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Synthetic sialylphosphatidylethanolamine derivatives bind to human influenza A viruses and inhibit viral infection

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We synthesized the sialylphosphatidylethanolamine (sialyl PE) derivatives Neu5Ac-PE, $(Neu5Ac)_2$-PE, Neu5Ac-PE (amide) and Neu5Ac-PE (methyl). We examined the anti-viral effects of the derivatives on human influenza A virus infection by ELISA/virus-binding, hemagglutination inhibition, hemolysis inhibition and neutralization assays. The sialyl PE derivatives that we examined bound to A/Aichi/2/68, A/Singapore/1/57 and A/Memphis/1/71 strains of H3N2 subtype, but not to A/PR/8/34 strain of H1N1 subtype. The derivatives inhibited viral hemagglutination and hemolysis of human erythrocytes with A/Aichi/2/68 and A/Singapore/1/57 (H3N2), but not with A/PR/8/34 (H1N1). The inhibitory activity of the $(Neu5Ac)_2$-PE derivative was the strongest of all sialyl PE derivatives (IC_{50}, 35 μM to 40 μM). Sialyl PE derivatives also inhibited the infection of A/Aichi/2/68 in MDCK cells. Complete inhibition was observed at a concentration between 0.3 to 1.3 mM. IC_{50} of $(Neu5Ac)_2$-PE was 15 μM in A/Aichi/2/68 strain. Taken together, the synthetic sialyl PE derivatives may be effective reagents against infection of some types of influenza A viruses.

Keywords: **Influenza virus; Sialic acid; Sialylphosphatidylethanolamine**

Introduction

Influenza is an infectious disease caused by influenza viruses. It is known that sialic acid-containing glycoproteins or glycolipids on cell surfaces are receptors for influenza viruses [14–18]. The interaction of influenza viruses with cellular receptors is mediated by two major virus glycoproteins, hemagglutinin and sialidase, which are responsible for the attachment to target cells and for release of progeny viruses from the surface of infected cells, respectively [22,23].

The binding of influenza viruses has been shown to depend on the molecular species of sialic acid [11], sialylgalactose (Gal) linkages, or carbohydrate core structures of gangliosides [18]. A/PR/8/34 (H1N1) predominantly recognizes the Neu5Acα2-3Gal linkage of sialo-glycoconjugates, whereas A/Aichi/2/68 (H3N2) preferentially recognizes the Neu5Acα2-6Gal linkage [10,15,17].

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Several studies reported that neoglycoproteins or synthetic co-polymers containing Neu5Ac strongly inhibited the binding of influenza A viruses to chicken erythrocytes [1,2,4,6,11]. These approaches could possibly allow us to develop drugs against infection of influenza A viruses.

In this study, we determined the anti-viral activity of synthetic sialyl PE derivatives containing Neu5Ac against human influenza A viruses by ELISA/virus-binding, hemagglutination inhibition, hemolysis inhibition, and nuetralization assays.

Materials and methods

Sialyl compounds

IV^3Neu5AcnLc4Cer and IV^6Neu5AcnLc4Cer were prepared from human red blood cells [25] and from human meconium, respectively [3]. Fetuin from fetal bovine serum was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Synthetic sialyl PE derivatives were prepared by the following methods (Fig. 1).

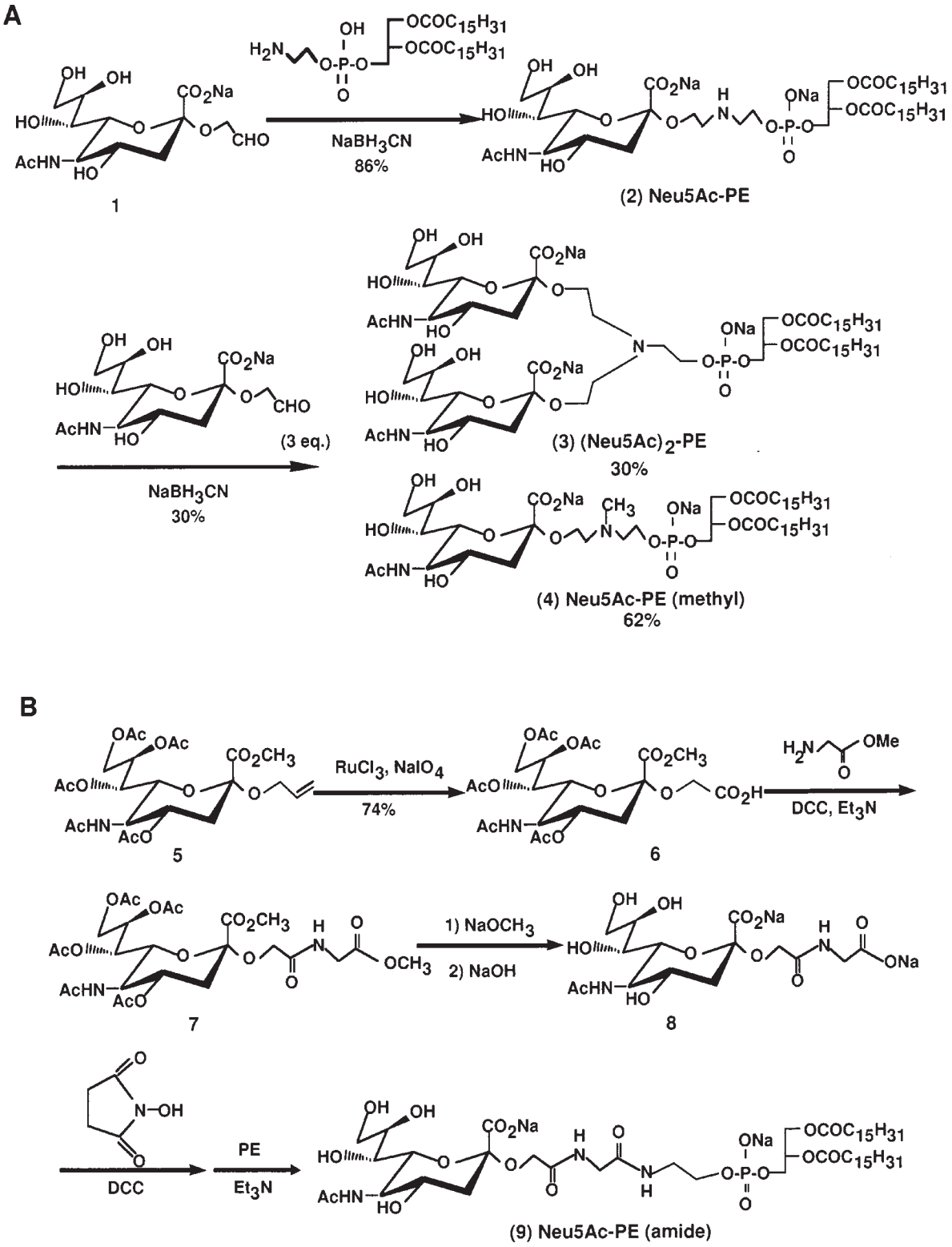

Figure 1. Synthesis of sialyl PE derivatives: (A). Synthesis of Neu5Ac-PE, $(Neu5Ac)_2$-PE and Neu5Ac-PE (methyl). (B). Synthesis of Neu5Ac-PE (amide).

Synthesis of Neu5Ac-PE (2)

As shown in Fig. 1A, a solution of aldehyde (**1;** 5.0 mg, 14.2 μmol) prepared by Roy's method [5] in H_2O (0.5 ml) was added to a solution of dipalmitoylphosphatidylethanolamine (49 mg, 71.2 μmol) in a mixture of $CHCl_3$ and MeOH (1 : 2, v/v, 6.0 ml). The reaction mixture was heated at 50 °C for 2 h, and a methanol solution (0.5 ml) of sodium cyanoborohydride (24 mg, 384 mmol) was added at room temperature. The reaction mixture was further heated at 50 °C for 2 h to complete the reaction. After being chilled to room temperature, it was loaded onto a column of Sephadex LH-20 (2 × 40 cm, $CHCl_3$/MeOH (1:1, v/v)). Each eluted fraction was checked by thin-layer chromatography (TLC), and the fractions stained with Dittmer reagent were collected and concentrated to dryness. The crude product thus obtained was further purified by silica gel chromatography ($CHCl_3$/MeOH/H_2O (7:3:0.4, v/v/v)) to give **2** (12.6 mg, 86%) as a white powder.

Figure 2A shows ^{1}H-NMR ($CDCl_3$/CD_3OD (1 : 1, v/v)) δ 0.89 (6H, t, J = 7.3 Hz), 1.28 (48H, m), 1.62 (4H, m), 1.67 (1H, t, J = 11.9 Hz), 2.04 (3H, s), 2.33 (2H, t, J = 7.6 Hz), 2.35 (2H, t, J = 7.6 Hz), 2.83 (1H, m), 3.23 (2H, m), 3.30 (2H, m), 3.48 (1H, dd, J = 8.9, 1.2 Hz), 3.59 (1H, d, J = 10.1 Hz), 3.64 (1H, dd, J = 11.8, 5.8 Hz), 3.69 (1H, t, J = 10.1 Hz), 3.74 (1H, dt, J = 4.6, 10.7 Hz), 3.83–3.88 (3H, m), 4.02 (3H, m), 4.14 (2H, m), 4.20 (1H, dd, J = 11.9, 6.7 Hz), 4.43 (1H, dd, J = 11.9, 3.1 Hz), 5.26 (1H, m). ^{13}C-NMR ($CDCl_3$/CD_3OD (1 : 1, v/v)) δ 14.3, 22.6, 23.2, 25.49, 25.54, 29.71, 29.74, 29.91, 29.93, 29.95, 30.11, 30.13, 30.23, 30.27, 32.5, 34.6, 34.8, 41.1, 48.2, 49.3, 53.4, 59.9, 61.2, 63.2, 64.2, 64.5, 68.4, 69.7, 71.1, 72.2, 74.1, 101.3, 173.5, 174.2, 174.6, 174.9. FAB MS: 1028 $[M+H]^+$, ($C_{50}H_{95}N_2O_{17}P$ MW, 1027).

Synthesis of $(Neu5Ac)_2$-PE (3) and Neu5Ac-PE (methyl) (4)

In Fig. 1A, a solution of aldehyde (**1;** 52.3 mg, 140 μmol) [5] in H_2O (0.6 ml) was added to a solution of compound **2** (50 mg, 46.7 μmol) in the mixture of $CHCl_3$ and MeOH (1 : 2, v/v, 18.0 ml). The reaction mixture was heated at 50 °C for 2 h, and a methanol solution (1.2 ml) of sodium cyanoborohydride (88 mg, 1.40 mmol) was added at room temperature. The reaction mixture was further heated at 50 °C for 2 h to complete the reaction. After being chilled to room temperature, it was loaded onto a column of Sephadex LH-20 (2 × 40 cm, $CHCl_3$MeOH (1 : 1, v/v)). Each eluted fraction was checked on TLC and the fractions stained with Dittmer reagent were collected and concentrated to dryness. The crude product thus obtained was further purified by silica gel chromatography ($CHCl_3$/MeOH/H_2O (7 : 3 : 0.4, v/v/v)) to yield **3** (20 mg, 30%) and **4** (30 mg, 62%) as a white powder. $(Neu5Ac)_2$-PE (**3**): ^{1}H-NMR ($CDCl_3$/CD_3OD (1:1, v/v)) δ 0.89 (6H, t, J = 7.0 Hz), 1.28 (48H, m), 1.61 (4H, m), 1.83 (2H, m), 2.03 (6H, s), 2.33 (2H, t, J = 7.6 Hz), 2.34 (2H, t, J = 7.6 Hz), 2.71 (2H, dd, J = 12.2, 3.6 Hz), 3.49 (2H, dd, J = 9.2, 1.2 Hz), 3.63 (10H, m), 3.77–3.88 (10H, m), 3.96 (2H, m), 4.04 (2H, t, J = 6.4 Hz), 4.21 (3H, m), 4.43 (1H, dd, J = 11.9, 3.1 Hz), 5.26 (1H, m). ^{13}C-NMR ($CDCl_3CD_3OD$ (1:1, v/v)) δ 14.3, 22.6, 23.2, 25.45, 25.49, 29.67, 29.69, 29.87, 29.89, 30.07, 30.10, 30.20, 30.24, 32.5, 34.6, 34.8, 39.9, 53.1, 53.5, 56.7, 58.5, 60.1, 63.1, 64.6, 64.7, 67.8, 69.7, 70.9, 71.5, 74.5, 99.4, 170.6, 174.2, 174.6, 174.8. FAB MS: 1363 $[M+H]^+$, ($C_{63}H_{116}N_3O_{26}P$ MW, 1362). Neu5Ac-PE (methyl) (**4**): ^{1}H-NMR ($CDCl_3$/CD_3OD (1:1, v/v)) δ 0.89 (6H, t, J = 7.2 Hz), 1.28 (48H, m), 1.61 (4H, m), 1.68 (1H, t, J = 11.8 Hz), 2.05 (3H, s), 2.33 (2H, t, J = 7.6 Hz), 2.35 (2H, t, J = 7.5 Hz), 2.81 (1H, dd, J = 11.8, 4.0 Hz), 2.98 (3H, s), 3.41 (4H, m), 3.51 (1H, d, J = 9.0 Hz), 3.62 (1H, d, J = 9.9 Hz), 3.66 (1H, dd, J = 11.0, 5.6 Hz), 3.72 (1H, t, J = 9.9 Hz), 3.77 (1H, dd, J = 10.4, 4.3 Hz), 3.89 (3H, m), 4.01 (2H, t, J = 5.9 Hz), 4.12 (1H, m), 4.19 (1H, dd, J = 12.1, 7.2 Hz), 4.20 (2H, m), 4.44 (1H, dd, J = 12.1, 2.9 Hz), 5.26 (1H, m). ^{13}C-NMR ($CDCl_3$/CD_3OD (1:1, v/v)) δ 14.4, 22.7, 23.3, 25.5, 25.6, 29.78, 29.80, 30.00, 30.03, 30.18, 30.21, 30.29, 30.30, 30.33, 32.6, 34.7, 34.8, 41.1, 41.8, 53.4, 56.8, 57.2, 58.8, 60.1, 63.3, 64.0, 64.5, 68.3, 69.7, 71.1, 72.2, 74.0, 101.1, 173.6, 174.2, 174.6, 175.0. FAB MS: 1042 $[M+H]^+$, ($C_{51}H_{97}N_2O_{17}P$ MW, 1041).

Synthesis of Neu5Ac-PE (amide) (9)

In Fig. 1B, compound **5** (2.00 g, 3.76 mmol) prepared by Roy's method [5] was oxidized with $RuCl_3$ and $NaIO_4$ to yield compound **6** (1.53 g, 74%). Compound **6** (100 mg, 0.18 mmol) was changed into compound **7** (78.4 mg, 70%) by reaction with glycine methylester hydrochloride (23 mg, 0.18 mmol) and dicyclohexylcarbodiimide (DCC; 38 mg, 0.18 mmol) in the presence of triethylamine (18 mg, 0.18 mmol). Compound **7** was deprotected with sodium methoxide in methanol and 0.1 N aqueous NaOH, successively, to make compound **8**. *N*-Hydroxysuccinimide (2.4 mg, 21 μmol) and DCC (4.8 mg, 23 μmol) were added to a solution of compound **8** (8 mg, 21 μmol) in THF and CH_2Cl_2 (1 : 1, v/v, 2 ml), and the reaction mixture was stirred for 18 h. A solution of dipalmitoylphosphatidylethanolamine (15 mg, 21 μmol) in $CHCl_3$ and EtOH (1 : 1, v/v, 1.0 ml) was added to the reaction mixture and heated at 40 °C for 21 h. After the reaction, the organic solvent was evaporated off. The crude product obtained was purified by silica gel chromatography ($CHCl_3$/MeOH/H_2O (7 : 3 : 0.4, v/v/v)) to yield **9** (14 mg, 61%) as a white powder. Figure 2B shows ^{1}H-NMR ($CDCl_3$/CD_3OD (1 : 1, v/v)) δ 0.89 (6H, t, J = 7.0 Hz), 1.28 (48H, m), 1.63 (4H, m), 1.91 (1H, t, J = 12.8 Hz), 2.03 (3H, s), 2.34 (2H, t, J = 7.7 Hz), 2.37 (2H, t, J = 7.3 Hz), 2.81 (1H, dd, J = 12.8, 4.4 Hz), 3.52 (3H, m), 3.65 (3H, m), 3.82 (3H, m), 3.92 and 3.98 (2H, each d, J = 16.8 Hz), 4.04–4.16 (5H, m), 4.20 (1H, dd, J = 12.1, 6.6 Hz), 4.34 (1H, d, J = 15.4 Hz), 4.41 (1H, dd, J = 12.1, 3.7 Hz), 5.26 (1H, m). FAB MS: 1121 $[M+Na]^+$, ($C_{52}H_{96}N_3O_{19}P$ MW, 1098).

Judged by both data from above NMR spectra (Fig. 2A, 2B) and chemical stained by resorcinol-HCl reagent of

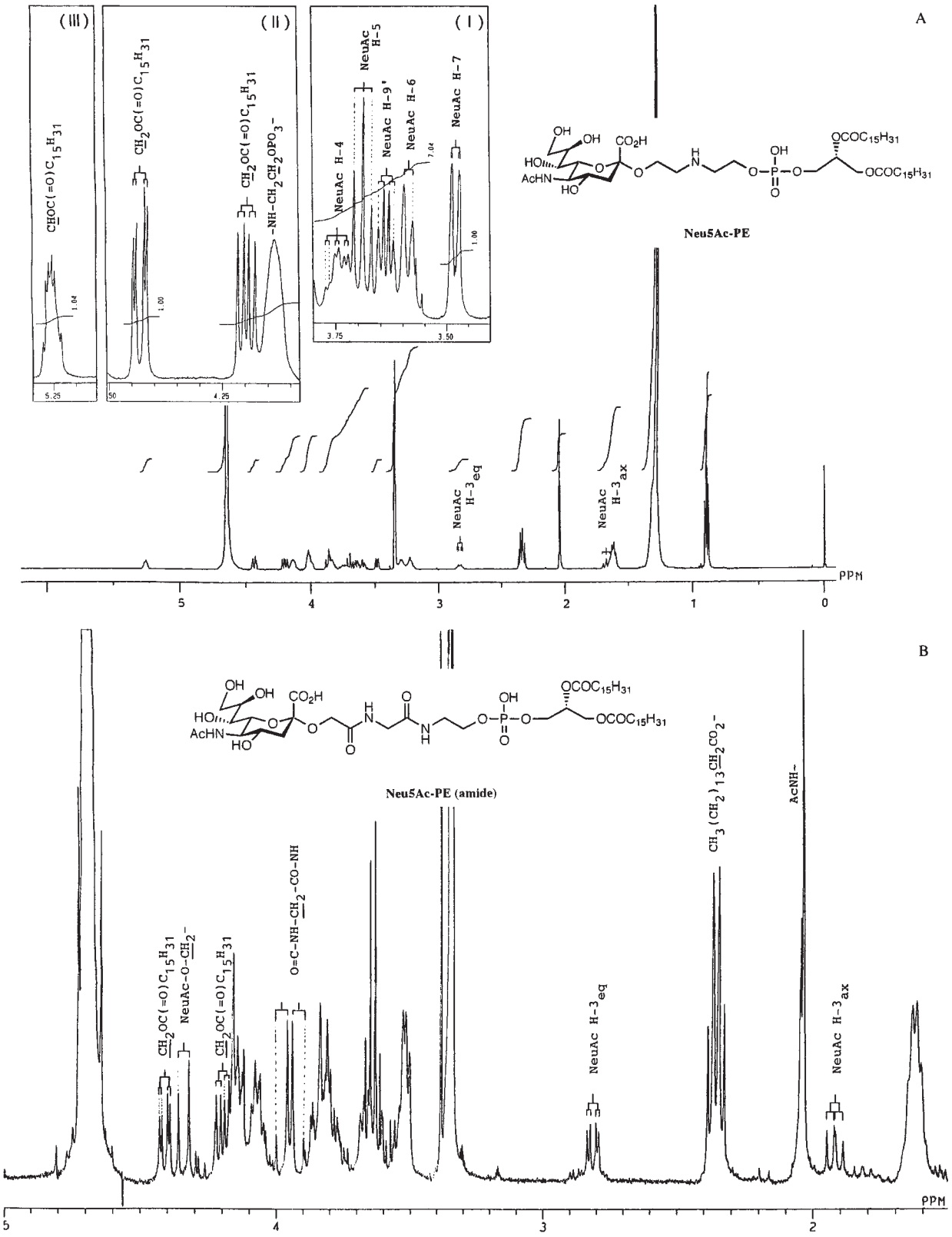

Figure 2. (A). a ^{1}H-NMR spectrum (500 MHz) for Neu5Ac-PE. Inlet (I) exhibits a spectrum magnified from 3.4 to 3.8 ppm; inlet (II) shows a spectrum magnified from 4.1 to 4.5 ppm; inlet (III) exhibits a spectrum magnified from 5.2 to 5.3 ppm. (B). a^{1}H-NMR spectrum (400 MHz) for Neu5Ac-PE (amide). (C). Detection of the sialyl PE derivatives by thin-layer chromatography. Silica gel plastic plate (Polygram Sic G; Macherey-Nagel, Germany) was developed with the sialyl PE derivatives using a solvent system containing chloroform/methanol/12 mM aqueous $MgCl_2$ (50 : 40 : 10). Visualization of the derivatives was performed with resorcinol-HCl reagent at 120 °C for 20 min. Lane 1 or 2 was 0.2 nmol of sialylparagloboside (Neu5Acα2-3 or Neu5Acα2-6) as a control. Lanes 3 to 6 were 1 nmol of NeuAc-PE, $(NeuAc)_2$-PE, NeuAc-PE (amide), and NeuAc-PE (methyl), respectively.

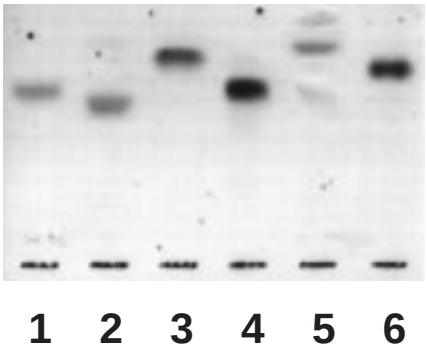

Figure 2. Continued.

those derivatives (Fig. 2C), all the derivatives were confirmed to be highly pure.

Influenza viruses and anti-influenza virus antibodies

Influenza viruses: A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2), A/Memphis/1/71 (H3N2), and A/Singapore/1/57 (H3N2) strain were each propagated in the allantonic cavity of 11-day-old chicken eggs at 35 °C for 48 h and purified by sucrose density gradient centrifugation [12]. Viral hemagglutination (HA) units were determined in microtiter plates using 0.5% chicken erythrocytes as described previously [9,13]. Rabbit anti-influenza virus antibodies were raised by immunization of A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2), A/Memphis/1/71 (H3N2), or A/Singapore/1/57 (H3N2) virus grown in eggs as described previously [17,21,26].

Enzyme-linked immunosorbent assay (ELISA)/virus-binding assay

The derivatives were diluted with methanol (0, 25, 50, 75 and 100 pmol/well) in the 96-well microtiter plates. Immunochemical detection of virions with ELISA was performed as described previously [17]. After having been blocked with phosphate buffer saline (PBS (pH 6.5); 131 mM NaCl, 15 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl) containing 1% egg albumin and 1% polyvinylpyrrolidone (solution A) at 4 °C for 2 h and washed, the plates were incubated with purified virus suspension at a concentration of 2^6 HA units at 4 °C overnight. After being washed with PBS 5 times, the plates incubated with anti-influenza virus antibody 1000-fold diluted with PBS containing 3% polyvinylpyrrolidone (the solution B) at 4 °C for 2 h. After being washed again, the plates were incubated with horseradish peroxidase-conjugated protein A at 4 °C for 2 h. After being washed with PBS 10 times, the plates were incubated with the substrate solution (100 mM citrate-phosphate buffer (pH 5.0) containing 400 mM *o*-phenylenedimine and 0.001% aqueous H_2O_2) for 10 min at room temperature. The reaction was stopped by 1N HCl. The absorbance was determined at 550 nm using a MTP-32 microplate reader (Coroa Electric Co., Ltd. Japan).

Hemagglutination inhibition assay

Hemagglutination inhibition (HAI) assay was carried out using 96-well microtiter plates as described previously [13]. Phosphate buffer saline (pH 6.5) containing 0.01% gelatin was used as a dilution buffer. Human erythrocytes were used as indicator cells. Virus suspension (2^4 HA units in 0.025 ml of PBS) was added to each well containing synthetic sialyl PE derivatives in two-fold serial dilutions with the dilution buffer. The plates were incubated at 4 °C for 1 h. After 0.05 ml of 0.5% (v/v) human erythrocytes in PBS were added to the plates, the plates were then kept at 4 °C for 1 h. The maximum dilution of the samples showing complete inhibition of the hemagglutination was defined as the hemagglutination inhibition titer.

Hemolysis inhibition assay

Hemolysis inhibition assay was performed as described previously [13,27,28]. Briefly, different concentrations of the synthetic sialyl PE derivatives (from 15 to 1000 μM) were reacted at 4 °C for 2 h with A/PR/8/34 (H1N1) or A/Aichi/2/68 (H3N2) virus (2^{10} HAU) in 1.0 ml of 20mM acetate buffered saline (pH 4.9). The reaction mixture was then used for the hemolysis assay. To estimate the level of virus-mediated hemolysis, 0.1 ml of the mixture was added to 1.0 ml of 20 mM acetate buffered saline (pH 4.9) containing 2.5% human erythrocytes. After incubation on ice for 10 min, the mixture was incubated at 37 °C for 30 min. One ml of PBS was added, and the mixture was then centrifuged at 400 × g for 5 min. The concentration of hemoglobin in the supernatant following the viral hemolysis was determined by measuring the absorbance at 540 nm as described previously [13–15].

Neutralization assay

The neutralization of influenza virus with synthetic sialyl PE derivatives was determined as previously described [10,20]. Madin Darby canine kidney (MDCK) cell monolayers were maintained in Eagle's minimum essential medium (EMEM) containing 5% fetal calf serum. One hundred microliters of $TCID_{50}$ (50% tissue-culture infectious dose) of A/Aichi/2/68 in the presence of sialyl PE derivatives (0.5 to 4000 μM) was inoculated at 34.5 °C for 5 h. After removal of the inoculum, the monolayers were washed 3 times with EMEM. The cells were examined using a light microscope for the progression of viral-induced cytopathic effects (CPE) after incubation at 34.5 °C for 20 h. The lactate dehydrogenase (LDH) that was released from MDCK cells was examined for virus neutralization by slightly modified colorimetric assay [20]. Fetuin and PE were used as controls. The LDH activities in the medium

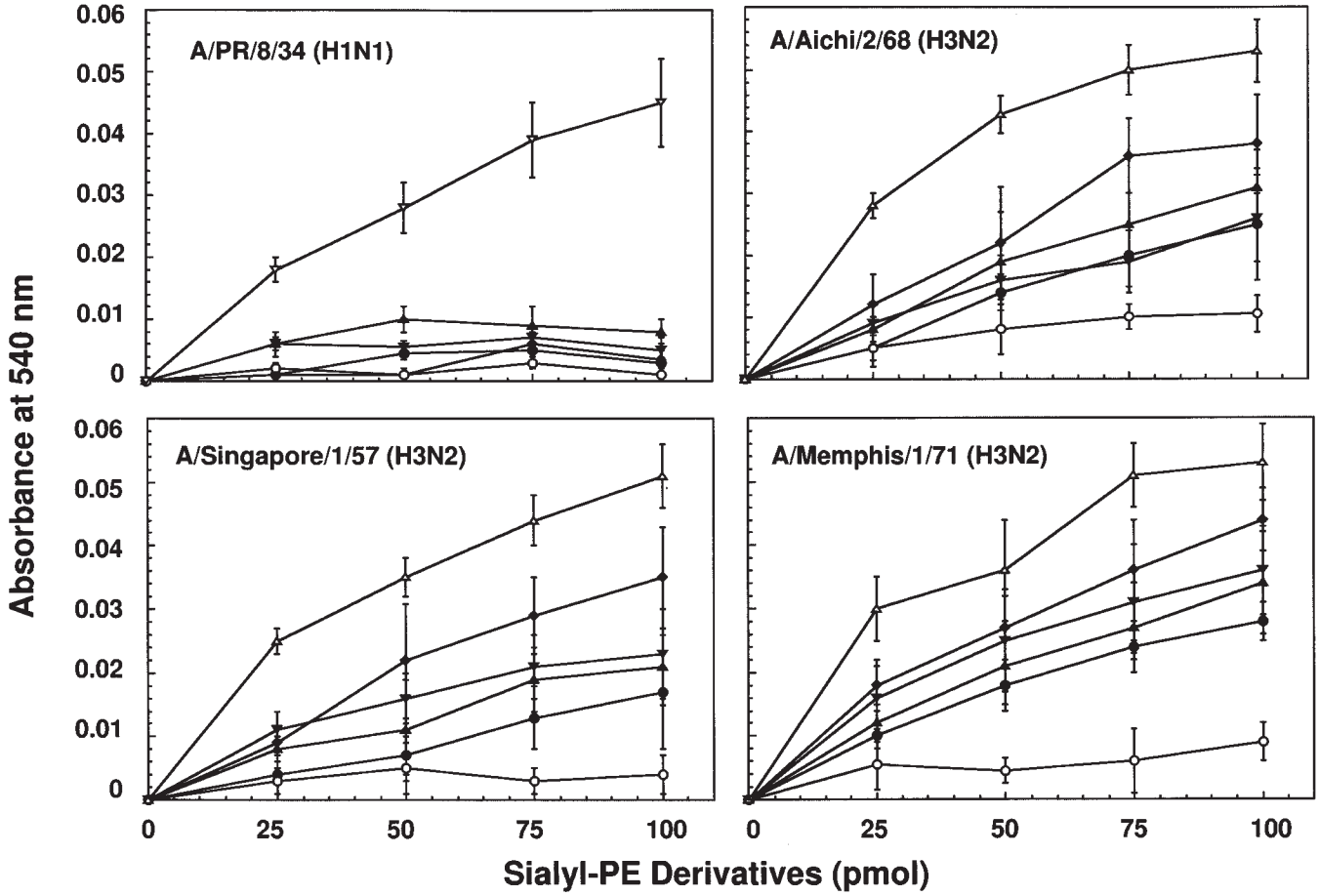

Figure 3. The binding activity of synthetic sialyl PE derivatives to human influenza A viruses: The binding of the sialyl PE derivatives (0, 25, 50, 75 and 100 pmol) to human influenza A viruses (A/PR/8/34, A/Aichi/2/68, A/Memphis/1/71, or A/Singapore/1/57) was determined by ELISA. IV^3 Neu5AcnLcCer (▽) and IV^6 Neu5AcnLcCer (△) were used as positive controls. PE (○) was used as a negative control. (●), Neu5Ac-PE; (◆), $(Neu5Ac)_2$-PE; (▲), Neu5Ac-PE (amide); (▼), Neu5Ac-PE (methyl).

were determined according to the manufacture's instructions. Briefly, the medium (0.0125 ml) was diluted to 1 : 4 with PBS and mixed with 0.05 ml of LDH reagent (Shinotest, Japan). The mixture was incubated at 37 °C for 10 min, and the reaction was stopped by the addition of 0.1 ml of 0.5 N HCl. Absorbance was measured at 550 nm (reference at 630 nm). The assays were performed in duplicate.

Results

Binding of synthetic sialyl PE derivatives to human influenza A virus

The binding activity of the synthetic sialyl PE derivatives to human influenza A viruses was examined with ELISA/virus-binding assay (Fig. 3). Our data showed that the 4 derivatives bound to these strains (A/Aichi/2/68, A/Singapore/1/57 and A/Memphis/1/71) with H3N2 subtype, but not to A/PR/8/34 strain with H1N1 subtype. The activity of the sialyl PE derivatives (2 nmol) was about 30% to 80% of the sialylparagloboside, which showed stronger binding to human influenza virus by ELISA. The non-sialylated PE did not bind to the influenza viruses that we examined. The effect of $(Neu5Ac)_2$-PE derivative was stronger than that of other derivatives that we tested.

Viral hemagglutination and hemolysis inhibition by synthetic sialyl PE derivatives

To measure biological responsiveness of the synthetic sialyl PE derivatives, we determined whether viral hemagglutination and hemolysis were inhibited in the presence of synthetic sialyl PE derivatives. We examined the effect of the synthetic sialyl PE derivatives on H3N2 subtype of viral hemagglutination and hemolysis. The synthetic sialyl PE derivatives inhibited not only hemagglutination, but also the hemolysis activity of A/Aichi/2/68 strain (Table 1 and Fig. 4), but not of the A/PR/8/34 strain. The inhibitory effect of the $(Neu5Ac)_2$-PE containing branched terminal

Table 1. Inhibition of hemagglutination of human influenza A viruses with synthetic sialyl PE derivatives

	Hemagglutination inhibition titer				
Viruses	*PE*	*Neu5Ac-PE*	*$(Neu5Ac)_2$-PE*	*Neu5Ac-PE (amide)*	*Neu5Ac-PE (methyl)*
A/PR/8/34	0	0	0	0	0
A/Aichi/2/68	0	128	256	64	64
A/Singapore/1/57	0	64	128	32	32

After incubation of both virus (2^4 HA units) and serial dilution of synthetic sialyl PE derivatives at 4 °C for 1 h, the reaction mixture was used for the hemagglutination assay as described in the Material and Methods section. The maximum dilution of the derivatives showing complete inhibition of the hemagglutination was defined as the hemagglutination inhibition titer.

Neu5Ac residues to A/Aichi/2/68 strain was stronger (IC_{50}, 150 μM) than that of other derivateives we examined, and was about half that of fetuin. The extent of the inhibitory effect of the derivatives corresponded well to the binding reactivity to influenza viruses that we tested.

Inhibitory effect of the synthetic sialyl PE derivatives against infection of A/Aichi12/68 (H3N2) strain

The inhibitory activity of the synthetic sialyl PE derivatives aganist infection of human influenza virus (A/Aichi/2/68) was assayed. Previous studies have shown that human influenza viruses can infect MDCK cells well [29–33]. We used MDCK cells as target cells for influenza A virus infection. The cells were inoculated with A/Aichi/2/68 (H3N2) in the presence or absence of the synthetic sialyl PE derivatives. After incubation for 20 h, the activity of lactate dehydrogenase (LDH) released from the cells was measured to estimate the cytopathic effects of the influenza virus A/Aichi/2/68 (H3N2) infection. As shown in Fig. 5, the activity of the enzyme was markedly reduced in the presence of sialyl PE derivatives. All of the synthetic sialyl PE derivatives, but not non-sialylated PE, showed inhibitory activity against infection of A/Aichi/2/68 strain in a dose dependent manner (IC_{50}, 15 μM to 200 μM). The effect of the $(Neu5Ac)_2$-PE derivative was stronger than that of other derivatives that we examined, and was about half that of fetuin, which has been reported to neutralize human influenza A viruses [11]. In according to the number of sialic acids, the effect of $(Neu5Ac)_2$-PE derivative was about 3.5-fold stronger than that of fetuin. These results

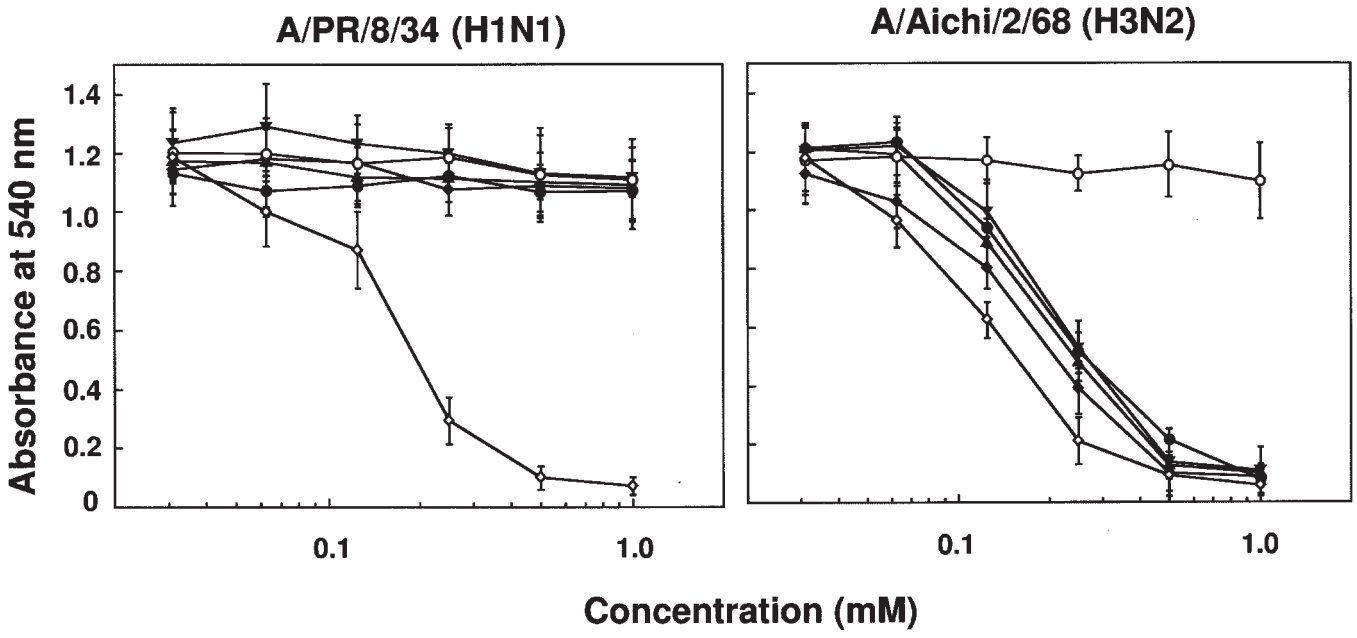

Figure 4. Hemolysis inhibition activity of the synthetic sialyl PE derivatives: Viral (A/Aichi/2/68 or A/Singapore/1/57) hemolysis activity was examined as described in the Materials and Methods section. Fetuin (◇) and PE (○) were used as controls. (●), Neu5Ac-PE; (◆), $(Neu5Ac)_2$-PE; (▲), Neu5Ac-PE (amide); (▼), Neu5Ac-PE (methyl).

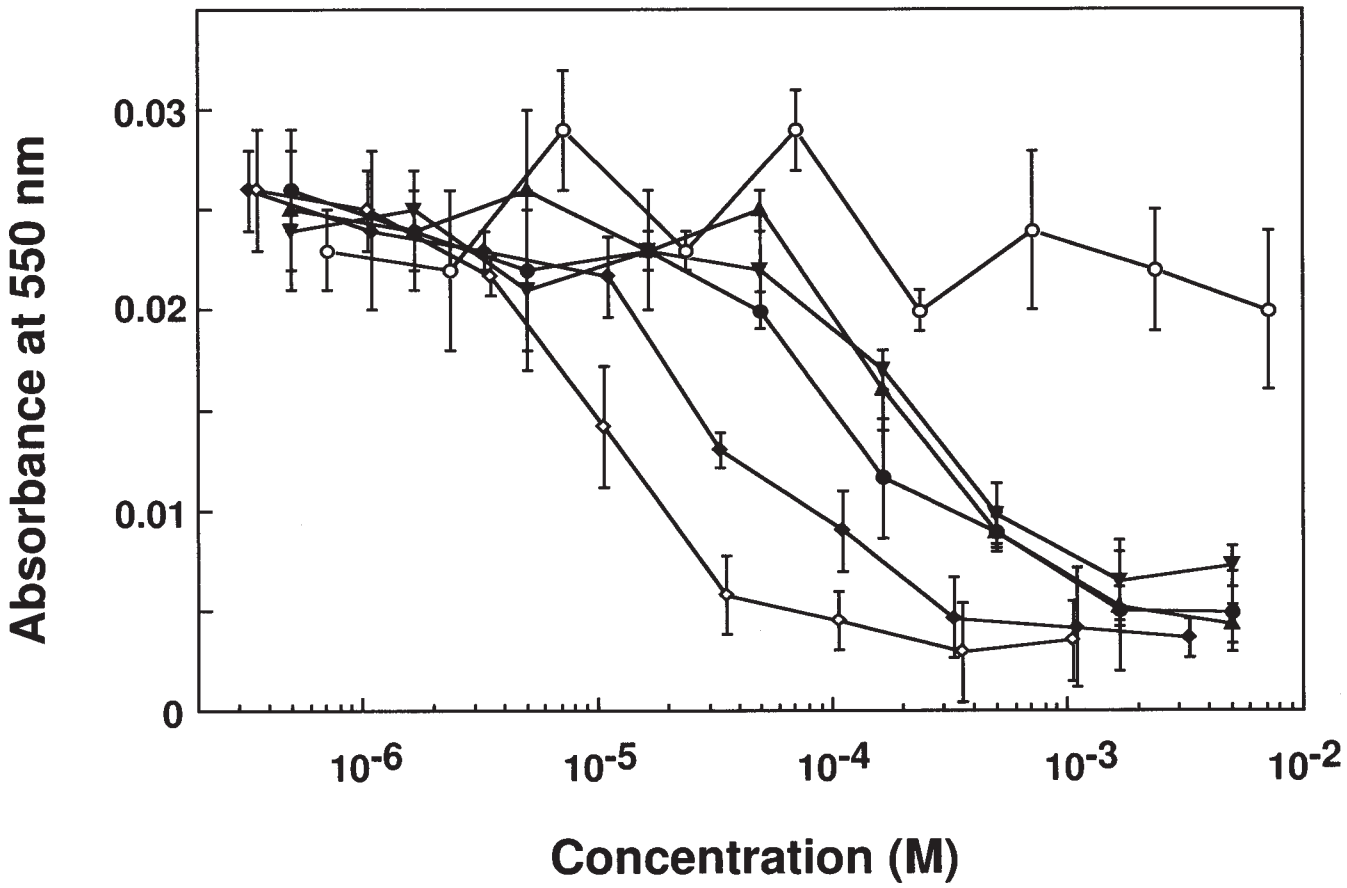

Figure 5. Neutralization of A/Aichi/2/68 (H3N2) infection by the sialyl PE derivatives: MDCK cells were inoculated for 5 h with A/Aichi/2/68 ($100TCID_{50}$) in the presence of various concentrations of the synthetic sialyl PE derivatives or control substrates (fetuin (◇) and PE (○)) as described in the Materials and Methods section. (●), Neu5Ac-PE; (◆), $(Neu5Ac)_2$-PE; (▲), Neu5Ac-PE (amide); (▼), Neu5Ac-PE (methyl).

indicated that synthetic sialyl PE derivatives are effective reagents for the neutralization of the A/Aichi/2/68 (H3N2) virus with complete inhibition of infection at a concentration of approximately 0.3 to 1.3 mM.

Discussion

The binding of influenza viruses to host cells has been shown to depend on the molecular species of sialic acid, the sialic acid-Gal linkage and the carbohydrate core structure of gangliosides [18]. Sialic acids found in animal cells were classified into 2 major species, Neu5Ac and Neu5Gc, in construction of the C-5 amino group [7]. Human influenza viruses recognize Neu5Ac residue but not Neu5Gc residue, which have not been detected in human tracheal epithelia [11]. Therefore, we designed and chemically synthesized compounds containing Neu5Ac and PE residues and tested the possibility that these compounds are inhibitors against human influenza viruses.

Our previous studies [10,15,17] have shown that A/PR/8/34 strain with H1N1 subtype bound most effectively to gangliosides containing the Neu5Acα2-3Gal linkage to the terminal galactose of their sugar chains, but showed a negative reaction with gangliosides containing the Neu5Acα2-6Gal linkage of the sialo-sugar chain. In contrast, A/Aichi/2/68 and A/Memphis/1/71 strains with H3N2 subtype preferentially recognized the Neu5Acα2-6Gal linkage of sialo-sugar chains and exhibited weak binding to the Neu5Acα2-3Gal linkage of sialo-sugar chains. Sialyl PE derivatives were recognized by the H3N2 subtype but not by the H1N1 subtype of human influenza A viruses, indicating that the structural assembly of Neu5Ac and PE residues may exhibit some similarity to the Neu5Acα2-6Gal linkage. The human influenza A viruses isolated after 1968 specifically bind to the Neu5Acα2-6Gal linkage [18]. Most human influenza B viruses were also specific to Neu5Acα2-6Gal linkage [19]. Therefore, we decided to develop synthetic anti-virus reagents showing similarity to the Neu5Acα2-6Gal linkage. In the TLC/virus-binding assay, the derivatives also bound a human influenza B virus (B/Lee/30 strain), which recognizes the Neu5Acα2-6Gal linkage (data not shown).

It is known that the interaction of influenza viruses with target cells is mediated by 2 major glycoproteins, hemagglutinin and sialidase, which are responsible for viral attachment to cells and for subsequent removal of viruses from cell surface sialyloligosaccharides, respectively [22,23]. The stability of the derivatives against the action of sialidase of viruses was demonstrated by incubating at 37 °C on TLC/virus-binding assay. The relative binding activity of the derivatives at 4 °C was not different from that at 37 °C (data not shown), suggesting that sialyl PE derivatives are resistant to hydrolysis with sialidases of influenza viruses. Rauvala [35] reported that the critical micelle concentration of ganglioside is 25 to 28 μM and demonstrated that the micelle form of substrate is resistant to the action of neuraminidase. In our case, since the IC_{50} of the sialyl PE derivatives to virus was 35 to 40 μM, the derivatives may exist as micelle forms.

Hemagglutinins of influenza viruses mediate the binding of the viruses to the host cell membrane receptors and the fusion between viral membrane and host cell lysosomal/endosomal membrane [24]. In this experiment, our results showed that synthetic sialyl PE derivatives inhibited viral hemagglutination, viral hemolysis and viral-induced cytopathic effect (CPE) *in vitro*. The effect of $(Neu5Ac)_2$-PE derivative was 2 to 3 times stronger than that of the other 3 derivatives. In contrast to Neu5Ac-PE derivative, the effect of Neu5Ac-PE (methyl) or Neu5Ac-PE (amide) was almost similar. Therefore, the inhibitory activity may be dependent upon the Neu5Ac residue content of derivative. Although the inhibitory concentration of the derivatives was higher than that of fetuin, the effect of the derivatives was about 2 - to 4-fold stronger than that of fetuin on the basis of the amount of sialic acid residue. Since there are different molecular species of sialic acid (NeuAc or NeuGc) and sialic acid linkage (α2-3, α2-6 or α2-8 linkage) on fetuin [13,34], the inhibitory effect of fetuin on an influenza virus (A/Memphis/1/71 (H3N2)) that only recognizes Neu5Acα2-6 linkage was lower.

In conclusion, synthetic sialyl PE derivatives bound to human influenza virus and inhibited both the hemagglutination and the hemolysis of H3N2 subtype of human influenza viruses. Furthermore, they inhibited the viral-induced cytopathic effect (CPE) *in vitro*. The synthetic sialyl PE derivatives may be considered a new type of anti-influenza virus agent.

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