2. A notional illustration of an experimental set-up.

Results and Discussion

Figure 3 showed the percentages of GM3 and GM3 lactone hydrolyzed by influenza virus (A/PR/8/34 (H1N1)). GM3 was hydrolyzed by influenza virus depending on the pH and the reaction temperature. Optimum conditions were pH 5.4 and 37 °C, and were well agreed with literature [15–17]. On the contrary, hydrolysis of GM3 lactone was very lower than that of GM3. Though hydrolytic cleavages were slightly observed, those were due to hydrolysis of GM3 as impurity in GM3 lactone sample. It has been reported that GD1b lactone and GT1b lactone showed the resistance to neuraminidase [18,19]. Generally, ganglioside lactones may not be substrates for neuraminidase.

Binding affinities of influenza virus for GM3 lactone were measured by the same method with the previous paper [13]. The frequency decrease (mass increase) of the QCM responding to the addition of influenza viruses (1.5×10^7 ml^{-1}) in the subphase of 10 mM Tris-HCl buffer (pH 7.4) was followed with time. Figure 4A shows the typical time courses of frequency decrease of the QCM attached to the monolayers of GM3 lactone (20 mol%) and GM3 (20 mol%) reconstituted in the GlcCer matrix responding to the addition of influenza virus into the subphase. Frequency decreases due to the binding of virions continued for 1–2 days. Binding rate of influenza virus for the GM3 lactone/GlcCer mixed monolayer was almost comparable to that for the GM3/GlcCer monolayer. We have found that sialyllactose (Neu5Acα2→3Galβ1→4Glc) that corresponds to oligosaccharide in GM3 inhibited the binding of

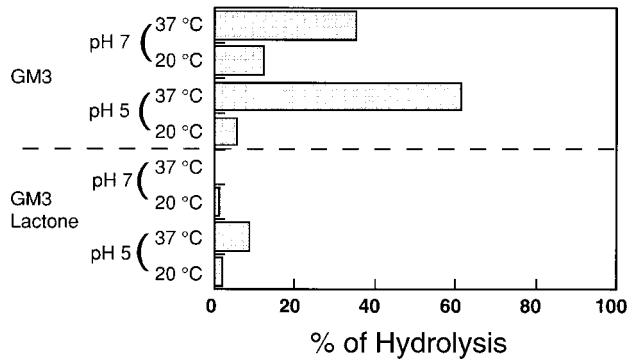

Figure 3. Percentages of GM3 and GM3 lactone hydrolyzed by influenza virus. Hydrolysis was carried out as described in "Materials and methods."

influenza virus (A/PR/8/34(H1N1)) to the GM3/GlcCer mixed membrane [13]. In this study, specific bindings of influenza virus to the GM3 lactone-containing monolayers were also completely inhibited by the addition of 2 mM sialyllactose. This result suggests that binding site of influenza virus for GM3 lactone is the same with that for the parent GM3.

Figure 4B showed the time courses of frequency decrease for GM3 lactone (20 mol%) and GM3 (20 mol%) reconstituted in the SM matrix responding to the addition of influenza virus. Binding affinity of influenza virus for the GM3 lactone/SM mixed monolayer was higher than that for the GM3/SM mixed monolayer. We have reported that the GM3-SM interaction causes conformational changes of oligosaccharide in GM3, and lowers the binding affinity of GM3 for influenza virus [13]. Lactonization of GM3, however, resulted in the recovery of the binding affinity in the SM matrix.

Binding rates (V_{bind}) of influenza virus at several mole percentages of GM3 lactone in monolayers were shown in Figure 5. In Figure 5, the V_{bind} values for GM3 lactone were compared with those for GM3 that were previously reported [13]. Figure 5A and 5B showed the binding rates of influenza virus for gangliosides (GM3 and GM3 lactone) reconstituted in GlcCer and SM matrices, respectively. In the GlcCer matrix, GM3 lactone gave slightly lower binding rates than GM3 in the range of mole percentages employed in this study. On the other hand, in the SM matrix, the V_{bind} values for GM3 lactone were higher than those for GM3, when the ganglioside contents were below 20 mol%.

It is known that GM3 binds to hemagglutinin presented on the membrane of influenza virus [8]. Thus, we investigated the interaction of GM3 lactone with influenza hemagglutinin extracted from influenza virus. Initial binding rates and maximum binding amounts of influenza hemagglutinin for 5 mol% GM3 and GM3 lactone in the GlcCer matrix were shown in Table 1. Though binding affinity of hemagglutinin for GM3 lactone was a little lower than those for GM3, specific binding was exactly observed. These results well agreed with the results of Figure 4A and Figure 5A. Furthermore, we measured the binding of neu-

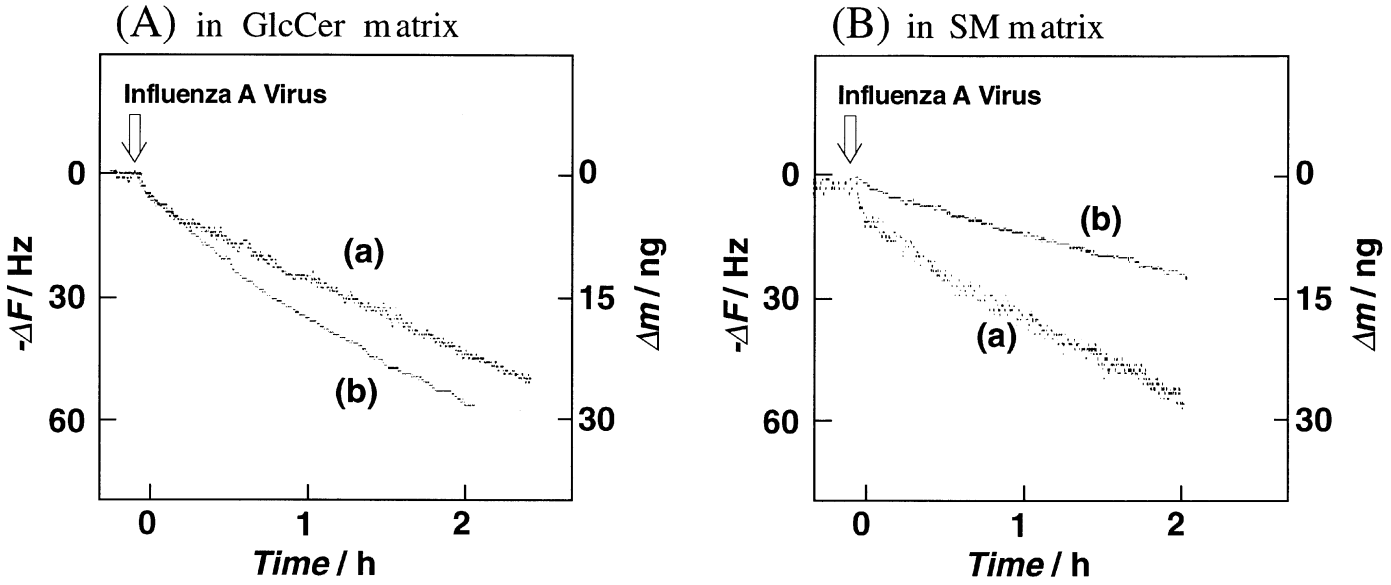

Figure 4. Typical frequency decreases of the QCM horizontally attached on the (a) 20 mol% GM3 lactone and (b) 20 mol% GM3 in (A) GlcCer and (B) SM matrices, responding to the addition of influenza A virus in 20 ml aqueous subphase at 4 °C and pH 7.6. The number of virus was 1.5×10^7 ml^{-1}.

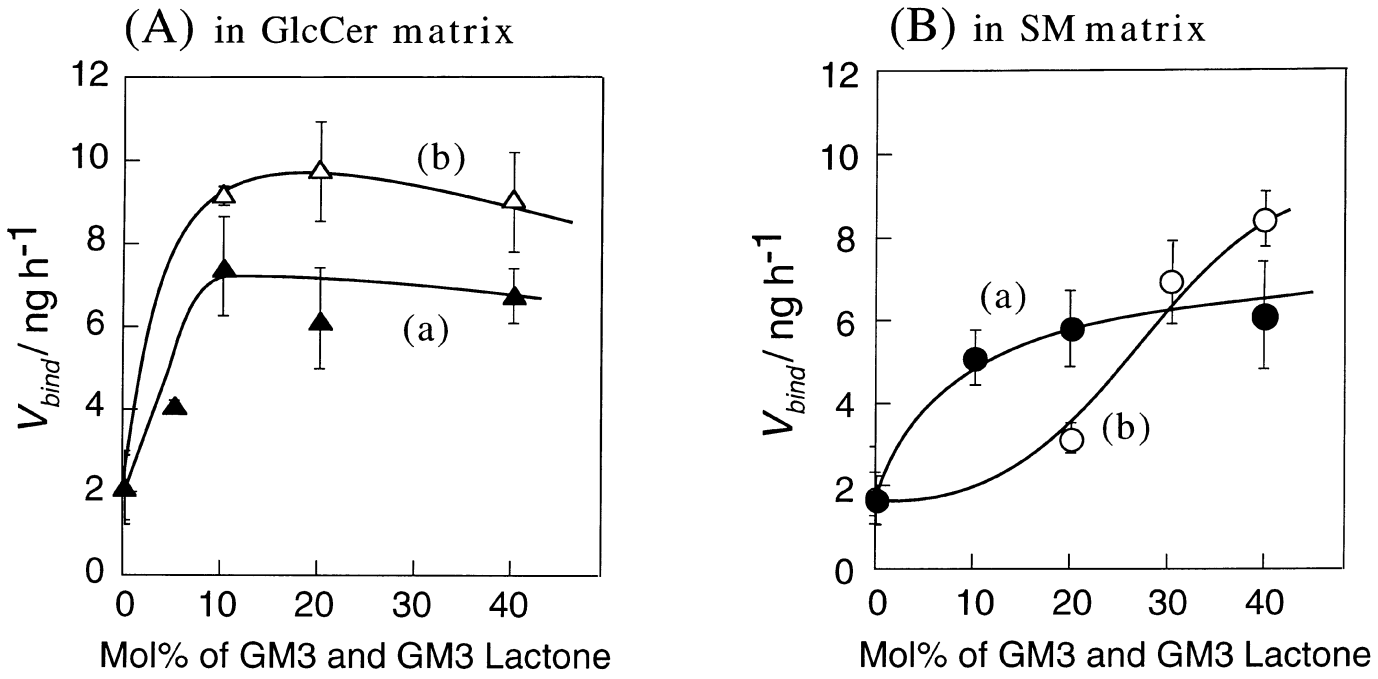

Figure 5. Binding rates (V_{bind}) of influenza virus to (a) GM3 lactone and (b) GM3 reconstituted in (A) GlcCer and (B) SM matrices as a function of GM_3 content. Binding rates(V_{bind}) are calculated from the initial slope of frequency decrease in Figure 4. The results for GM3 were partly cited from our previous paper [13].

raminidase to the GM3- or GM3 lactone-containing monolayers by the QCM method as shown in Figure 2. No bindings, however, were observed. These results suggest that binding affinity of influenza virus for GM3 lactone is due to the specificity of hemagglutinin.

According to literature, it has been considered that the carboxylate anion in sialic acid is indispensable to bind with influenza virus [8]. Thus, binding affinity of GM3 lactone for influenza virus was unexpected. The fact that GM3 lactone has binding affinity with influenza virus and its hemagglutinin, will lead to new research subject involving the analysis of binding manner between ganglioside and hemagglutinin, design of transfection inhibitors, and receptor function of ganglioside lactone.

Table 1. Initial binding rates (V_{bind}) and maximum binding amounts (Δm_{max}) of influenza hemagglutinin for GM3, GM3 lactone, and GlcCer.

	V_{bind} / 10^{-6} nmol s^{1}cm^{-2}	Δm_{max} / ng cm^{-2}
5% GM3 in GlcCer	6	1300
5% GM3 lactone in GlcCer	4.5	1100
GlcCer as control	1.5	490

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Received 22 September 1998, revised and accepted 8 December 1998.