

Inhibition of influenza A virus sialidase activity by sulfatide

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Received 20 May 2003; revised 1 September 2003; accepted 12 September 2003

First published online 25 September 2003

Edited by Hans-Dieter Klenk

Abstract Sulfatide, which binds to influenza A viruses and prevents the viral infection, was found to inhibit the sialidase activities of influenza A viruses in a pH-dependent manner. The kinetic parameters of the effect of sulfatide on the sialidase activities of human influenza A viruses using fluorometric assay indicated that sulfatide was a powerful and non-competitive type inhibitor in low-pH conditions.

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Key words: Sulfatide; Sialidase; Influenza virus; Neuraminidase; Inhibitor

1. Introduction

Neuraminidase (NA) is one of the two surface glycoproteins that play distinct roles in the life cycle of influenza A virus [1]. NA prevents the aggregation of influenza virus by removing sialic acids from the sugar chain on the newly synthesized hemagglutinin (HA) and NA. NA also promotes the release of the progeny virus from infected cells by removing sialic acids from host cell receptors [2]. Studies using NA-deletion mutant viruses have indicated that viral sialidase activity is not required for viral attachment, entry, replication or assembly [3,4]. Furthermore, characterization of influenza A virus mutants lacking sialidase activity that undergo multiple cycles of replication [5,6] and HA mutants of fowl plague viruses differing only in NA subtype [7] has indicated that a balance between HA and NA activities is important for effective replication of influenza virus. In contrast to the biological roles of NA in virus infection, the molecular mechanism by which the sialidase activity of NA in the virus infection is regulated is still not well understood.

We previously found that sulfatide binds to influenza A viruses and inhibits viral infection [8]. Sulfatide, a non-sialyl-glycolipid, is abundantly expressed at high levels in human and animal neural tissues and in the trachea and lung [9]. This sulfate-containing glycolipid is also found in the kidney, spleen, platelets, and gastrointestinal tract [10–12] and in cells of Madin–Darby canine kidney (MDCK) [13], bovine kidney (MDBK) [14], and monkey kidney (Vero), which are used for the primary isolation and cultivation of influenza A viruses [15]. Sulfatide is known to interact with extracellular matrix proteins [12,16] and adhesion molecules [10]. Sulfatide is also involved in the adhesion of human immunodeficiency virus, bacteria, and malaria parasites [9,17,18].

In the present work, we studied the effects of sulfatide on the sialidase activities of influenza A viruses. We found that sulfatide inhibited the sialidase activities of influenza A viruses in a pH-dependent manner and that the degree of inhibition by sulfatide was at least 10 times greater than that by 2-deoxy-2,3-didehydro-*N*-acetyl-*D*-neuraminic acid (Neu5Ac2en).

2. Materials and methods

2.1. Viruses

Influenza A viruses tested were propagated in the allantoic sac of 10-day-old embryonated eggs and were purified as described previously [8].

2.2. Lipids and NA inhibitors

Sulfatide and galactosylceramide (galactocerebroside) were prepared from bovine brain gray matter as described previously [8]. 2,3-Dihexadecanoyl-sn-glycero-1-phosphocholine (phosphatidylcholine), cholesterol, and Neu5Ac2en were purchased from Sigma, St. Louis, MO, USA. 4-Guanidino-2-deoxy-2,3-didehydro-*N*-acetyl-*D*-neuraminic acid (4G-Neu5Ac2en) was supplied by Glaxo Wellcome Research and Development, Stevenage, UK.

2.3. Sialidase inhibition assay

Five µl of each influenza A virus suspension (500 ng protein) in 10 mM acetate buffer (pH 4.0–6.0) or 10 mM phosphate buffer (pH 6.0–8.0) was incubated with 5 µl of sulfatide (3–200 µM), Neu5Ac2en (78–5000 µM), and 4G-Neu5Ac2en (0.03–2 µM) at 37°C for 30 min and then incubated with 5 µl of 4 mM 2'-(4-methylumbelliferyl)- α -*D*-N-acetylneuraminic acid (4-MU-Neu5Ac) (Sigma) at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of 100 mM carbonate buffer (pH 10.7). The fluorescence of released 4-methylumbelliferone (4-MU) was measured using a fluorescence spectrophotometer (Hitachi F-4010) with excitation at 355 nm and emission at 460 nm. The concentration causing 50% inhibition (IC₅₀) of sialidase activity using 4-MU-Neu5Ac was calculated graphically by plotting percent inhibition versus inhibitor concentration.

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Abbreviations: 4G-Neu5Ac2en, 4-guanidino-2-deoxy-2,3-didehydro-*N*-acetyl-*D*-neuraminic acid; HA, hemagglutinin; MDCK, Madin–Darby canine kidney; 4-MU-Neu5Ac, 2'-(4-methylumbelliferyl)- α -*D*-*N*-acetylneuraminic acid; 4-MU, 4-methylumbelliferone; NA, neuraminidase; Neu5Ac2en, 2-deoxy-2,3-didehydro-*N*-acetyl-*D*-neuraminic acid

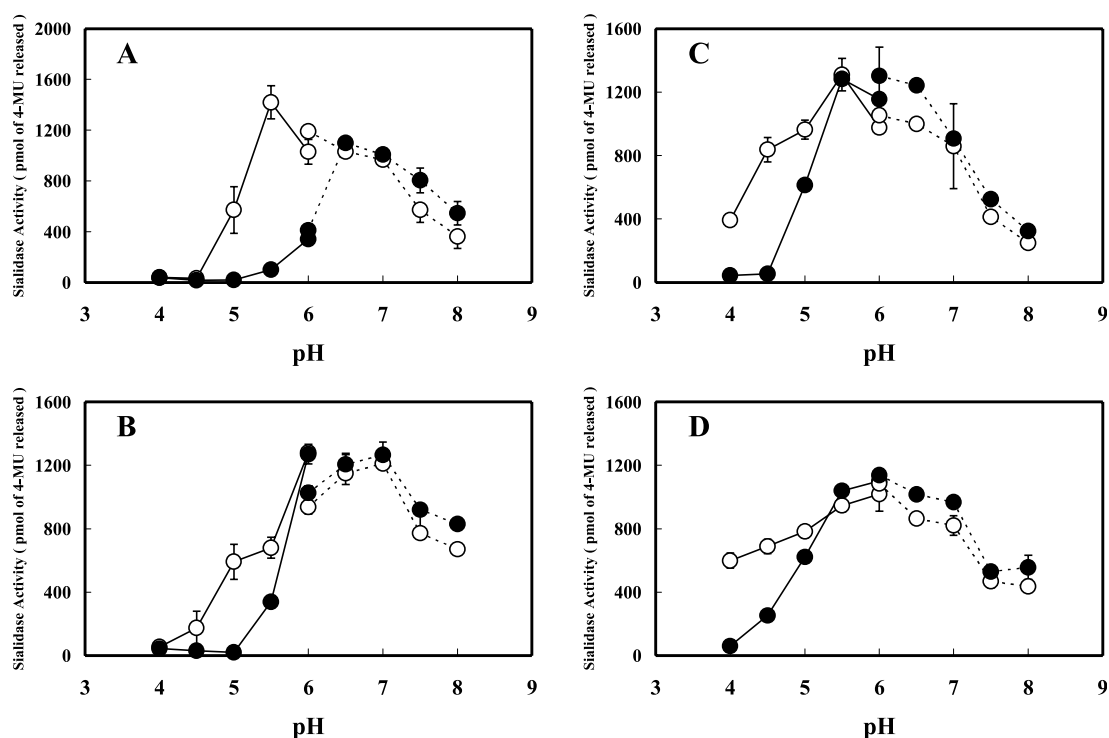


Fig. 1. Effect of pH on the inhibition of influenza A virus sialidase activity by sulfatide. Influenza A virus suspension (500 ng protein) in 10 mM acetate buffer (pH 4.0–6.0; solid lines) or 10 mM phosphate buffer (pH 6.0–8.0; dotted lines) was incubated with 200 μ M sulfatide (closed circles) at 37°C for 30 min and then incubated with 5 μ l of 4-MU-Neu5Ac at 37°C for 30 min. The amount of released 4-MU was measured as described in Section 2. Controls were determined under the same conditions in the absence of sulfatide (open circles). The values are the means for duplicate experiments. A: A/Memphis-Bel (H3N1); B: A/Memphis/1/71 (H3N2); C: A/duck/Hong Kong/849/3/80 (H4N1); D: A/duck/276/2/78 (H2N2).

2.4. Preparation of liposomes bearing sulfatide

Liposomes bearing sulfatide were prepared by a modified method previously described [16]. Sulfatide (320 nmol), phosphatidylcholine (32 nmol), and cholesterol (320 nmol) were mixed in 500 μ l of a solvent containing chloroform and methanol (2:1) and evaporated to form the thin lipid film. The lipid film was vortexed in 100 mM sodium acetate buffer (pH 5.0) and sonicated for 2 min. The inhibitory potency of the liposome solution was examined as described above. As controls, liposomes without glycolipids and bearing galactosylceramide (galactocerebroside) were prepared in the same manner. The assays were performed in duplicate.

3. Results

3.1. Inhibition of influenza A virus sialidase activity by sulfatide

We examined the effects of sulfatide on the sialidase activities of N1 and N2 NA subtypes of human and avian influenza A virus strains by fluorometric assays using 4-MU-Neu5Ac. Sulfatide inhibited the viral sialidase activities in a pH-dependent manner. The sialidase activities of the two human viruses were completely inhibited by 200 μ M sulfatide at pH

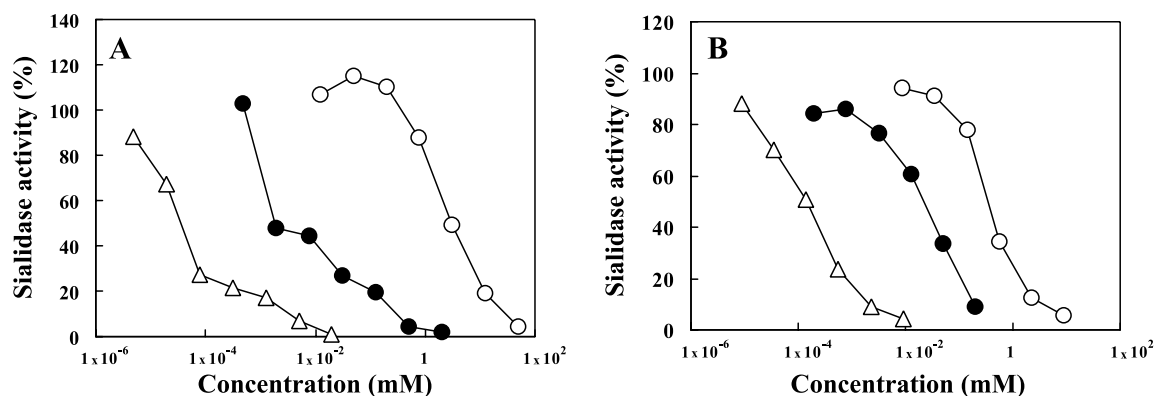


Fig. 2. Inhibition of sialidase activities of influenza A viruses by sulfatide. Human influenza A virus suspension in 10 mM acetate buffer (pH 5.2) was incubated with sulfatide (3–200 μ M; closed circles), Neu5Ac2en (0.03–2 μ M; open circles), and 4G-Neu5Ac2en (0.03–2 μ M; open triangles) at 37°C for 30 min and then incubated with 4-MU-Neu5Ac at 37°C for 30 min. The sialidase activity of each influenza A virus is expressed as the percentage of activity relative to the activity in the absence of sulfatide. A: A/Memphis-Bel (H3N1); B: A/Memphis/1/71 (H3N2).

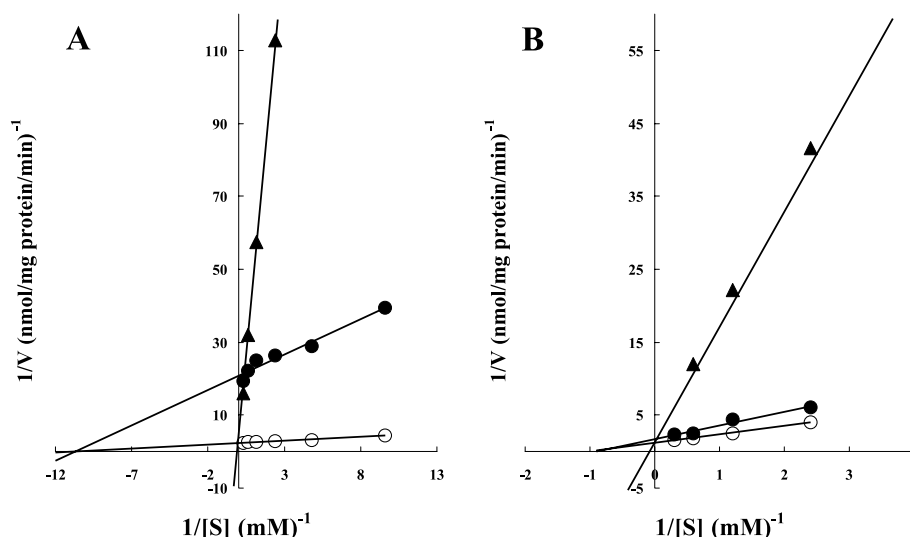


Fig. 3. Lineweaver–Burk plots of inhibition of influenza A virus sialidase activity by sulfatide. The sialidase activity of each influenza A virus with various concentrations of 4-MU-Neu5Ac in the presence of sulfatide (closed circles) or Neu5Ac2en (closed triangle) or in the absence of the inhibitor (open circles) was measured as described in Section 2. A: A/Memphis-Bel (H3N1); B: A/Memphis/1/71 (H3N2).

values lower than pH 5.2 (Fig. 1A,B). The sialidase activities of the two avian viruses were also completely inhibited at pH 4.0 but not at pH 5.0 (Fig. 1C,D). The kinetic parameters of sulfatide inhibition of the viral NAs on the hydrolysis of 4-MU-Neu5Ac were determined and compared with those of the known competitive inhibitors of sialidases, Neu5Ac2en [19] and 4G-Neu5Ac2en [20]. The concentration causing 50% inhibition (IC_{50}) of sialidase activity using 4-MU-Neu5Ac was calculated graphically by plotting percent inhibition versus inhibitor concentration. Sulfatide proved to be a potent inhibitor of the human influenza virus N1 and N2 sialidases with IC_{50} values of 1.79×10^{-4} and 1.88×10^{-2} mM, respectively, at pH 5.2 (Fig. 2). The inhibitory potency of sulfatide was weaker than that of 4G-Neu5Ac2en (7.18×10^{-6} and

1.32×10^{-4} mM) but was about 10 – 10^3 -fold more potent than that of Neu5Ac2en (2.84×10^{-1} and 3.37×10^{-1} mM). The kinetic parameters of the effect of sulfatide on the sialidase activities of human influenza A viruses were determined by the double-reciprocal (Lineweaver–Burk) method. The linear double-reciprocal plots indicated that sulfatide acted as a non-competitive inhibitor (Fig. 3).

We examined whether membrane-associated sulfatide affects the sialidase activity. The sialidase activity of human influenza A virus was distinctly inhibited by the liposomes containing sulfatide, relative to the liposomes without glycolipids and bearing galactosylceramide (Fig. 4).

3.2. Inhibition of the sialidase activities of human, avian, swine, and equine influenza A viruses by sulfatide

We examined the inhibitory effect of sulfatide on NA subtypes of influenza A virus isolated from humans, pigs, horses, and birds at pH 5.2 using 4-MU-Neu5Ac as a substrate (Table 1). The inhibition of viral sialidase activities by sulfatide at pH 5.2 varied depending not only on the NA subtype but also on the host animal species from which the virus was isolated. Sialidase activities of most human, swine, and equine viruses, with the exception of four early human viruses isolated in 1957 and 1968, were efficiently inhibited by sulfatide at pH 5.2, while avian viruses varied in sensitivity to sulfatide at pH 5.2. The sialidase activities of avian N3 and N6 NA subtypes, similar to those of the mammalian viruses, were efficiently inhibited by sulfatide at pH 5.2. In contrast, the sialidase activities of avian N2 and N8 NA subtypes, with the exception of H9N2 viruses, retained more than 50% of the activity under the same condition. However, the sialidase activities of all avian viruses tested, including N2 and N8 NA subtypes, were efficiently inhibited by sulfatide at pH 4.0 (data not shown).

4. Discussion

Previous studies have shown that glycosaminoglycans and sulfated compounds inhibited many human lysosomal enzymes and bacterial sialidases [21–24]. In the present study,

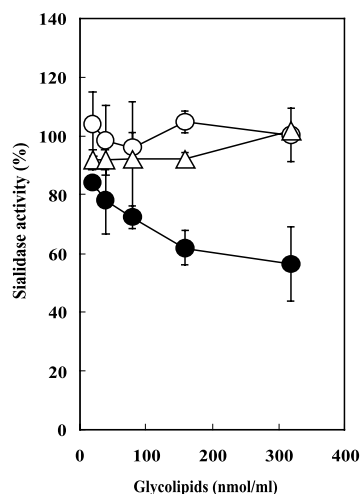


Fig. 4. Inhibition of the sialidase activity of influenza A virus by the liposomes bearing sulfatide. Human influenza virus A/Memphis/1/71 (H3N2) suspension in 10 mM acetate buffer (pH 5.2) was incubated with liposomes containing sulfatide (20–320 nmol/ml; closed circles), galactosylceramide (20–320 nmol; open circles), and without glycolipids (open triangle) at 37°C for 30 min and then incubated with 4-MU-Neu5Ac at 37°C for 30 min. The sialidase activity of each influenza A virus is expressed as the percentage of activity relative to the activity in the absence of the liposomes.

Table 1
Inhibition of the sialidase activities of influenza A viruses at pH 5.2 by sulfatide

Virus	Sialidase activity (4-MU released pmol/min){ {decimal:split}} ± S		Sialidase inhibition (%)
	Control	Sulfatide	
Human isolates			
A/Singapore/1/57 (H2N2)	33.6±0.4	23.5±0.1	30.0
A/Japan/305/57 (H2N2)	120.7±1.3	109.3±0.2	9.4
A/Hong Kong/1/68 (H3N2)	43.2±0.6	25.0±0.4	42.2
A/Aichi/2/68 (H3N2)	31.4±0.4	13.0±0.1	58.6
A/Memphis/1/71 (H3N2)	20.2±0.7	1.1±0.1	94.7
A/Memphis/102/72 (H3N2)	28.1±0.4	0.5±0.0	98.1
A/Tokyo/6/73 (H3N2)	39.3±2.0	0.4±0.1	99.1
A/Kumamoto/55/76 (H3N2)	32.3±3.1	0.2±0.0	99.3
A/Texas/1/77 (H3N2)	23.0±1.7	0.4±0.0	98.4
A/Yamanashi/2/77 (H3N2)	9.7±0.7	0.6±0.2	94.1
A/Bangkok/1/79 (H3N2)	25.0±1.6	6.8±0.5	72.8
A/Victoria/2/82 (H3N2)	13.0±0.9	0.3±0.0	98.1
Avian isolates			
A/duck/Alberta/35/76 (H1N1)	177.2±2.5	61.0±4.4	65.6
A/duck/Hong kong/36/4/76 (H1N1)	66.2±0.8	23.4±0.8	64.7
A/duck/Hong kong/849/3/80 (H4N1)	125.5±19	114.8±3.8	8.6
A/duck/Hong kong/13/2/76 (H6N1)	159.7±1.3	14.0±1.4	91.2
A/duck/Hong kong/33/3/76 (H10N1)	294.0±0.3	149.5±17	49.1
A/duck/Hong kong/273/8/78 (H2N2)	234.8±0.9	198.1±0.4	15.6
A/duck/Hong kong/276/2/78 (H2N2)	44.4±1.1	33.4±0.2	24.7
A/duck/Hong kong/24/5/76 (H3N2)	175.6±2.2	130.5±0.0	25.7
A/duck/Hong kong/47/5/76 (H7N2)	171.6±2.1	119.4±4.3	30.4
A/duck/Hong kong/86/1/76 (H9N2)	107.2±1.6	4.2±0.2	96.0
A/duck/Hong kong/92/1/76 (H9N2)	47.0±0.2	5.5±0.1	88.4
A/duck/Hong kong/717/8/79 (H1N3)	72.5±4.2	10.0±0.9	86.1
A/duck/Hong kong/313/4/78 (H5N3)	177.2±5.4	2.6±0.5	98.5
A/duck/Hong kong/44/3/76 (H11N3)	89.5±0.9	0.9±0.2	99.0
A/teal/Alberta/69/87 (H1N4)	32.9±0.6	9.7±0.7	70.4
A/mallard/Alberta/58/89 (H6N4)	12.5±0.3	7.5±0.2	39.9
A/mallard/Alberta/7/87 (H8N4)	15.5±0.9	4.4±0.3	71.8
A/duck/Hong kong/668/3/79 (H4N5)	175.6±0.9	10.9±0.4	93.8
A/duck/Hong kong/862/5/80 (H12N5)	33.7±1.1	29.5±0.1	12.6
A/duck/Hong kong/526/3B/79 (H3N6)	10.3±0.6	2.0±0.1	80.7
A/mallard/Alberta/25/85 (H4N6)	18.6±0.5	2.0±0.1	89.3
A/duck/Hong kong/200/1/77 (H4N6)	125.5±0.6	41.5±1.2	66.9
A/duck/Hong kong/219/1/77 (H4N6)	72.5±9.5	5.9±0.1	91.8
A/duck/Ukraine/1/63 (H3N8)	63.6±6.5	40.1±0.8	36.9
A/duck/Hokkaido/8/80 (H3N8)	66.2±5.4	40.3±3.1	39.2
A/mallard/Alberta/23/89 (H3N8)	40.5±1.9	20.8±1.2	48.5
A/duck/Hong kong/278/5/78 (H2N9)	63.6±2.8	13.4±0.5	78.9
A/mallard/Alberta/24/92 (H11N9)	9.6±0.3	4.0±0.7	58.1
Swine isolates			
A/swine/Wisconsin/15/30 (H1N1)	3.8±0.1	0.8±0.0	80.1
A/swine/Hong Kong/168/93 (H1N1)	144.1±1.9	10.5±0.4	92.7
A/swine/Colorado/1/77 (H3N2)	14.6±0.6	1.3±0.1	90.9
A/swine/Hokkaido/10/85 (H3N2)	22.1±0.6	5.1±0.5	76.8
Equine isolates			
A/equine/Prague/56 (H7N7)	27.5±1.3	13.5±0.3	50.9
A/equine/London/1416/73 (H7N7)	18.0±0.2	5.9±0.1	67.2
A/equine/Fontainebleau/79 (H3N8)	12.3±0.9	0.4±0.0	97.1
A/equine/Tennessee/5/86 (H3N8)	16.8±0.3	2.0±0.1	87.9

Each influenza A virus suspension (500 ng protein) in 10 mM acetate buffer (pH 5.2) was incubated with 1 nmol sulfatide at 37°C for 30 min and then the mixture was incubated with 4-MU-Neu5Ac at 37°C for 30 min as described in Section 2. The amount of released 4-MU was measured using a fluorescence spectrophotometer. The degree of sialidase inhibition is expressed as a percentage inhibition of each enzyme activity.

we found that sulfatide efficiently inhibited sialidase activities of influenza A virus NAs under conditions of less than pH 6.0. In contrast, galactosylceramide, the biosynthetic precursor of sulfatide, did not inhibit the sialidase activities of the viruses. In the basic amino acids, only histidine ($pK_R = 6.0$) carries an imidazolium moiety and ionizes under conditions of less than pH 6.0. The results suggest that the inhibition of the sialidase activity was caused by interaction of the sulfate galactose of sulfatide with the side chain of histidines in NAs. For example, histidines at positions 144, 150, 155, 164, 189,

191, 264, and 274 in human N2 NAs are highly conserved among human N2 NAs tested. Above all, the binding of sulfatide to histidine at position 150, which is located by the enzyme active site, may affect the enzymatic activity of the NA. We found that chondroitin sulfates (A and B) and dextran sulfate also inhibited the sialidase activity of influenza A virus. On the other hand, the sialidase activity was little affected by sulfated monosaccharides under the same conditions (data not shown). These results show that the inhibitory effect of sulfated glycoconjugates on the sialidase activity is remark-

ably dependent on the topology of sulfate sugar in the molecules.

Duck strains varied in sensitivity to sulfatide at pH 5.2; however, the sialidase activities of all duck viruses tested were efficiently inhibited by sulfatide at pH 4.0. In previous studies, we found that NAs of 1957 and 1968 human pandemic influenza A virus strains as well as duck viruses retained their sialidase activities even at pHs of less than 4.5, but not NAs of the epidemic strains isolated after 1968 and swine strains tested [25,26]. Effect of pH values on the sialidase inhibition of the mammalian and duck N2 NAs by sulfatide correlated with low-pH stability of their sialidase activities. On the other hand, the effect of pH values on other duck sialidase inhibition by sulfatide did not coincide with their pH stability. The results indicate that the mechanism of NA inhibition by sulfatide is different from the mechanism contributing to their low pH stability.

Duck viruses replicate in the intestinal tract through the digestive tract of ducks. The difference in their sensitivity to sulfatide between duck and mammalian viruses may therefore depend on their target tissues.

Sulfatide was synthesized through the endoplasmic reticulum and Golgi apparatus, from which it was transported by membrane vesicle to the plasma membrane and other intracellular organelles, like lysosomes [27,28]. Interestingly, some of the main sorting organelles, such as lysosomes and the trans-Golgi network, in cells are known to be acidified. For example, lysosomes maintained a pH 4.0–5.0 [29]. The pH in the trans-Golgi network in virus-infected chicken embryo fibroblasts or MDCK cells has been shown to be approximately 5.2 and 5.6 [30]. The studies suggest that sulfatide can affect NA in lysosomes or trans-Golgi network. Further studies are needed to characterize the role of sulfatide in influenza virus infections.

Acknowledgements: We thank Yoshihiro Kawaoka and Kennedy F. Shortridge for providing virus strains. Support for this work was provided by grants-in-aid for scientific research (Nos. 13557207 and 13470066) from the Japan Society for the Promotion of Science and National Institutes of Health Grant NS 09626.

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