FEBS Letters 404 (1997) 192–196 FEBS 18272

# Swine influenza virus strains recognize sialylsugar chains containing the molecular species of sialic acid predominantly present in the swine tracheal epithelium

Takashi Suzuki[a], Goh Horiike[a], Yasuhiro Yamazaki[a], Kaoru Kawabe[a], Hiroyuki Masuda[a], Daisei Miyamoto[a], Masao Matsuda[b], Shin-Ichiro Nishimura[b], Tatsuya Yamagata[c], Toshihiro Ito[d], Hiroshi Kida[d], Yoshihiro Kawaoka[e], Yasuo Suzuki[a,*]

[a]*Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Science, 52-1 Yada, Shizuoka-shi 422, Japan*
[b]*Division of Biological Science, Graduate School of Science, Hokkaido University, Sapporo 060, Japan*
[c]*Department of Biomolecular Engineering, Tokyo Institute of Technology, 4259 Nagatsuta, Midoriku, Yokohama 226, Japan*
[d]*Department of Veterinary and Hygiene and Microbiology, School of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan*
[e]*Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, Memphis, TN 38101, USA*

Received 29 December 1996; revised version received 27 January 1997

**Abstract** **We determined the ratio of *N*-glycolylneuraminic acid (Neu5Gc) to *N*-acetylneuraminic acid (Neu5Ac) in swine respiratory epithelia by fluorometric high-performance liquid chromatography, and examined the binding specificity of swine influenza virus strains for gangliosides containing different molecular species of sialic acid (Neu5Ac and Neu5Gc), and for bovine erythrocyte sialoglycoprotein 2 (GP-2) containing Neu5Gc as its predominate sialic acid (96% of total sialic acids). The presence of Neu5Gc, which had not been detected in human tracheal epithelia, and Neu5Ac in swine tracheal epithelia was observed in a 1:1 ratio. The swine influenza virus H1 and H3 isolates tested, except for A/swine/Iowa/15/30 (H1N1), displayed a marked binding ability for sialylsugar chains containing Neu5Gc compared with that of the human influenza virus strains. These results suggest that swine influenza viruses recognize sialylsugar chains containing the molecular species of sialic acid present predominantly in the swine tracheal epithelium.**



*Key words:* Influenza virus; Sialic acid

## 1. Introduction

Influenza A viruses infect many animal species including horses, birds, pigs, and humans. The interaction of influenza A virus and a host cell is mediated by a viral spike glycoprotein, the hemagglutinin which recognizes glycoproteins and glycolipids containing terminal sialic acid residues [1–5]. The binding specificity of influenza A virus for a host cell receptor is an important factor in the host range and tissue tropism. Human strains preferentially recognize the terminal *N*-acetylneuraminic acid (Neu5Ac)-α-2,6-D-galactose (Gal) linkage in sialo-sugar chains, whereas the hemagglutinin of avian and equine strains preferentially recognizes terminal Neu5Ac-α-2,3-Gal sequences [6–9]. It was reported that the presence of a Neu5Ac-α-2,6-Gal sequence which was found on the surface of the human tracheal epithelium would select for the receptor specificity of human influenza A virus strains, and would be a determinant of the host range of viruses [10].

Sialic acids which were found in animals were classified into 2 major species, Neu5Ac and *N*-glycolylneuraminic acid (Neu5Gc), in construction of the C-5 amino group [11]. The ratio between Neu5Gc and Neu5Ac in tissues varies among animal species and their tissues. Neu5Ac is widely found in various animal tissues. By contrast, Neu5Gc has not been detected in human tissues under normal conditions [11–13]. We determined the ratio of Neu5Gc to Neu5Ac in pig respiratory epithelia by fluorometric high-performance liquid chromatography (HPLC), and examined the binding specificity of swine influenza viruses for gangliosides containing different molecular species of sialic acid, and for bovine sialoglycoprotein 2 (GP-2) containing I-active oligosaccharides with Neu5Gcα-2,3-Gal [14–16]. In addition, the binding specificity of swine influenza virus isolates for sialic acid linkage was determined using water-soluble polymers containing different sialic acid linkages. Human H1N1 and H3N2 strains preferentially recognized oligosaccharides with Neu5Ac as previously reported [3,5,17]. On the other hand, 4 swine H1N1 and H3N2 strains tested, except for A/swine/Iowa/15/30 (H1N1), recognized sugar chains containing Neu5Gc, which were detected in the mucosal epithelia of swine trachea, as well as Neu5Ac. The results indicate that swine influenza viruses preferentially recognize the oligosaccharides containing the principal molecular species of sialic acid which is existent on the host cells, and that the binding specificity of influenza A virus for sialic acid molecular species may be an important factor for restricting the virus host range.

## 2. Materials and methods

### 2.1. Influenza virus

Influenza virus A/swine/Kanagawa/1/92 (H1N2), A/swine/Italy/309/83 (H3N2), A/swine/Hokkaido/2/81 (H1N1), A/swine/Colorado/77 (H3N2), A/swine/Iowa/15/30 (H1N1), A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2), and A/Memphis/1/71 (H3N2) were propagated in the allantoic cavity of 11-day-old chicken eggs for 48 h at 35°C and purified by sucrose density gradient centrifugation [18]. Viral hemagglutination units (HAU) were determined at 4°C in microtiter plates as previously described [2].

### 2.2. Swine and human tracheal epithelia

Human tracheal epithelia were obtained from normal tracheas of patients (4 males and 1 female, 67–72 years old) who died of disease (2 gastric cancers, 2 pancreatic cancers, and diabetes). Swine tracheal epithelia were from the Shizuoka municipal meat works.

*Corresponding author. Fax: (81) (54) 264-5721.


*PII* S0014-5793(97)00127-0

*2.3. Glycoconjugate polymer*

Glycoconjugate polymer having a Neu5Acα-2,3-Galβ-1,4-GlcNAc (Neu5Acα2,3LacNAcPA) linkage was enzymatically prepared by stepwise sugar-elongation on a water-soluble GlcNAc-bearing polyacrylamide as previously described [19]. The polymer having Neu5Acα-2,6-Galβ-1,4-GlcNAc (Neu5Acα2,6LacNAcPA) linkage was prepared by sialylation with rat liver sialyltransferase (Sigma).

*2.4. TLC/virus overlay assay*

Gangliosides (0.5–4 nmol) were developed on silica gel plastic plates (Polygram Sil G, Macherey-Nagel, Germany) using a solvent system of chloroform/methanol/12 mM $MgCl_2$ (5:4:1, v/v). Gangliosides were visualized by spraying the plate with the resorcinol-HCl reagent [20]. Immunochemical detection of virions on thin-layer plates was performed as described in [5,6,21].

*2.5. Fluorometric HPLC method for determination of sialic acid species*

Fluorometric determination of Neu5Ac and Neu5Gc was conducted by the HPLC method using 1,2-diamino-4,5-methylenedioxybenzene (DMB) as previously described [22]. Human and swine tracheal epithelia were washed with cold PBS and lyophilized. Each tracheal epithelium (10 mg) was hydrolyzed with 200 μl of 25 mM sulfuric acid. The hydrolysate was reacted with DMB reagent and heated at 60°C for 2.5 h in the dark to develop the fluorescence of sialic acids. A 10 μl aliquot of the resulting solution was used for determination of sialic acids. For the establishment of calibration curves, standard mixtures of Neu5Ac and Neu5Gc (Sigma, USA) were used.

*2.6. Sialoglycoprotein 2 (GP-2)*

GP-2 was isolated from bovine erythrocyte membranes as described [14–16].

*2.7. Hemagglutination inhibition assay*

A hemagglutination inhibition (HAI) assay with glycoconjugate polymers was performed according to [15,17].

*2.8. Virus hemadsorption assay with GP-2 immobilized microplates*

PBS (50 μl) containing GP-2 (20 μg/ml) was added to each microtiter well (Immuno plate MaxiSorp, Nunc, Denmark) and incubated at 37°C for 5 h. The solution was removed and the remaining binding sites on the wells were blocked with 250 μl of PBS containing 0.01% of a blocking reagent (Block Ace, Snow Brand, Japan) at 4°C for 12 h. After washing with PBS, serial 2-fold dilutions of influenza virus (50 μl containing $2^{10}$ HAU) in 0.01% (w/v) gelatin-PBS were added to the wells. The microplate was incubated at 4°C for 12 h. After washing with PBS 5 times, 25 μl of 0.01% (w/v) gelatin-PBS and 25 μl of 0.5% (v/v) chicken erythrocytes were added to each well, mixed and allowed to settle at 4°C for 1 h. The maximum dilution of virus showing hemadsorption was defined as the virus hemadsorption (VH) titer. As a negative control, each influenza virus was added to the asialo-GP-2 immobilized wells instead of GP-2. Asialo-GP-2 was prepared by treatment with proteinase-free sialidase (*Arthrobacter ureafaciens*, Nacalai Tesque) as previously described [17].

## 3. Results

*3.1. Determination of Neu5Ac and Neu5Gc in swine and human tracheal epithelia*

As shown in Fig. 1, peaks ascribable to Neu5Ac and Neu5Gc were detected in the chromatogram obtained for swine tracheal epithelia. The concentrations of Neu5Ac and Neu5Gc in the epithelia were 74.6 ± 17.9 and 84.0 ± 17.6 pmol/mg (mean values ± standard deviation, $n = 5$), and the ratio of Neu5Ac and Neu5Gc was approx. 1:1 as shown in Table 1. On the other hand, only the peak ascribable to Neu5Ac was detected in the chromatogram obtained for human tracheal epithelia. In addition, no trace of the peak ascribable to Neu5Gc was detected in those, even at higher detector sensitivities (data not shown).

*3.2. Binding of swine influenza viruses to gangliosides containing different molecular species of sialic acid*

TLC/virus overlay assays with $II^3$Neu5AcLacCer (Neu5AcGM3) and $II^3$Neu5GcLacCer (Neu5GcGM3) were used to determine the binding activity of 3 swine influenza viruses. As shown in Fig. 2, 2 swine H1 influenza virus strains, A/swine/Hokkaido/2/81 (H1N1) and A/swine/Kanagawa/1/92 (H1N2) except for A/swine/Iowa/15/30 (H1N1) recognized not only Neu5AcGM3 but also Neu5GcGM3. In contrast with these isolates, human strains, A/PR/8/34 (H1N1) and A/Memphis/1/71 (H3N2) preferentially bound to Neu5AcGM3, but not to Neu5GcGM3, which contains terminal Neu5Gc as reported previously [3,5,6,17].

*3.3. Sialic acid linkage specificity for swine influenza viruses*

The binding specificity of 5 swine and 3 human influenza virus H1 and H3 isolates was determined by HAI assay using glycoconjugate polymers containing 2 types of sialo-sugar linkage Neu5Acα2,3LacNAcPA or Neu5Acα2,6LacNAcPA. As shown in Table 2, hemagglutination of the swine H1 and H3 isolates, except for A/swine/Iowa/15/30 (H1N1), was sensitive to inhibition by Neu5Acα2,6LacNAcPA. As compared with the 4 isolates, A/swine/Iowa/15/30 (H1N1) was preferentially sensitive to inhibition by Neu5Acα2,3LacNAcPA. Human influenza virus A/PR/8/34 preferentially recognizes the Neu5Acα2,3Gal linkage in sialo-sugar chains over the Neu5Acα2,6Gal linkage, whereas A/Aichi/2/68 and A/Memphis/1/71 preferentially recognize the Neu5Acα2,6Gal linkage, as described previously [3,5,6,17]. The binding specificity of the human strains determined by HAI assay using the polymers concurred with previous data.

*3.4. Binding of swine influenza viruses to sialoglycoprotein 2 containing N-glycolylneuraminic acid*

Differences in the binding specificity of swine and human influenza viruses for structures of sialic acid residues were confirmed by the ability of the viruses to bind bovine erythrocyte sialoglycoprotein 2 (GP-2) containing the Neu5Gcα2-3Gal sequence and I-active oligosaccharides with Neu5Gc as its predominate sialic acid (96% of the total sialic acids) [14–16,23]. Swine and human influenza A virus isolates exhibited marked differences in their ability to bind GP-2 (Table 3). Swine H1 and H3 isolates except for A/swine/Iowa/15/30 bound to the microplate coated with GP-2. In contrast, human strains had a poor binding ability for GP-2.

Table 1
Concentration of Neu5Ac and Neu5Gc in swine and human respiratory epithelia

| Animal | Neu5Ac (pmol/mg)[a] | Neu5Gc (pmol/mg) | Neu5Ac/Neu5Gc (mol/mol) |
|---|---|---|---|
| Pigs | 74.6 ± 17.9 | 84.0 ± 17.6 | 47/53 |
| Human | 124 ± 48.1 | ND[b] | 100/0 |

[a]The concentration is expressed as the mean value ± standard deviation ($n$=5).
[b]Not detected.

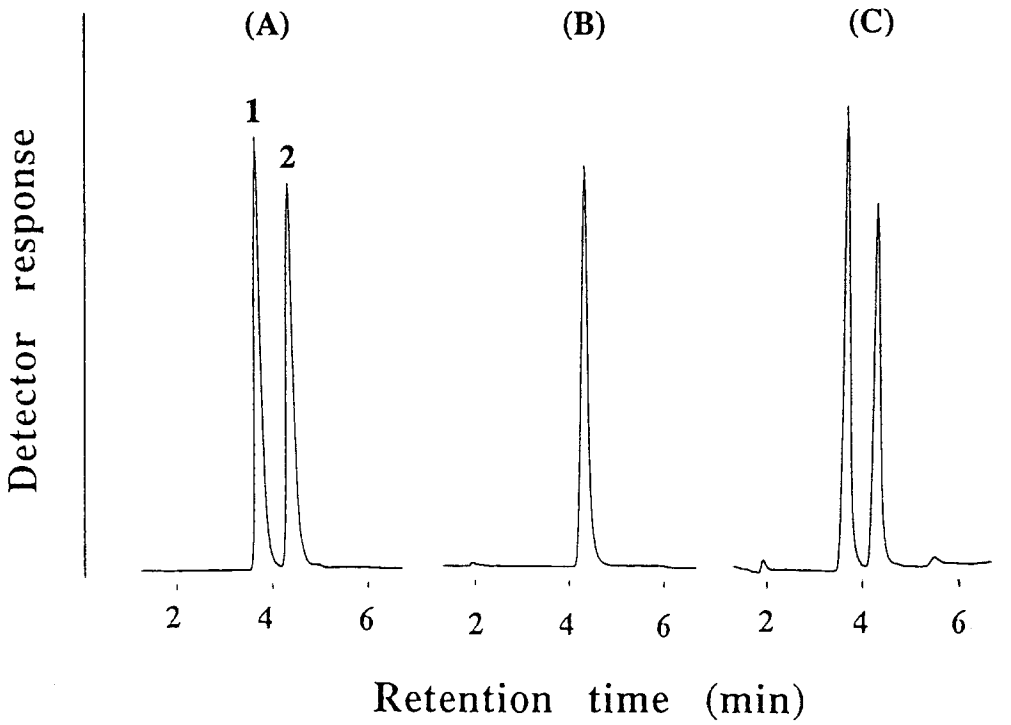


Fig. 1. Chromatograms of DMB derivatives of Neu5Ac and Neu5Gc obtained from tracheal epithelia of humans and pigs. The standard mixtures of Neu5Ac and Neu5Gc (A) and tracheal epithelia from humans (B) and pigs (C) were treated as described in Section 2. The fluorescence of DMB derivatives was detected at an excitation wavelength of 373 nm and an emission wavelength of 448 nm. Peak 1, Neu5Gc; peak 2, Neu5Ac.

## 4. Discussion

In this study, we investigated whether influenza viruses isolated from swine recognize the major molecular species of sialic acid expressed on host respiratory epithelia. As shown in Table 1, the predominant presence of Neu5Gc, which had not been detected in human tracheal epithelia, was observed in swine tracheal epithelia. Accordingly, the binding activity of swine influenza viruses toward gangliosides containing different molecular species of sialic acid (Neu5Ac and Neu5Gc) was examined by a virus overlay assay on TLC. Swine influenza viruses except for A/Iowa/15/30 (H1N1) exhibited obvious binding activity toward Neu5GcGM3 as well as Neu5AcGM3. Similarly, the swine isolates bound to $IV^3$Neu5GcnLc$_4$Cer containing longer sialyloligosaccharides than Neu5GcGM3 (data not shown), whilst human strains exhibited poor binding activity for Neu5GcGM3. Previously, we demonstrated that the hemagglutination of human influenza virus A and B strains was not inhibited by bovine sialylglycoprotein, GP-2 containing I-active oligosaccharides with Neu5Gc as its predominate sialic acid, however, they were very sensitive to a GP-2 derivative to which Neu5Ac was reattached in a single and defined linkage by sialyltransferase, and which exhibited a specific inhibitory effect on the hemagglutination induced by A/PR/8/34 (H1N1) and A/Aichi/2/68 (H3N2), which is 5–16 times higher than that of human glycophorin [17]. Accordingly, GP-2 was used to determine whether swine virus strains can bind sialylglycoproteins with

Table 2
Inhibition of swine and human influenza virus hemagglutination by glycoconjugate polymers containing 2 types of sialo-sugar linkage

| Virus preparation | HAI titer[a] | |
|---|---|---|
| | Neu5Acα2,3LacNAcPA | Neu5Acα2,6LacNAcPA |
| Swine isolates | | |
| A/swine/Iowa/15/30 (H1N1) | 16 | 2 |
| A/swine/Colorado/77 (H3N2) | 4 | 16 |
| A/swine/Hokkaido/2/81 (H1N1) | 2 | 32 |
| A/swine/Italy/309/83 (H3N2) | 4 | 16 |
| A/swine/Kanagawa/1/92 (H1N2) | 2 | 16 |
| Human isolates | | |
| A/PR/8/34 (H1N1) | 128 | 2 |
| A/Aichi/2/68 (H3N2) | 4 | 64 |
| A/Memphis/1/71 (H3N2) | 4 | 32 |

The glycoconjugate polymers (1 mg/ml) were serially diluted (2-fold) with 25 μl of 0.02% (w/v) gelatin-PBS. The hemagglutination inhibition (HAI) assay was performed as described previously [15,17].
[a]HAI titers are expressed as the maximum dilution required to give complete inhibition of hemagglutination.

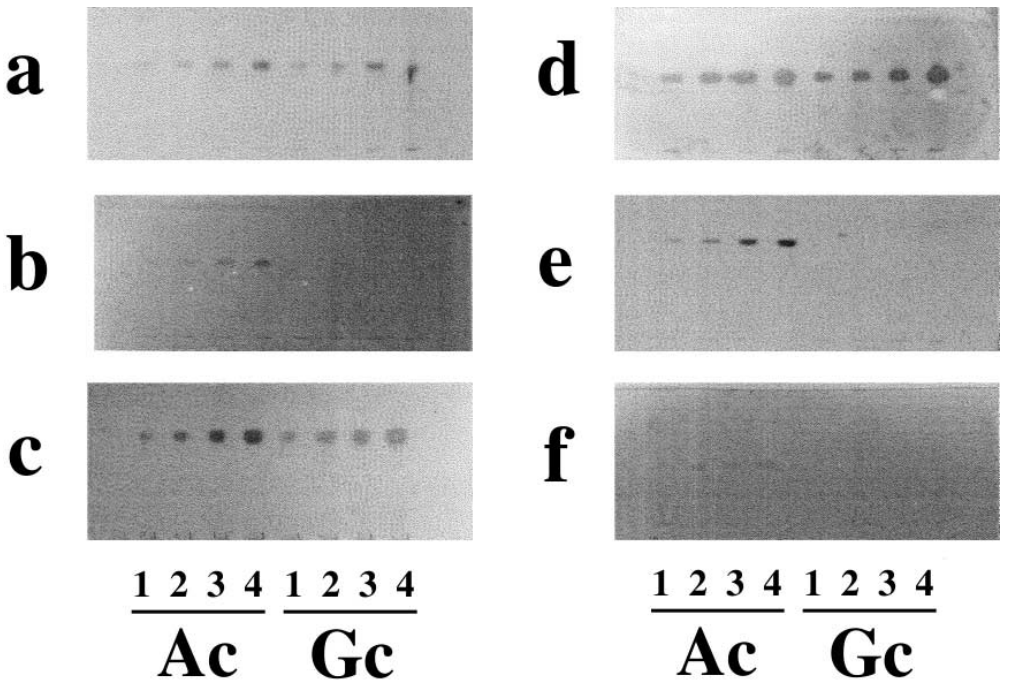


Fig. 2. Binding specificity of swine and human influenza virus strains to gangliosides containing different molecular species of sialic acid. Various amounts of each gangliosides (Ac: Neu5AcGM3 and Gc: Neu5GcGM3), 0.5 nmol (lane 1), 1 nmol (lane 2), 2 nmol (lane 3), 4 nmol (lane 4) were applied to a TLC plate. The plates were incubated with influenza virus suspension of $2^8$ HAU in PBS at 4°C for 12 h, and then stained by a TLC/virus overlay assay as described previously. (a) Detected by resorcinol reagent; (b) A/swine/Iowa/15/30 (H1N1); (c) A/swine/Hokkaido/2/81 (H1N1); (d) A/swine/Kanagawa/1/92 (H1N2); (e) A/PR/8/34 (H1N1); (f) A/Memphis/1/71 (H3N2).

Neu5Gc-sugar chains as well as gangliosides. Three swine H1 and 2 swine H3 isolates except for A/swine/Iowa/15/30 had remarkable binding abilities for GP-2 as compared with that of human influenza virus strains. These results suggest that the influenza A virus may preferentially recognize oligosaccharides containing the molecular species of sialic acid which is presented predominantly in host cells. A proposal has been made that selection of receptor specific variants could result from selective binding of viruses towards host cells expressing complementary sialyloligosaccharide sequences [24–26]. In addition, it has been reported that the presence of Neu5Acα2,6-Galactose linkages on human ciliated cells and Neu5Ac2,3Galactose linkages in bronchial mucus secretions may combine and select for the hemagglutinin receptor specificity of human influenza A virus strains [10]. A/swine/Iowa/15/30 exhibited marked differences in their recognition of sialyl linkages and molecular species of sialic acid. The binding specificity of A/swine/Iowa/15/30 might be changed by selective pressure on the allantoic membranes in a long-term passage.

Recently, we have found that equine influenza viruses preferentially bound to the sialyloligosaccharides containing Neu5Gc. In addition, the ratio of Neu5Gc to Neu5Ac in equine respiratory epithelia was higher than that of swine (described elsewhere). These results suggest that the binding specificity of influenza A virus for molecular species of sialic acid may be an important factor in restricting the virus host range.

*Acknowledgements:* This work was supported in part by a Grants-in-aid 07670349 (T.S.), 06454211, 05274101, 08457098, 07044286,

Table 3
Comparison of GP-2 binding activity of swine and human influenza A virus isolates

| Virus preparation | VH titer[a] | |
|---|---|---|
| | GP-2 | asialo-GP-2 |
| Swine isolates | | |
| A/swine/Iowa/15/30 (H1N1) | <2 | <2 |
| A/swine/Colorado/77 (H3N2) | 32 | <2 |
| A/swine/Hokkaido/2/81 (H1N1) | 16 | <2 |
| A/swine/Italy/309/83 (H3N2) | 8 | <2 |
| A/swine/Kanagawa/1/92 (H1N2) | 8 | <2 |
| Human isolates | | |
| A/PR/8/34 (H1N1) | <2 | <2 |
| A/Aichi/2/68 (H3N2) | <2 | <2 |
| A/Memphis/1/71 (H3N2) | <2 | <2 |

[a]The maximum dilution of virus showing hemadsorption was defined as the VH titer. As a control, each influenza virus was tested with asialo-GP-2 immobilized in the wells instead of GP-2.
Data represent the average of 3 determinations.

07557166 (Y.S.) for Scientific Research from the Ministry of Education, Science and Culture of Japan, and Leading Research Utilizing Potential of Regional Science and Technology (Y.S.).

## References

[1] Carroll, S.M., Higa, H. and Paulson, J.C. (1981) J. Biol. Chem. 256, 8357–8363.
[2] Suzuki, Y., Matsunaga, M. and Matsumoto, M. (1985) J. Biol. Chem. 260, 1362–1365.
[3] Suzuki, Y., Nagao, Y., Kato, H., Matsumoto, M., Nerome, K., Nakajima, K. and Nobusawa, E. (1986) J. Biol. Chem. 261, 17057–17061.
[4] Suzuki, Y., Kato, H., Naeve, C.W. and Webster, R.G. (1989) J. Virol. 63, 4298–4302.
[5] Suzuki, Y., Nakao, T., Ito, T., Watanabe, N., Toda, Y., Xu, G., Suzuki, T., Kobayashi, T., Kimura, Y., Yamada, A., Sugawara, K., Nishimura, H., Kitame, F., Nakamura, K., Deya, E., Kiso, M. and Hasegawa, A. (1992) Virology 189, 121–131.
[6] Suzuki, T., Sometani, A., Yamazaki, Y., Horiike, G., Mizutani, Y., Masuda, H., Yamada, M., Tahara, H., Xu, G., Miyamoto, D., Oku, N., Okada, S., Kiso, M., Hasegawa, A., Ito, T., Kawaoka, Y. and Suzuki, Y. (1996) Biochem. J. 318, 389–393
[7] Rogers, G.N. and Paulson, J.C. (1983) Virology 127, 361–373.
[8] Rogers, G.N., Daniels, R.S., Skehel, J.J., Wiley, D.C., Wang, X-F., Higa, H.H. and Paulson, J.C. (1985) J. Biol. Chem. 260, 7362–7367.
[9] Connor, J.R., Kawaoka, T., Webster, R.G. and Paulson, J.C. (1994) Virology 205, 17–23.
[10] Couceiro, J., Nelson, S.S., Paulson, J.C. and Baum, L.G. (1993) Virus Res. 29, 155–165.
[11] Schauer, R. (1982) Sialic Acids: Chemistry, Metabolism, and Function, Springer, New York.
[12] Hara, S., Kakemori, Y., Yamaguchi, M., Nakamura, M. and Ohkura, Y. (1987) Anal. Biochem. 164, 138–145.
[13] Varki, A. (1992) Glycobiology 2, 25–40
[14] Feizi, T., Gooi, H.C., Loomes, L.M., Suzuki, Y., Suzuki, T. and Matsumoto, M. (1984) Biosci. Rep. 4, 743–749.
[15] Suzuki, Y., Suzuki, T. and Matsumoto, M. (1983) J. Biochem. 93, 1621–1633.
[16] Hirabayashi, Y., Suzuki, T., Suzuki, Y., Taki, T., Matsumoto, M., Higashi, H. and Kato, S. (1983) J. Biochem. 94, 327–330.
[17] Suzuki Y., Nagao, Y., Kato, H., Suzuki, T., Mastumoto, M. and Murayama, J. (1987) Biochim. Biophys. Acta 903, 417–424.
[18] Maeda, A., Suzuki, Y. and Matsumoto, M. (1978) Microbiol. Immunol. 22, 775–784.
[19] Nishimura S., Lee, K.B., Matsuoka, K. and Lee, Y.C. (1994) Biochem. Biophys. Res. Commun. 199, 249–254.
[20] Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604–611.
[21] Susan, M. and Kidwell, D.A. (1991) Anal. Biochem. 192, 207–211.
[22] Hara, S., Takemori, Y., Yamaguchi, M., Nakamura, M. and Ohkura, Y. (1987) Anal. Biochem. 164, 138–145.
[23] Suzuki, Y., Hirabayashi, Y., Suzuki, T. and Matsumoto, M. (1985) J. Biochem. 98, 1653–1659.
[24] Naeve, C.W., Hinshaw, V.S. and Webster, R.G. (1984) Virology 53, 567–569.
[25] Rogers, G.N., Pritchett, T.J., Lane, J.L. and Paulson, J.C. (1983) Virology 131, 394–408.
[26] Rogers, G.N., Daniels, R.S., Skehel, J.J., Wiley, D.C., Wang, X.-F., Higa, H.H. and Paulson, J.C. (1985) J. Biol. Chem. 260, 7362–7367.