Analysis of *N*-glycans in embryonated chicken egg chorioallantoic and amniotic cells responsible for binding and adaptation of human and avian influenza viruses

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Abstract The initial step essential in influenza virus infection is specific binding of viral hemagglutinin to host cell-surface glycan receptors. Influenza A virus specificity for the host is mediated by viral envelope hemagglutinin, that binds to receptors containing glycans with terminal sialic acids. Human viruses preferentially bind to $\alpha2 \rightarrow 6$ linked sialic acids on receptors of host cells, whereas avian viruses are specific for the $\alpha2 \rightarrow 3$ linkage on the target cells. Human influenza virus isolates more efficiently infect amniotic membrane (AM) cells than chorioallantoic membrane (CAM) cells. *N*-glycans were isolated from AM and CAM cells of 10-day-old chicken embryonated eggs and their structures were analyzed by multi-dimensional HPLC mapping and MALDI-TOF-MS techniques. Terminal *N*-acetylneuraminic acid contents in the two cell types were similar. However, molar percents of $\alpha2 \rightarrow 3$ linkage prefer-

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entially bound by avian influenza virus were 27.2 in CAM cells and 15.4 in AM cells, whereas those of $\alpha2\rightarrow6$ linkage favored by human influenza virus were 8.3 (CAM) and 14.2 (AM). Molar percents of sulfated glycans, recognized by human influenza virus, in CAM and AM cells were 3.8 and 12.7, respectively. These results have revealed structures and molar percents of N-glycans in CAM and AM cells important in determining human and avian influenza virus infection and viral adaptation.

Keywords Influenza virus · N-glycans · HPLC-MALDI-TOF mapping · Chorioallantoic membrane · Amniotic membrane · Host cell receptor specificity

Abbreviations

AM	amniotic membrane
CAM	chorioallantoic membrane
DEAE	diethylamino ethanol
Gal	galactose
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GU	glucose unit
HA	hemagglutinin
LacNAc	N-acetyllactosamine
MALDI-	matrix-assisted laser desorption
TOF-MS	ionization time-of-flight mass spectrometry
MDCK	Madin Darby canine kidney
Neu5Ac	5-N-acetylneuraminic acid
Neu5Gc	5-N-glycolylneuraminic acid
ODS	octadecyl silica
PA	pyridylamino
SiaLac	sialyl-N-acetyllactosamine

Introduction

Influenza is caused by enveloped single-stranded negative-sense RNA viruses, including A, B and C types that differ in their nucleoproteins and matrix proteins. Influenza B and C viruses can cause epidemic influenza mainly in humans, whereas influenza A viruses have caused epidemics and sometimes pandemics of influenza in both humans and animals [1, 2]. Influenza viruses are enveloped by glycoproteins with antigenic hemagglutinins (HAs; H1–H16 subtypes), which have an important role in binding to oligosaccharide (glycan) receptors on glycoproteins or glycolipids of host cell surfaces, triggering endocytosis of the virus into host cells [1, 3]. Results of a recent *in vivo* study have shown that N-glycans are required for influenza virus infection and entry into host cells of influenza viruses, at least influenza A (H1N1 and H3N2) and influenza B viruses [4]. Antigenic HA of human and avian influenza A

isolates recognizes sialic acid with $\alpha2\rightarrow6$ and $\alpha2\rightarrow3$ linkages respectively, and HA of type B viruses prefers the $\alpha2$-6-linked sialic acid [2, 5–7]. HA of influenza C viruses requires sialic acid with a 9-O-acetyl group for attachment [8, 9]. Importantly, HAs have been known to be sugar (glycan)-recognizing proteins that determine transmission and virulence of influenza viruses [1, 2, 10].

Due to the difficulty of obtaining sufficient amounts of influenza viruses isolated from humans and avians for studies such as studies on viral biology, vaccine production and exploration of new antiviral drugs, cultivation of viruses is needed. Viruses isolated from avian and human hosts have traditionally been grown in chorioallantoic and amniotic cavities, respectively, of chicken embryonated eggs. This is because isolated human influenza viruses replicate less efficiently if they are not adapted, whereas isolated avian influenza viruses replicate more efficiently in a chorioallantoic cavity [11–13]. What is responsible for the replication requirement of these isolated viruses in their respective cavity is not known.

Mammalian Madin Darby canine kidney (MDCK) cells have become routinely used for cultivation of isolated human influenza viruses, because the newly formed viruses are antigenically similar to the original isolates [14–16]. Human influenza viruses grown in embryonated chicken eggs select variants with amino acid mutations in the receptor-binding site of the HA molecule (host adaptation) in order to enable the viruses to grow well in these particular host cells [13, 17–19].

Several studies have shown that different cell types contain different amounts, types and linkages of sugar chains by using sialyl linkage-specific lectins [20–24]. Chorioallantoic membrane (CAM) cells were found to contain Neu5Ac($\alpha2\rightarrow3$) Gal (5-N-acetylneuraminic acid (Neu5Ac) linked to galactose (Gal) by $\alpha2$-3 linkage), and amniotic membrane (AM) and MDCK cells contain both Neu5Ac$\alpha2\rightarrow$Gal and Neu5Ac$\alpha2\rightarrow$Gal [25]. However, there has been no report in which the quantity and structure of N-glycans present on these two types of cells are described.

By using a multi-dimensional high-performance liquid chromatography (HPLC) mapping technique [26–28], we have been able to carry out N-glycosylation profiling in a quantitative manner at molecular, cellular, and organ levels. This prompts us to characterize the N-glycans expressed on CAM and AM cells of 10-day-old chicken embryonated eggs.

Materials and methods

Preparation of N-glycans from CAM and AM cells

CAM and AM cells of 10-day-old chicken embryonated eggs were removed carefully using fine forceps from the

inner shell membrane and the embryo, respectively, washed thoroughly with cold PBS to remove blood cells, and lyophilized. Dried CAM (22.5 mg) and AM (20.3 mg) was taken and their lipid was sequentially extracted from the cells with 80% ethanol, 100% ethanol, chloroform/methanol (2:1, *v/v*), chloroform/methanol/H$_2$O (1:2:0.8, *v/v/v*), and 80% acetone. The cell residues were proteolyzed with pepsin and further digested with glycoamidase A to release *N*-glycans. The resultant peptidic materials were hydrolyzed by treatment with pronase [28, 29]. The glycan fraction was then purified by a Bio-Gel P-2 column (1 cm i.d.×30 cm) and evaporated to dryness.

Fluorescent derivatization of *N*-glycans with 2-aminopyridine and HPLC mapping

The reducing ends of *N*-glycans were labeled with a fluorescent reagent, 2-aminopyridine [30]. The pyridylamino-labeled glycan (PA-glycan) mixture was then purified by gel filtration on a Sephadex G-15 column (1 cm i.d.× 30 cm) to remove excess reagents. The purified PA-glycan mixture was firstly subjected to an anion exchange chromatography [TSKgel diethylamino ethanol (DEAE)-5PW column; 7.5 mm i.d.×75 mm; Tosoh, Tokyo, Japan]. Each peak fraction from the DEAE column was collected, evaporated, and analyzed by reverse-phase HPLC using a Shim-pack HRC-octadecyl silica (ODS) column (6.0 mm i.d.×150 mm, Shimadzu, Kyoto, Japan). Individual peak fractions from the ODS column were then isolated using a size fractionation column, TSK-gel amide-80 (Tosoh, Tokyo, Japan) as conditions reported previously [28, 29]. The elution times of the individual peaks from the amide-silica and ODS columns were normalized with respect to PA-derivatized isomalto-oligosaccharides of polymerization degree and represented in units of glucose (GU). The identification of *N*-glycan structures was based on their elution positions on three kinds of HPLC columns in comparison with PA-glycans in the GALAXY database (http://www.glycoanalysis.info/galaxy2/ENG/systemin1. jsp) [27].

Exo-glycosidase digestion and matrix-assisted laser desorption ionization time-of-flight mass spectrometric (MALDI-TOF-MS) analysis

PA-glycans, which did not agree with any of the *N*-glycans so far registered in GALAXY, were trimmed by exoglycosidase (α-sialidase, α2,3-sialidase, α-fucosidase, β-galactosidase and β-*N*-acetylglucosaminidase) treatment according to previously described [29] to become identical to known ones. Then the reaction products were subjected to MALDI-TOF-MS spectrometric analysis and operated as described previously [31].

Results and discussion

N-glycans released from CAM and AM cells by glycoamidase A and labeled with PA were separated by a DEAE column. Four peaks were eluted at 2, 10–15.5, 21–25.5 and 27–28.5 min (Fig. 1a). These peak fractions were identified as a neutral glycan (peak 1) and three kinds of acidic glycans, namely, monosialyated (peak 2), disialyated (peak 3) and disulfated (peak 4) glycan. Each DEAE peak fraction was further analyzed by ODS column. As shown in Fig. 1b–e, 13 major peaks (N1–N12′), 11 major peaks (M1-11), 5 major peaks (D1–D5) and 1 major peak (D6) were separated from DEAE peaks 1, 2, 3 and 4, respectively.
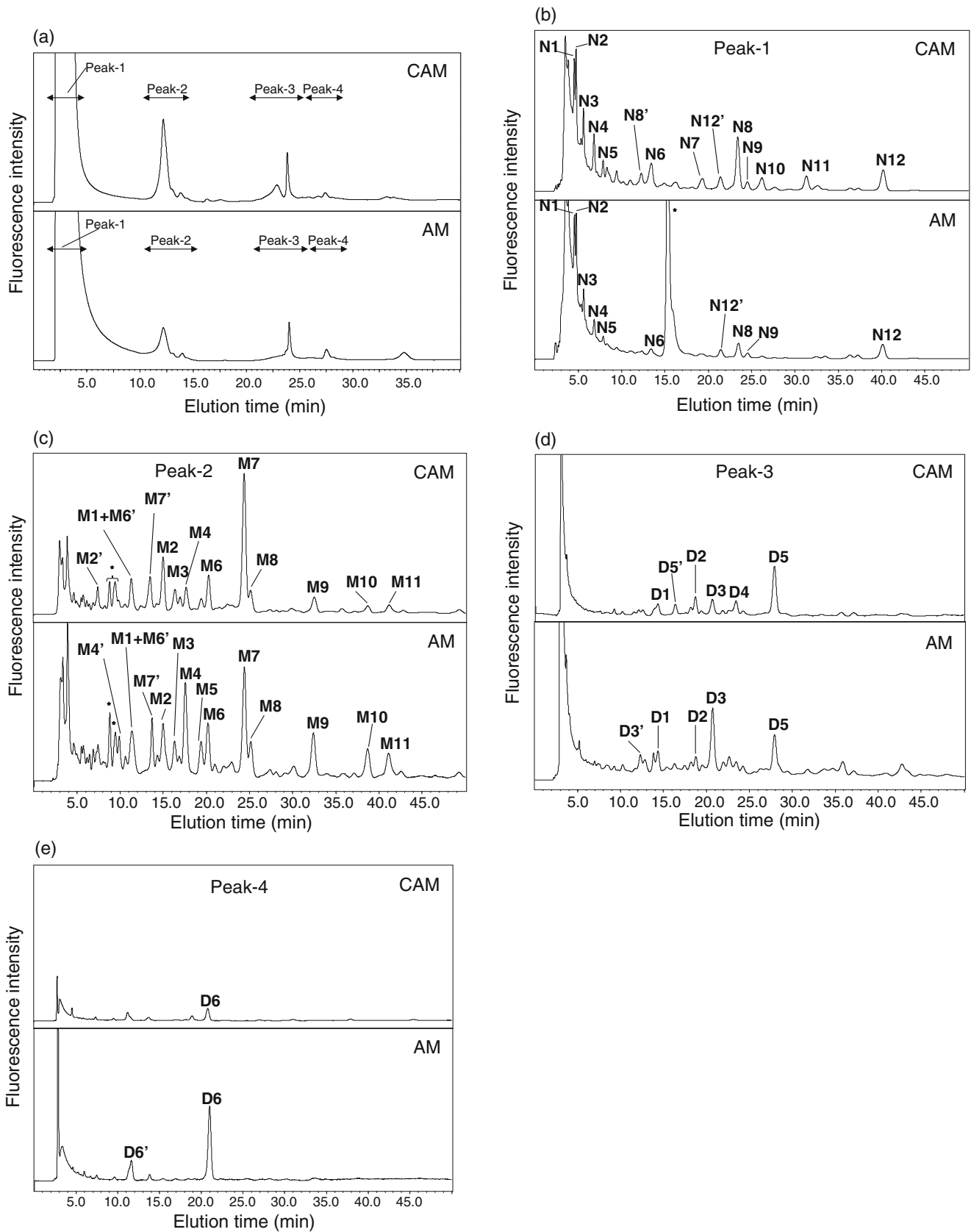
Based on the peak areas in the chromatograms shown in Fig. 1b–e, molar percents of peaks 1, 2, 3 and 4 from CAM cells were 59.7, 29.5, 9.3 and 1.5, respectively, and those from AM cells were 56.7, 29.4, 9.5 and 4.4, respectively. The ratio of molar percent of neutral to acidic glycans was 1.5:1.0 for both CAM and AM cells (Fig. 2). However, the total amount of *N*-glycans derived from CAM cells (114.6 pmol mg^{-1} dry cells) was 2.4-times than that derived from AM cells (47.0 pmol mg^{-1} dry cells).

The PA-oligosaccharide was identified on the basis of coincidence of elution time normalized in GU with those on the HPLC map. For example, the major sialo-*N*-glycan corresponding to peak M7 was eluted at 14.8 GU on the ODS column and at 6.8 GU on the amide column. The elution data set was in good agreement with a known reference α2→3 sialyl glycan, Galβ1→4GlcNAcβ1→ 2Manα1→6(Neu5Acα2→3Galβ1→4GlcNAcβ1→2-Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc-PA (code no. 1A3-210.4 in the GALAXY database). By co-chromatography and the MALDI-TOF-MS analyses, we confirmed the structure of this PA-oligosaccharide.

The sialylated PA-glycans corresponding to the fractions M9, M10, M11 and D4 did not agree with any of the PA-glycans so far registered in the GALAXY. These PA-glycans were trimmed by exoglycosidase treatments to become identical to known ones. Taking into account the specificities of the exoglycosidases used, the original structures of these PA-glycans were uniquely determined.

In a similar way, we identified the remaining 27 kinds of the *N*-glycans derived from CAM and AM cells, which consist of neutral and sialyl oligosaccharides, along with sulfated glycans.

The molar percents of neutral *N*-glycans detected, divided into high-mannose-type, galactose-terminal, *N*-acetylglucosamine (GlcNAc)-terminal and others, were 18.6, 29.1, 8.2 and 3.8, respectively, in CAM cells, and 30.1, 21.5, 0.0 and 5.1, respectively, in AM cells (Table 1 and Fig. 2). A previous study has shown that human influenza viruses can react with mannose-binding lectins of

the collectin family and infect murine macrophages expressing the mannose receptor and that the infection was inhibited by yeast mannan [32].

Negatively charged glycans, including sialylated and sulfated glycans, are the major viral receptors [6]. Influenza viruses preferentially bind to glycans terminated by sialic acid, mostly Neu5Ac derivative, either Neu5Ac($\alpha2\rightarrow3$)Gal or Neu5Ac($\alpha2\rightarrow6$)Gal; human isolates predominantly bind to Neu5Ac($\alpha2\rightarrow6$)Gal, while avian isolates mainly bind to Neu5Ac($\alpha2\rightarrow3$)Gal [7, 10, 33–40]. Glycan microarray analyses detected differences in human and avian influenza virus HA specificity, such as preferences for fucosylation and sialylation at positions 2 (Gal) and 3 (GlcNAc, GalNAc) of the terminal trisaccharide [41], and also showed that highly pathogenic avian influenza H5N1 viruses bind preferentially to Sia($\alpha2\rightarrow3$)Gal structure [42]

and highly pathogenic avian H7N7 viruses from The Netherlands in 2003 maintain the classic avian-binding preference for $\alpha2\rightarrow3$ linked sialic acids [43]. Recently, it was reported that a characteristic structural topology enables specific binding of HA to $\alpha2\rightarrow6$ sialylated glycans and human adapted H1N1 and H3N2 viruses specifically bind to long sialylated glycans containing tandem lactosamine structure such as Sia($\alpha2\rightarrow6$)Gal($\beta1\rightarrow4$)GlcNAc ($\beta1\rightarrow3$)Gal($\beta1\rightarrow4$)GlcNAc($\beta1\rightarrow3$)Gal-structures [10]. In CAM and AM, terminal short sialylated trisaccharide structure of *N*-glycans, Neu5Ac($\alpha2\rightarrow3$)Gal($\beta1\rightarrow4$) GlcNAc-and Neu5Ac($\alpha2,6$)Gal($\beta1,4$)GlcNAc-, were detected, but long tandem *N*-acetyllactosamine structure was not found. Some neutral and sialyl-sugar chains of *N*-glycans in CAM and AM were fucosylated. The molar percents of terminal Neu5Ac($\alpha2\rightarrow3$)Gal and Neu5Ac ($\alpha2\rightarrow6$)Gal derived from CAM cells were significantly different to those from AM cells: 27.2 and 8.3, respectively, for CAM cells, and 15.4 and 14.2 respectively, for AM cells (Table 1 and Fig. 2). This is in agreement with the results of a previous study using a qualitative lectin assay [25] and explains why CAM cells are susceptible to avian but not human influenza viruses, while AM cells are recognized by human influenza viruses. Moreover, the presence of similar molar percents of Neu5Ac($\alpha2\rightarrow3$)Gal and Neu5Ac($\alpha2\rightarrow6$) Gal in AM cells may explain why human influenza viruses grown in AM cells are easily adapted from human-receptor to avian-receptor specificity with amino acid substitutions that cluster around the receptor-binding site of the HA molecule as described previously [13, 18, 19, 25]. Three subtypes of avian influenza viruses, H9N2, H7N7 and highly pathogenic H5N1, have been reported in humans in recent years [44, 45]. Human lower respiratory tissues and

Fig. 2 Comparison of percent contents of *N*-glycans of cells derived from chorioallantoic (CAM) and amniotic (AM) membranes. The data plotted correspond to percent glycan content in Table 1
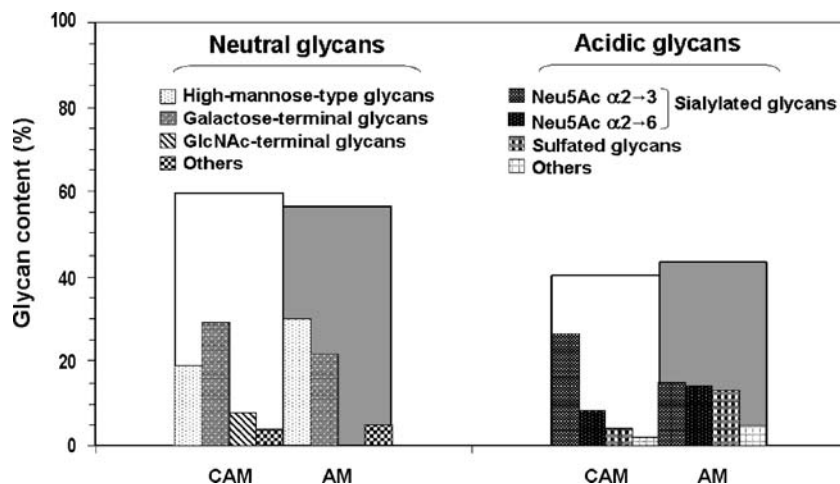
Table 1 Structures and molar percents of *N*-linked glycans of chorioallantoic membrane (CAM) and amniotic membrane (AM) cells isolated from 10-day-old embryonated eggs

Peak Code No.[a]	Structure	Relative quantity (mol %)[b]	
		CAM	AM
Neutral glycans			
Peak 1			
High-mannose-type glycans			
N1 M8.1	Manα2Manα6 \ Manα6 \ / Manβ4GlcNAcβ4GlcNAc / Manα3 / Manα2Manα2Manα3	4.3	9.7
N2[c] M7.2	Manα2Manα6 \ Manα6 \ / Manβ4GlcNAcβ4GlcNAc / Manα3 / Manα2Manα3	1.6	3.7
N2[c] M9.1	Manα2Manα6 \ Manα2Manα3 / Manα6 \ Manβ4GlcNAcβ4GlcNAc / Manα2Manα2Manα3	4.3	8.0
N3 M6.1	Manα6 \ Manα6 \ / Manβ4GlcNAcβ4GlcNAc / Manα3 / Manα2Manα3	3.0	3.9
N4 M5.1	Manα6 \ Manα6 \ / Manβ4GlcNAcβ4GlcNAc / Manα3 / Manα3	3.7	3.3
N5 H5.12	Manα6 \ Manα6 \ / Manβ4GlcNAcβ4GlcNAc / Manα3 / Galβ4GlcNAcβ2Manα3	1.7	1.5
Galactose-terminal glycans			
N6 200.4	Galβ4GlcNAcβ2Manα6 \ Manβ4GlcNAcβ4GlcNAc / Galβ4GlcNAcβ2Manα3	5.4	2.1
N8+8' 210.4	Galβ4GlcNAcβ2Manα6 \ Fucα6 \ Manβ4GlcNAcβ4GlcNAc / Galβ4GlcNAcβ2Manα3	13.0	4.3
N9 201.4	Galβ4GlcNAcβ2Manα6 \ GlcNAcβ4-Manβ4GlcNAcβ4GlcNAc / Galβ4GlcNAcβ2Manα3	1.6	2.0
N12+N12' 211.4	Galβ4GlcNAcβ2Manα6 \ Fucα6 \ GlcNAcβ4-Manβ4GlcNAcβ4GlcNAc / Galβ4GlcNAcβ2Manα3	9.1	13.1
N-acetylglucosamine-terminal glycans			
N7[d] 210.1	GlcNAcβ2Manα6 \ Fucα6 \ Manβ4GlcNAcβ4GlcNAc / GlcNAcβ2Manα3	1.0	-

Table 1 (continued)

Peak Code No.[a]	Structure	Relative quantity (mol %)[b] CAM	AM
N7[d] 201.1	GlcNAcβ2Manα6 　　　GlcNAcβ4-Manβ4GlcNAcβ4GlcNAc GlcNAcβ2Manα3	1.9	-
N10 501.1	GlcNAcβ6 GlcNAcβ4 — Manα6 GlcNAcβ2　　GlcNAcβ4-Manβ4GlcNAcβ4GlcNAc GlcNAcβ4 　　　Manα3 GlcNAcβ2	1.9	-
N11 301.1	GlcNAcβ2Manα6 　　　GlcNAcβ4-Manβ4GlcNAcβ4GlcNAc GlcNAcβ4 　　Manα3 GlcNAcβ2	3.4	-
Others		3.8	5.1
Total		59.7	56.7

Acidic glycans
Peak 2
Monosialylated glycans

		CAM	AM
M1 1A1-200.4	Galβ4GlcNAcβ2Manα6 　　　Manβ4GlcNAcβ4GlcNAc NeuAcα6Galβ4GlcNAcβ2Manα3	1.3	1.3
M2 + M2' 1A3-200.4	Galβ4GlcNAcβ2Manα6 　　　Manβ4GlcNAcβ4GlcNAc NeuAcα3Galβ4GlcNAcβ2Manα3	4.2	2.0
M3 1A4-200.4	NeuAcα3Galβ4GlcNAcβ2Manα6 　　　Manβ4GlcNAcβ4GlcNAc Galβ4GlcNAcβ2Manα3	1.5	0.8
M5 1A1-201.4	Galβ4GlcNAcβ2Manα6 　　　GlcNAcβ4-Manβ4GlcNAcβ4GlcNAc NeuAcα6Galβ4GlcNAcβ2Manα3	-	1.4
M6 + M6' 1A1-210.4	Galβ4GlcNAcβ2Manα6　　Fucα6 　　　Manβ4GlcNAcβ4GlcNAc NeuAcα6Galβ4GlcNAcβ2Manα3	3.1	3.1
M7 + M7' 1A3-210.4	Galβ4GlcNAcβ2Manα6　　Fucα6 　　　Manβ4GlcNAcβ4GlcNAc NeuAcα3Galβ4GlcNAcβ2Manα3	12.8	7.0
M8 1A4-210.4	NeuAcα3Galβ4GlcNAcβ2Manα6　　Fucα6 　　　Manβ4GlcNAcβ4GlcNAc Galβ4GlcNAcβ2Manα3	1.4	1.7
M9 1A1-211.4	Galβ4GlcNAcβ2Manα6　　Fucα6 　　　GlcNAcβ4-Manβ4GlcNAcβ4GlcNAc NeuAcα6Galβ4GlcNAcβ2Manα3	0.8	2.5
M9 1A4-310.8	Galβ4GlcNAcβ2Manα6　　Fucα6 Galβ4GlcNAcβ4　　Manβ4GlcNAcβ4GlcNAc 　　　Manα3 NeuAcα3Galβ4GlcNAcβ2	0.8	-

Table 1 (continued)

Peak Code No.[a]	Structure	Relative quantity (mol %)[b]	
		CAM	AM
M10 1A3-211.4	Galβ4GlcNAcβ2Manα6 GlcNAcβ4-Manβ4GlcNAcβ4GlcNAc Fucα6 NeuAcα3Galβ4GlcNAcβ2Manα3	0.5	1.8
M11 1A2-211.4	NeuAcα6Galβ4GlcNAcβ2Manα6 GlcNAcβ4-Manβ4GlcNAcβ4GlcNAc Fucα6 Galβ4GlcNAcβ2Manα3	0.7	1.3
Monosulfated glycans			
M4 + M4'	$(Hex)_4(HexNAc)_5(DeoxyHex)_1(HSO_3)_1$[e]	1.6	4.8
Others		0.8	1.7
Total		29.5	29.4
Peak 3			
Disialyated glycans			
D1 2A1-200.4	NeuAcα6Galβ4GlcNAcβ2Manα6 Manβ4GlcNAcβ4GlcNAc NeuAcα6Galβ4GlcNAcβ2Manα3	0.7	0.8
D2 2A4-200.4	NeuAcα3Galβ4GlcNAcβ2Manα6 Manβ4GlcNAcβ4GlcNAc NeuAcα3Galβ4GlcNAcβ2Manα3	1.0	0.5
D4 2A3-210.4	NeuAcα3Galβ4GlcNAcβ2Manα6 Manβ4GlcNAcβ4GlcNAc Fucα6 NeuAcα6Galβ4GlcNAcβ2Manα3	0.8	-
D5 + D5' 2A4-210.4	NeuAcα3Galβ4GlcNAcβ2Manα6 Manβ4GlcNAcβ4GlcNAc Fucα6 NeuAcα3Galβ4GlcNAcβ2Manα3	4.6	1.6
Monosialylated and monosulfated glycan			
D3 + D3'	$(Hex)_4(HexNAc)_5(DeoxyHex)_1(HSO_3)_1(NeuAcα2,6)_1$[e]	1.3	3.8
Others		0.9	2.8
Total		9.3	9.5
Peak 4			
Disulfated glycan			
D6 + D6'	$(Hex)_3(HexNAc)_6(DeoxyHex)_1(HSO_3)_1$[e]	0.9	4.1
Others		0.6	0.3
Total		1.5	4.4

Hex hexose, *HexNAc* N-acetylhexosamine, *DeoxyHex* deoxyhexose, *HSO₃* sulfate group

[a] PA-oligosaccharides are coded according to the literature [27]

[b] Molar percent of glycan content in CAM and AM cells was calculated on the basis of peak area in Fig. 1b–e by comparison with total glycan content in CAM and AM cells, respectively

[c] Fraction N2 from the ODS column was separated into two subfractions on the amide column. The molar percent of each glycan was calculated on the basis of peak areas in the elution profile on the amide column

[d] Molar percent of each glycan was calculated on the basis of peak intensity in the MALDI-TOF-MS spectrum

[e] Carbohydrate composition was estimated on the basis of MALDI-TOF-MS data

lungs to which mainly these viruses attached have been shown to contain both 2→3 and 2→6 linkages by lectin staining [21–24]. Although several factors may be required for crossing host restriction [44, 45], surveillance of transmission between humans or emergence of new pandemic strains has to be increased because they are RNA viruses capable of rapid evolution [1, 2].

Another sialic acid derivative, *N*-glycolylneuraminic acid (Neu5Gc), an additional receptor of some human and animal influenza A viruses [38, 46, 47], could not be detected in *N*-linked glycans of both CAM and AM cells.

The sialic acid with 9-*O*-acetyl, which serves as a specific primary receptor for influenza C viruses [8, 9] and is recognized by avian (duck) influenza A virus [46], could not be detected in *N*-linked glycans of both CAM and AM cells. However, AM cells have been shown to be susceptible to influenza C viruses [25, 48]. These findings indicate that 9-*O*-acetyl sialic acid may be carried on *O*-linked glycoproteins or glycolipids, such as gangliosides [8, 49], in AM cells of chicken embryonated eggs.

Unlike sialic acid, little is known about the relationship between sulfated glycans and influenza viruses. There is evidence that some chicken and mammalian influenza A viruses display a high binding affinity for sulfated sialyl-glycan receptor, and this binding affinity is decreased after treatment of cells with sulfatase [50]. The presence of sulfated Neu5Ac(α2→6)Gal in CAM and AM cells (1.3% and 3.8%, respectively, Table 1) may facilitate human influenza virus infection of AM cells. A 6′-HSO$_3$ LacNAc probe without sialic acid was also shown to bind to human influenza type A and B viruses with affinity comparable to that of a 6′-SiaLac probe [51]. The difference in molar percents of sulfated glycans detected in CAM cells (2.5) and AM cells (8.9; Fig. 2) may be a reason why human influenza viruses are more efficiently cultivated in AM cells than in CAM cells.

In summary, by using highly sensitive and efficient analytical techniques, we have identified *N*-glycan structures and have confirmed the presence of both α2→3 and α2→6 linkages in *N*-glycans, known to be important for efficient virus entry and infection. CAM and AM cells have different ratios of molar percent of Neu5Ac(α2→3)Gal to Neu5Ac(α2→6)Gal (3.3:1.0 and 1.1:1.0 in CAM and AM cells, respectively) reflecting distinctions in susceptibility of these cells to different influenza virus species and accounting for the binding of viruses cultivated in chicken embryonated eggs to shift to Neu5Ac(α2→3)Gal specificity. Our data have also shown that CAM and AM cells contain *N*-glycans with terminal mannose and sulfate residues capable of binding to influenza viruses. However, Neu5Gc and 9-*O*-Acetyl sialic acid, recognized by some influenza viruses, were not detected in *N*-glycans of CAM and AM cells.

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