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Sialidase of swine influenza A viruses: variation of the recognition specificities for sialyl linkages and for the molecular species of sialic acid with the year of isolation

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The sialidase of swine influenza A viruses of N1 and N2 subtypes, isolated from 1930 to 1992, was studied for substrate specificity with ganglio-series, lacto-series type II and GM3 gangliosides containing Neu5Acα2-3Gal, Neu5Gcα2-3Gal and Neu5Acα2-6Gal linkages. All viral sialidases tested showed that the activity for hydrolysing substrates with Neu5Acα2-3Gal was higher than the activities with Neu5Gcα2-3Gal and Neu5Acα2-6Gal linkages. When GM1b, GM3 and sialylparagloboside were used as substrates, the earliest strain (A/Wisconsin/15/30 H1N1, isolated in 1930) showed the activity ratio of Neu5Acα2-6Gal to Neu5Acα2-3Gal to be 0.13:0.2, and the ratio Neu5Gcα2-3Gal/Neu5Acα2-3Gal to be 0.19:0.37, while those strains isolated from 1978 to 1992 exhibited ratios of 0.29:0.58 for Neu5Acα2-6Gal/Neu5Acα2-3Gal and 0.51:0.76 for Neu5Gcα2-3Gal/Neu5Acα2-3Gal. The above results indicate that the substrate specificities of sialidases from swine influenza A viruses towards sialyl linkages and the molecular species of sialic acid are related to the year of isolation, i.e. strains isolated after 1978 exhibited higher activity towards Neu5Acα2-6Gal and Neu5Gcα2-3Gal linkages when compared with strains isolated in an earlier year, 1930.

Keywords: Influenza virus, sialidase specificity, gangliosides

Abbreviations: Neu5Ac, 5-*N*-acetylneuraminic acid; Neu5Gc, 5-*N*-glycolyneuraminic acid; Gal, D-galactose; Glc, D-glucose; Cer, Ceramide; II^3(Neu5Ac)Lac, Neu5Acα2-3Galβ1-4Glc; GM3(Neu5Acα2-3Gal), Neu5Acα2-3Galβ1-4Glcβ1-Cer; GM3(Neu5Gcα2-3Gal), Neu5Gcα2-3Galβ1-4Glcβ1-Cer; GM1b(Neu5Acα2-3Gal), Neu5Acα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer; GM1b(Neu5Gcα2-3Gal), Neu5Gcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer; IV^3(Neu5Ac)nLc4Cer, Neu5Acα2-3Galβ1-3GlcNAcβ1-4Galβ1-4Glcβ1-Cer; IV^3(Neu5Gc)nLc4Cer, Neu5Gcα2-3Galβ1-3GlcNAcβ1-4Galβ1-4Glcβ1-Cer; IV^6(Neu5Ac)nLc4Cer, Neu5Acα2-6Galβ1-3GlcNAcβ1-4Galβ1-4Glcβ1-Cer; TDC, taurodeoxycholate.

Introduction

It is known that sialic acid-containing glycoproteins and glycolipids on cell-surfaces are receptors for influenza viruses [1–4]. The interaction of influenza virus with the cellular receptor is mediated by two major glycoproteins, haemagglutinin and sialidase, which are responsible, respectively, for viral attachment to target cells and for subsequent removal of virus from cell surface sialyloligosaccharides [5–8]. The structures of glycoproteins and glycolipids on the surfaces of human or animal cells are very different [9]. The human influenza virus isolates exhibited different binding specificities for the carbohydrate residues and different hydrolysing specificities for sialyl linkages due to the action of the haemagglutinin and sialidase, respectively [10–12].

To study if there is any relationship for sialyl-linkage substrate specificities between the haemagglutinin and sialidase, several workers [13–15], including our laboratory [3, 16], have studied the specificities of haemagglutinin and sialidase of human influenza A and B isolates towards various sialyloligosaccharides.

In the case of animal viruses, particularly swine virus there is little information about specificity towards sialyloligosaccharides of diverse structure. Swine cells carry two major molecular species of sialic acid (Neu5Ac, Neu5Gc) in glycoproteins

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and glycolipids [17]. In this work we report the activities of sialidase from swine influenza A viruses, isolated from 1930 to 1992, on gangliosides with a diversity of sialyl-linkages and of molecular species of sialic acid (Neu5Ac, Neu5Gc).

Materials and methods

Viruses A/Swine/Wisconsin/15/30 (H1N1) was supplied by the National Institute of Health, Tokyo, Japan. A/Swine/Niigata/78 (H1N1) was from Chuo Livestock Hygiene Service Center, Niigata Prefecture, Japan. A/Swine/Shizuoka/1/78 (H1N1) was supplied by Shizuoka Prefectural Institute of Animal Health, Japan. A/Swine/Kanagawa/285/91 (H1N2) and A/Swine/Kanagawa/188/92 (H1N2) were from Kanagawa Prefectural Veterinary Diagnostic Laboratory, Japan. The viruses were grown in 11-day-old-embryonated chicken eggs, and were purified by sucrose density gradient centrifugation as described previously [18]. Protein was determined by the method of Lowry *et al.* [19] using bovine albumin as the standard.

Substrates The mixture of sialyllactose containing Neu5Acα2-3Gal and Neu5Acα2-6Gal linkages was purchased from Sigma (St Louis, Mo). The pure sialyllactose with Neu5Acα2-3Gal linkage was separated from the mixture by thin-layer chromatography in our laboratory [16]. GM 3(Neu5Acα2-3Gal) was from human liver [20], GM3(Neu5Gcα2-3Gal) was from equine erythrocytes [21]. GM1b(Neu5Acα2-3Gal) was purified from bovine brains by anion exchange and Iatrobeads column chromatography [22]. Native lacto-series gangliosides containing type II sugar chain, IV^3(Neu5Ac)nLc4Cer and IV^6(Neu5Ac)nLc4Cer, were prepared from human red blood cells [23], and from human meconium [24], respectively. IV^3(Neu5Gc)nLc4Cer was from bovine red blood cells [25]. Synthetic GM3(Neu5Acα2-6Gal) and GMlb(Neu5Acα2-6Gal) were prepared as described previously [26].

Assay of enzyme activity Standard assays for sialidase activity from human influenza B virus [16] were carried out using sialyllactose with a Neu5Acα2-3Gal linkage as substrate. One unit of sialidase activity was defined as the amount of activity required to release 1 μmol of Neu5Ac from the substrate per minute at 37°C.

Each of the reaction mixtures contained 0.1 mM substrate and 10 mU ml^{-1} of viral sialidase activity in a total volume of 50 μl in 20 mM of sodium acetate buffer, pH 5.4 (the optimum pH was determined using sialyllactose with a Neu5Acα2-3Gal linkage). The mixtures were incubated at 37°C for an appropriate time (usually for 10 min) and the reaction was stopped by immersing the mixture in boiling water for 1 min. After the enzyme reaction, incubation mixtures were directly applied to a TLC plate, which was developed in the solvent system isopropanol:butanol-water, 5:3:3 (v/v/v). Free sialic acid was determined by spraying with resorcinol/HCl reagent [27] and scanning at 580 nm (reference at 780 nm) with a dual-wavelength TLC scanner (CS 910 Shimadzu, Kyoto, Japan), using sialic as the standard.

Results

The five influenza A viruses of N1 and N2 subtypes were isolated from pigs in America and Japan. The three strains of N1 serotypes included A/Wisconsin/15/30, A/Niigata/78, and A/Shizuoka/1/78. The two isolates of N2 serotypes were A/Kanagawa/285/91 and A/Kanagawa/188/92. The substrate specificities of these strains were examined with eight gangliosides, including GM3, ganglio- and lacto-series gangliosides containing different molecular species of sialic acid (Neu5Ac, Neu5Gc) and different sialyl-linkages (Neu5Acα2-3Gal, Neu5Acα2-6Gal), for various incubation times (0–30 min). The rates of sialic acid hydrolysis with gangliosides containing different sialyl-linkages (Neu5Acα2-3Gal and Neu5Acα2-6Gal) were assayed for each strain. All strains were similar in their enzymatic properties. They showed a preferential sialidase activity for the Neu5Acα2-3Gal linkage of the ganglioside, GM3(Neu5Acα2-3Gal) (II^3(Neu5Ac)LacCer), and exhibited a weak hydrolysing activity for GM3(Neu5Acα2-6Gal) (Fig. 1).

A/Wisconsin/15/30 showed a marked substrate specificity preference for the Neu5Acα2-3Gal linkage, and remarkably low reactivity with the Neu5Acα2-6Gal linkage of GM3 (Fig. 1a). However the reactivity of A/Wisconsin/15/30 on the Neu5Acα2-3Gal linkage was similar to the other four strains under the same conditions (Table 1).

Lacto-series type II sugar chain gangliosides gave similar results (Fig. 2). These result demonstrate that the sialidase of swine influenza A virus shows remarkably preferential recognition for the linkage Neu5Acα2-3Gal in gangliosides relative to the Neu5Acα2-6Gal linkage.

The rate of sialic acid released by the sialidase from our five strains was also compared using the same GM3 gangliosides with different molecular species of sialic acid. Sialidases from all swine A viruses tested preferentially cleave the Neu5Acα2-3Gal rather than the Neu5Gcα2-3Gal linkages of GM3 (Fig. 1) and sialylparagloboside (lacto-series type II ganglioside) (Fig. 2).

The sialidase activities towards the substrate containing different sialyl linkages (2-3, 2-6) and different molecular species of sialic acid (Neu5Ac, Neu5Gc) were determined for viruses isolated in the 1930–1992 period (Fig. 3). A dramatic increase in sialidase activity towards the Neu5Acα2-6Gal linkage and towards the *N*-glycolyl type of sialic acid (Neu5Gcα2-3Gal) was found in the viruses isolated between 1930 and 1978. When GM1b, GM3 and sialylparagloboside were respectively used as substrates, the earliest strain, A/Wisconsin/15/30 H1N1 isolated in 1930, showed the activity ratio of Neu5Acα2-6Gal to Neu5Acα2-3Gal to be 0.13 ± 0.03 to 0.2 ± 0.05 (Fig. 3, panel 1), and the ratio of Neu5Gcα2-3Gal/Neu5Acα2-3Gal to be 0.19 ± 0.02 to 0.37 ± 0.04 (Fig. 3, panel 2). In contrast, A/Niigata/78 (H1N1), A/Shizuoka/1/78

Table 1. Sialidase activity of swine influenza A viruses on gangliosides containing the Neu5Acα2-3Gal linkage.

Virus	*% of Neu5Ac released* (n = 6)		
	GM1b(2-3) % ± SD	*GM3(2-3)* % ± SD	*IV^3(Neu5Ac)nLc4Cer* % ± SD
A/Wisconsin/15/30	61.8 ± 1.7	76.0 ± 3.7	79.8 ± 5.3
A/Niigata/78	58.8 ± 3.2	68.0 ± 1.4	67.4 ± 1.4
A/Shizuoka/1/78	53.1 ± 1.1	57.1 ± 2.3	72.6 ± 4.1
A/Kanagawa/285/91	58.5 ± 5.8	72.0 ± 1.6	82.3 ± 4.9
A/Kanagawa/188/92	73.2 ± 6.2	64.1 ± 4.3	71.4 ± 5.7

Sialidase activity was assayed by measuring the release of sialic acid from the GM1b, GM3 and lacto-series type II sugar chain ganglioside (sialylparagloboside, IV^3(Neu5Ac)nLc4Cer) which contain Neu5Acα2-3Gal linkages. Reaction mixtures contained 10 mU ml^{-1} viral sialidase activity, 0.1 mM substrate, in 20 mM sodium acetate buffer, (pH 5.4), and were incubated with 2 mM of TDC at 37°C for 10 min. The percentage of sialic acid released from the Neu5Acα2-3Gal linkage is expressed relative to the ganglioside added.

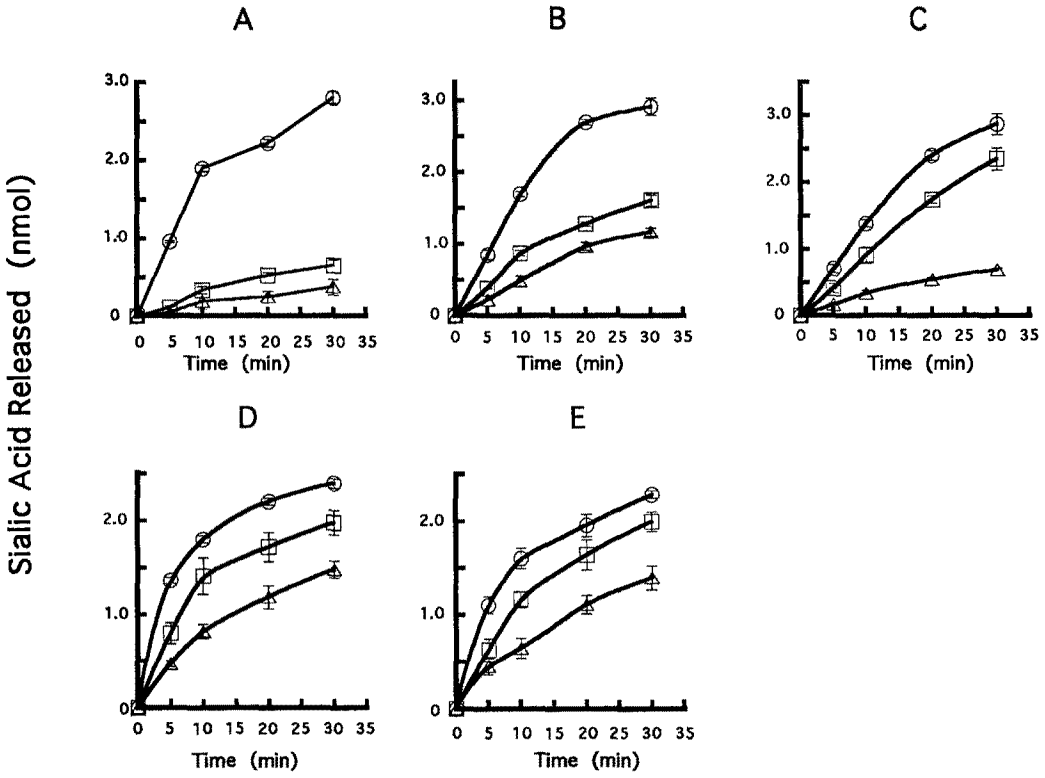

Figure 1. Sialidase specificity of swine influenza A virus on GM3 carrying different molecular species of sialic acid and sialyl linkages. The release of sialic acid from GM3 containing Neu5Acα2-3Gal, (-○-), Neu5Gcα2-3Gal (-□-) and Neu5Acα2-6Gal (-Δ-) linkages was compared in five strains. Reaction mixtures contained 10 mU ml^{-1} of viral sialidase activity and 0.1 mM of GM3 in 20 mM of sodium acetate buffer, pH 5.4, and were incubated with 2 mM of TDC at 37°C for the indicated times. The sialic acid released was measured as described in Materials and methods. Each line represents the mean ± SD of six determinations.
A, A/Wisconsin/15/30(H1N1); B, A/Niigata/78(H1N1); C, A/Shizuoka/1/78(H1N1); D, A/Kanagawa/258/91(H1N2); E, A/Kanagawa/188/92(H1N2).

(H1N1), A/Kanagawa/285/91 (H1N2), and A/Kanagawa/188/92 (H1N1) isolated between 1978 and 1992 exhibited ratios of 0.29 ± 0.06 to 0.58 ± 0.02 for Neu5Acα2-6Gal/Neu5Acα2-3Gal (Fig. 3, panel 1) and 0.51 ± 0.02 to 0.76 ± 0.05 for Neu5Gcα2-3Gal/Neu5Acα2-3Gal (Fig. 3, panel 2). The above results indicate that the sialidase specificities of swine influenza A viruses towards sialyl linkages and the molecular species of sialic acid are related to the year of isolation,

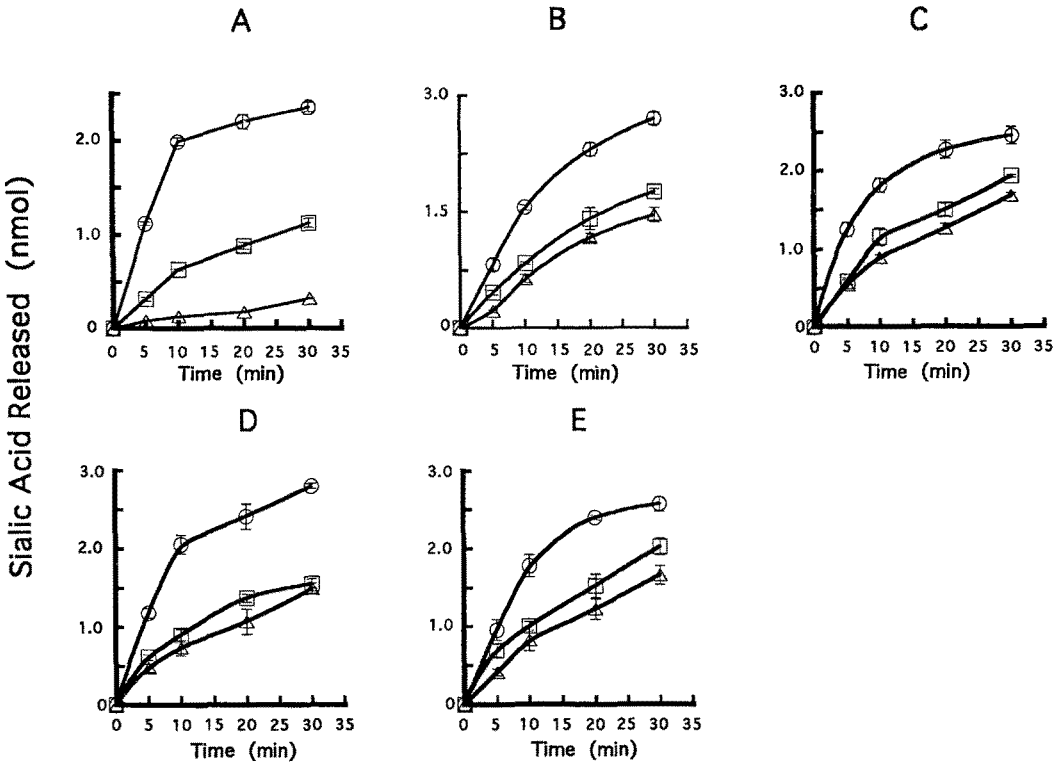

Figure 2. Sialidase specificity of swine influenza A virus on lacto-series type II sugar chain gangliosides, sialyparaloboside, carrying different molecular species of sialic acid and sialyl linkages. The release of sialic acids from lacto-series gangliosides containing the Neu5Acα2-3Gal (IV^3(Neu5Ac)nLc4Cer), (–○–), the Neu5Gcα2-3Gal (IV^3(Neu5Gc)nLc4Cer) (–□–) and Neu5Acα2-6Gal (IV^6(Neu5Ac)nLc4Cer) (–Δ–) linkages was compared in five strains. Reaction mixtures contained 10 mU ml^{-1} of viral sialidase activity and 0.1 mM of ganglioside in 20 mM of sodium acetate buffer, pH 5.4, and were incubated with 2 mM of TDC at 37°C for the indicated times. The sialic acid released was measured as described in Materials and methods. Each line represents the mean ± SD of six determinations.
A, A/Wisconsin/15/30(H1N1); B, A/Niigata/78(H1N1); C, A/Shizuoka/1/78(H1N1); D, A/Kanagawa/285/91(H1N2); E, A/Kanagawa/188/92(H1N2).

i.e. later strains isolated after 1978 exhibited higher activity towards Neu5Acα2-6Gal and Neu5Gcα2-3Gal linkages when compared with strains isolated in an earlier year, 1930.

Discussion

Gangliosides, essentiai components of cell surface membranes, are the native substrates of viral sialidase, and are also the native receptors of haemagglutinin of influenza viruses, as previously reported [1–4, 16, 28]. It is evident that swine cells contain gangliosides carrying different molecular species of sialic acid (Neu5Ac, Neu5Gc), linkages and lengths of sugar chains [17]. Gangliosideswith different sialyl-linkages (α2-3, α2-6), different molecular species of sialic acid (Neu5Ac, Neu5Gc) and different sugar chain lengths (GM1b, GM3) were therefore used as substrates to determine the specificity of swine virus sialidases.

From the data obtained it may be concluded that the diversities of sialyl-linkage and molecular species of sialic acid influence the enzymatic activity of sialidases from swine influenza A virus. For gangliosides with the same molecular species of sialic acid but with different forms of sialyl-linkage sialidase exhibited a high activity towards the Neu5Acα2-3Gal linkage, whereas a low activity was shown towards the Neu5Acα2-6Gal linkage for the same virus strain. In the case of similar sialyl-linkage and different substitutions of the 5-amino group acid of sialic acid (Neu5Ac, Neu5Gc), the release of *N*-acetylneuraminic acid was faster than the release of sialic acid carrying the 5-*N*-glycolyl group for the same virus strain. We did not find any significant effect the sugar chain length had on sialidase activity.

TDC is the optimum detergent for enhancing enzymatic activity [16], because ganglioside contains a hydrophobic ceramide group.

The drift in linkage specificity of the sialidase has been studied by analysing human influenza A isolated from 1957 to 1987 [12] and also influenza B viruses from 1940 to 1990 [16]. In this work, especially for the period of 48 years between 1930 (A/Wisconsin/15/30) and 1978 (A/Niigata/78, A/Shizuoka/1/78), the sialidase specificity towards the Neu5Acα2-6Gal linkage (activity ratio of Neu5Acα2-6Gal/Neu5Acα2-3Gal) increased remarkably. The ratio for two other strains (A/Kanagawa/285/91, A/Kanagawa/188/92) from the nineties was also markedly higher than that of A/Wisconsin/15/30 isolated in 1930 using GM1b, GM3, and

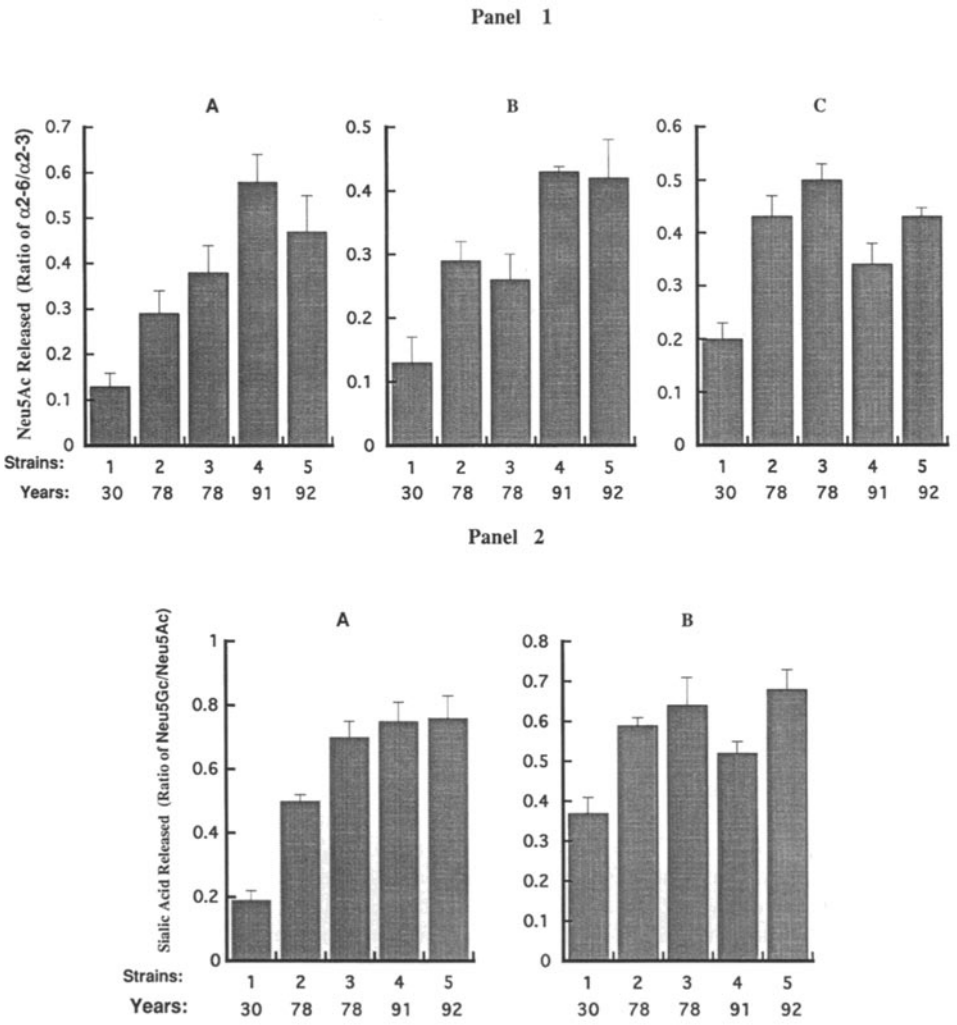

Figure 3. The change in the activity of swine influenza A virus sialidase towards sialyl linkages (α2-6/α2-3) (panel 1) and the molecular species of sialic acid (Neu5Gc/Neu5Ac) (panel 2) relative to the year of isolation. The five strains isolated from 1930 to 1992 were compared for sialidase activity assayed by measuring the release of sialic acids from gangliosides containing Neu5Acα2-3G al, Neu5Acα2-6Gal, and Neu5Gcα2-3Gal linkages. Reaction mixtures contained 10 mU ml^{-1} of viral sialidase activity and 0.1 mM of substrates in 20 mM of TDC at 37°C for 10 min. Each column represents the mean ± SD of six determinations. The substrates used in panel 1 were GM1b(Neu5Acα2-3Gal), GM1b(Neu5Acα2-6Gal) (A); GM3(Neu5Acα2-3Gal), GM3(Neu5Acα2-6Gal) (B); and sialylparagloboside (lacto-series type II ganglioside), IV^3(Neu5Ac)nLc4Cer, IV^6(Neu5Ac)nLc4Cer (C); and in panel 2 were GM3(Neu5Acα2-3Gal), GM3(Neu5Gcα2-3Gal) (A) and sialylparagloboside (lacto-series type II gangliosides) IV^3(Neu5Ac)nLc4Cer, IV^3(Neu5Gc)nLc4Cer (B). 1, A/Wisconsin/15/30(H1N1); 2, A/Niigata/78(H1N1); 3, A/Shizuoka/1/78(H1N1); 4, A/Kanagawa/285/91(H1N2); 5, A/Kanagawa/188/92(H1N2).

sialylparagloboside ganglioside as substrates. The above results indicated that the tendency for sialidase from the later swine strains to show an increased specificity for the Neu5Acα2-6Gal linkage was consistent with previous reports on human influenza A [12] and B [16] viruses. Additional isolates from the period 1930–1978 were not available to us and therefore could not be tested.

Major sialic acid species in swine cells are Neu5Ac and Neu5Gc [17] although only Neu5Ac is found in human cells. Human influenza virus haemagglutinin binds specifically to Neu5Ac-sugar chains and the Neu5Gc species of sialic acid is very poorly recognized by human influenza virus haemagglutinin [3]; however, haemagglutinin of influenza virus isolated from pigs can bind to Neu5Gc-sugar chains at almost the same rate as to Neu5Ac-sugar chains (Suzuki Y. *et al.*, manuscript in preparation) indicating that there are correlations between the sialic acid species recognized by the influenza virus haemagglutinin as host cell receptors and sialic acid species predominantly present in the host. In this report, we found that the sialidase of swine influenza A viruses showed significant hydrolysis of Neu5Gc species in gangliosides and also that the viruses isolated after 1978 exhibited preferential sialidase activity towards Neu5Gc species gangliosides rather than Neu5Ac when compared with strains isolated in an earlier

year, 1930. The results suggest that the sialic acid species recognized by the influenza virus sialidase may correlate, in part, with the sialic acid species occurring in host cells, as is the case for influenza virus haemagglutinin.

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