Title

Recombinant production and characterization of human anti-influenza virus monoclonal antibodies identified from hybridomas fused with human lymphocytes

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Keywords:

Human antibody production; SPYMEG; Chinese hamster ovary; Glycosylation

Abstract

In previous studies, hybridomas producing human immunoglobulin G, the antibodies 5E4 and 5A7 against influenza A and B virus were established using a novel human lymphocyte fusion partner, SPYMEG. In the present study, we succeeded in achieving the recombinant production and secretion of 5E4 and 5A7 in Chinese hamster ovary cells. Our *N*-glycan analysis by intact-mass detection and liquid chromatography mass spectrometry showed that recombinant 5E4 and 5A7 have one *N*-glycan and the typical mammalian-type *N*-glycan structures similar to those in hybridomas. However, the glycan distribution was slightly different among these antibodies. The amount of high-mannose-type structures was under 10% of the total *N*-glycans of recombinant 5E4 and 5A7, compared to 20% of the 5E4 and 5A7 produced in hybridomas. The amount of galactosylated *N*-glycans was increased in recombinants. Approximately 80% of the *N*-glycans of all antibodies was fucosylated, and no sialylated *N*-glycan was found. Recombinant 5E4 and 5A7 neutralized pandemic influenza A virus specifically, and influenza B virus broadly, quite similar to the 5E4 and 5A7 produced in hybridomas, respectively. Here we demonstrated that recombinants of antibodies identified from hybridomas fused with SPYMEG have normal *N*-glycans and that their neutralizing activities bear comparison with those of the original antibodies.

1. Introduction

Monoclonal antibodies (mAbs) are commonly used biopharmaceutical proteins because of their specificity and high-affinity against antigens. Since the 1980s, the production of mouse mAbs using mammalian cells has contributed to the development of mAb production systems [1]. Compared to human immunoglobulins, mouse mAbs

exhibit short half-lives after being administered to humans, and they frequently cause immunogenicity [2]. To solve these problems, attempts have been made to develop chimeric mouse-human and humanized antibodies by genetic engineering with fusion of the variable region of the mouse antibodies to the constant region of human antibodies and grafting of the complementarity-determining region (CDR) of mouse antibodies on human antibodies, respectively. However, allergic reactions and the induction of anti-drug antibodies are not completely eliminated, although approximately 95% of the antibody framework is derived from human antibodies [3]. Thus, the production of recombinant human- but not humanized-antibodies has been attempted.

A novel human lymphocyte fusion partner, SPYMEG, was established in previous work by the cell fusion of MEG-01 human megakaryoblastic leukemia cells with a murine myeloma cell line [4,5]. SPYMEG is available for the simple and easy generation of recombinant therapeutic human mAbs (HuMabs) because of no human chromosome deletion. Hybridoma cell lines producing HuMabs against pandemic A(H1N1)2009 influenza virus and influenza B virus were recently established using SPYMEG and peripheral lymphocytes from vaccinated volunteers, respectively [6,7]. An anti-pandemic A(H1N1)2009 antibody, 5E4, recognized a classical antigenic site Sb on the hemagglutinin (HA) protein. An anti-influenza B virus antibody, 5A7, broadly reacted with influenza B virus with the recognition of a highly conserved region on the HA protein. These antibodies also showed effective neutralizing activities against their target virus in mice. In particular, it is expected that because of its broad neutralizing activity, 5A7 will be a promising candidate for the development of one or more the next-generation universal therapeutics against influenza B virus.

Chinese hamster ovary (CHO) cells are an important host for the industrial

production system of recombinant pharmaceuticals, and CHO cells have been widely used in research worldwide [8,9]. Meanwhile, the post-translational modification of recombinant proteins has received increasing attention. An important factor in the quality of proteins is glycosylation, because of its contribution to protein stability, biological activity, and more [10,11]. Glycan residues not found in humans, i.e., *N*-glycolylneuraminic acid, α 1,3-linked galactose (Gal), α 1,3-linked fucose, and β 1,2-linked xylose, also cause immunogenicity in the human body [12-14]. CHO cells can produce glycans with *N*-glycolylneuraminic acid [15]. It is therefore necessary to not only examine the activity but also determine the detailed glycan structures of recombinant proteins for industrial production.

In previous research, we identified the nucleotide sequences of 5E4 and 5A7 from hybridomas, but to our knowledge there is no report about the production of recombinant HuMabs isolated from SPYMEG-derived hybridomas. In the present study, we produced recombinant 5E4 and 5A7 using CHO cells, examined their neutralizing activities against influenza virus, and analyzed their *N*-glycan structures. We then compared their characteristics with those of 5E4 and 5A7 derived from hybridomas.

2. Materials and methods

2.1. Cloning and plasmids

The coding region of variable regions of H- and L-chains from HuMabs 5E4 and 5A7 was cloned as described [6,7]. A polymerase chain reaction (PCR) with the following primers was performed for the construction of expression vectors: 5'-ATTGCGGCCGCCATGAAACACCTGTGGTTCTTC-3' (forward primer for 5E4 H-chain), 5'-ATTGCGGCCGCCATGGAGTTTGGGCTGAG-3' (forward primer for

5A7 H-chain), 5'-ATACTCGAGGGTGCCAGGGGGAAGACCGATG-3' (reverse primer for 5E4 or 5A7 H-chain), 5'-ATTGCGGCCGCCATGGCCTGGGTCTCATT-3' (forward primer for 5E4 L-chain), 5'-ATTGCGGCCGCCATGGCCTGGGCTCTGCT-3' (forward primer for 5A7 L-chain), and 5'-ATACTCGAGGGCGGGAACAGAGTGACCGTGG-3' (reverse primer for 5E4 or 5A7 L-chain). The PCR products of the variable region gene from the H- and L-chains were digested using *NotI* and *XhoI*, and ligated to pQCXIP (Takara Bio Inc., Shiga, Japan) with an H-chain constant region and pQCXIH (Takara Bio Inc., Shiga, Japan) with an L-chain constant region, respectively. The gene encoding constant regions were gifted from MBL (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan).

2.2. Cells and reagents

CHO-K1 cells were cultured in 5% CO₂ at 37°C in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA) or Ham's F12 Nutrient Mixture (DMEM; Life Technologies, Carlsbad, CA) containing 10% heat-incubated fetal calf serum (FCS). Transfection to cells grown on 6-well plates was performed using Lipofectamine2000 transfection reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Cells stably expressing both H- and L-chains were selected with DMEM containing 10% FCS, 1 µg/mL puromycin dihydrochloride, and 100 µg/mL hygromycin B.

2.3. Enzyme linked immunosorbent assay (ELISA)

For the enzyme linked immunosorbent assay (ELISA), 96-well ELISA plates were

pre-coated with 50 μ L of 0.1 μ g/mL influenza B antigen or 0.1 μ g/mL H1N1 antigen or 10 μ g/mL goat anti-human IgG (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) and incubated overnight at 4°C. After removal of the antigens, cell-cultured media were applied on the wells and the plates were then incubated at room temperature for 1 h. After the plates were washed with 0.05% Tween-20 in phosphate buffered saline (PBS), horseradish peroxidase-conjugated goat anti-human IgG was applied on the wells and the plates were incubated at room temperature for 1 h. After the plates were washed with 0.05% Tween-20 in PBS again, SIGMAFAST™ OPD (Sigma-Aldrich, St. Louis, MO) was applied on the wells as a substrate. The plates were incubated at room temperature for 30 min, and 3 M HCl was then added to stop the reaction. The productivity of the antibody was measured at 450 nm with a microplate reader (model 680 spectrophotometer, Bio-Rad Laboratories, Hercules, CA).

2.4. Antibody preparation

H- and L-chain-expressing cells were subcultured on 150-mm dishes in DMEM containing 10% FCS, 1 μ g/mL puromycin dihydrochloride, and 100 μ g/mL hygromycin B. At 90% confluency, the medium was removed and cells were washed well with PBS and incubated in serum-free Ham's F12 nutrient mixture (Sigma-Aldrich, St. Louis, MO) in 5% CO₂ at 37°C for 1 week. The medium was centrifuged at 1,500g for 5 min and the supernatant was applied to a Protein G Sepharose™ 4 Fast Flow column (GE Healthcare UK Ltd., Buckinghamshire, UK) equilibrated with a binding buffer, 20 mM sodium phosphate buffer, pH 7.0. IgG was eluted by 100 mM glycine buffer, pH 2.7 after washing with the binding buffer, and a neutralization buffer, 1 M Tris-HCl buffer, pH 9.0 was added to the eluted fractions. The eluted fractions were collected and

purified antibodies were concentrated by ultrafiltration using an Ultrafilter Mole-cut II NK (Merck-Millipore, Darmstadt, Germany). The production and purification of the antibodies were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 5% stacking gel and a 12.5% resolving gel with Coomassie Brilliant Blue (CBB) staining.

2.5. Nano liquid chromatography-mass spectrometry (nanoLC-MS) analysis of recombinant IgGs

The purified IgGs were denatured in 50 mM dithiothreitol at 50°C for 30 min and then subjected to micrOTOF-QII (Bruker Daltonics, Bremen, Germany) equipped with a nano-liquid chromatography (nanoLC) system (1200 series, Agilent Technologies, Santa Clara, CA). A trapping column (5 μ m, 0.3 mm $\times$ 5 mm) and an analytical column (3.5 mm, 75 μ m $\times$ 150 mm) (both ZORBAX 300SB-C18, Agilent Technologies, Santa Clara, CA) were used for the liquid chromatography. The mobile phase for the nanoLC consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The peptides were separated by linearly increasing the solvent B concentration in solvent A from 5%–20% for 5 min, 20%–40% for 5 min, and 40%–90% for 5 min at the flow rate of 0.8 mL/min at 35°C. A tandem mass spectrometry (MS/MS) analysis was performed with the following parameters: m/z 50–2500 of scan range, 1.0 bar nebulizer flow, 4.0 L/min dry gas flow rate, and 180°C dry temperature in the positive-ion mode. The MS data were analyzed using DataAnalysis 4.0 software (Bruker Daltonics, Bremen, Germany).

2.6. Preparation of N-Glycans

N-Glycans were released from 500 µg of purified antibody by Glycopeptidase F (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The enzymatic reaction mixture was dissolved in equilibration buffer (butanol:ethanol:0.6 M acetate = 4:1:1, v/v) and subjected to a cellulose column equilibrated with the equilibration buffer [16]. After the column was washed with equilibration buffer, *N*-glycans were eluted with elution buffer (ethanol:75 mM NH₄HCO₃ = 1:2, v/v) and lyophilized. The obtained sugar chains were labeled with pyridylamine (PA) as described [17]. Phenol/chloroform extraction was performed to purify the PA-sugar chains with the removal of unreacted PA.

2.7. High-performance liquid chromatography (HPLC)

PA-sugar chains were monitored on a high-performance liquid chromatography (HPLC) apparatus (Hitachi 7000 HPLC system, Hitachi, Tokyo) at the excitation and emission wavelengths of 310 nm and 380 nm, respectively, and fractionated and purified using a 7.5 × 75 mm column (TSK-GEL DEAE-5PW, Tosoh, Tokyo), a 4.6 × 250 mm column (Cosmosil 5C18-P column, Nacalai Tesque, Kyoto, Japan) for anion-exchange chromatography, and reversed-phase (RP-) HPLC, respectively. In the anion-exchange chromatography, PA-sugar chains were eluted by linearly increasing the concentration of 3% acetic acid/acetonitrile, pH 7.3 in 10% acetonitrile with triethylamine, pH 9.5 from 0% to 15% for 30 min at the flow rate of 0.7 mL/min. In the RP-HPLC, the PA-sugar chains were eluted by linearly increasing the acetonitrile concentration in 0.02% trifluoroacetic acid from 0% to 8% for 35 min at the flow rate of 0.7 mL/min.

2.8. LC-MS/MS analysis of *N*-glycans

We estimated the molecular mass and composition of the PA-sugar chains by using an LC-MS/MS system (1200 series, Agilent Technologies, Santa Clara, CA) equipped with HCT plus-77 software (Bruker Daltonics, Bremen, Germany). The mobile phase consisted of solvent A (acetonitrile:acetic acid = 98:2, v/v) and solvent B (water:acetic acid:triethylamine = 92:5:3, v/v/v) for the LC. The PA-sugar chains were separated using a 2.0 × 150 mm column (Shodex Asahipak NH2P-50, Showa Denko, Tokyo) by linearly increasing the solvent B concentration from 20% to 55 % for 35 min at the flow rate of 0.2 mL/min. The MS/MS was performed with the following parameters: scan 350-2750 *m/z*, 5.0 psi nebulizer flow, 3.0 L/min dry gas flow rate, 300°C dry temperature, 200,000 target count, and the MS/MS Frag. Ampl. of 1.0 V in the positive-ion mode. The relative amount of detected PA-sugar chains was calculated on the basis of the peak area of the LC.

2.9. Neutralizing activity assay

Three viruses (B/Florida/4/2006, B/Malaysia/2506/2004, and A/Suita/1/2009) were used for the assay. The B/Florida/4/2006 and B/Malaysia/2506/2004 strains were kindly provided by the National Institute of Infectious Diseases, Tokyo. The A/Suita/1/2009 strain was established in our previous study [6]. MDCK-7 cells (1.5×10^5 cells/mL) were grown on a 96-well plate overnight. Viruses (100 PFU/well) preincubated with 30 µL of serially four-fold diluted antibodies at 37°C for 1 h were added to the cells, which were then incubated at 37°C for 1 h, washed with PBS, incubated with Minimum Essential Medium (Life Technologies, Carlsbad, CA) overnight at 37°C, and fixed with ethanol at room temperature for 2 min before being subjected to an immunofluorescence assay.

Serum from a mouse infected with B/Ibaraki/2/1985 was used as the primary antibody [7]. Cells were incubated with the serum diluted in PBS (1:500) at 37°C for 30 min. After being washed with PBS three times, the cells were incubated with Alexa Fluor-conjugated goat anti-mouse IgG (Life Technologies, Carlsbad, CA) diluted in PBS (1:1,000) at 37°C for 30 min. The cells were then washed with PBS three times and observed using a fluorescent microscope (ECLIPSE Ti, Nikon, Tokyo).

3. Results

3.1. Production of recombinant human IgGs

We introduced a gene encoding the variable region of the H- and L-chains of 5A7 and 5E4 to pQCXIP with the human H-chain constant region and pQCXIH with the L-chain constant region, respectively. H- and L-chain expression vectors were simultaneously transfected to CHO-K1 cells. After the establishment of recombinant 5A7- and 5E4-stably expressing cells, the 2-day-old cultured medium was applied to ELISA (Fig. 1A). Both IgGs were produced in CHO-K1 cells as human IgGs. The recombinant 5A7 bound to influenza virus type B antigen but not type A(H1N1)pdm. In contrast, the recombinant 5E4 recognized type A(H1N1)pdm antigen but not type B. No antibody and no antigenicity against influenza were detected in HAM media without transformed CHO cells. The productivities of 5A7 and 5E4 calculated using a standard curve of human IgG were 55 ng/mL and 50 ng/mL, respectively (data not shown).

3.2. Purification of recombinant IgGs

To purify the recombinant IgGs, a scaling-up of cell culture was done. Cells were subcultured on 150-mm dishes in DMEM with serum. Cultured medium was exchanged

to serum-free DMEM at 90% of cell confluency, and the cells were then cultured for 1 week. Affinity column chromatography using Protein G Sepharose resulted in the productivity values of 2.4 µg/mL of 5A7 and 2.9 µg/mL of 5E4, respectively. Purified 2.0 µg of recombinant and hybridoma-derived IgGs were separated by SDS-PAGE and stained with CBB to examine the quality. As shown in Fig. 1B, 5A7 and 5E4 produced in CHO cells consisted of H- and L-chains, and their molecular weights corresponded to those of the IgGs produced in hybridomas.

3.3. Intact MS analysis by nano-LC/MS

Forty nanograms of DTT-denatured 5A7 and 5E4 were directly analyzed by nano-LC/MS. Multi-peaks corresponding to the different charge states were detected in the mass spectra of the intact H- and L-chains of 5A7 as shown in Fig. 2A and 2B, respectively. Their molecular weights were calculated from the charge states $[M+28H]^{+28}$ and $[M+11H]^{+11}$ through $[M+50H]^{+50}$ and $[M+21H]^{+21}$ as 50995.3 and 22718.4 Da, respectively. In the same way, the molecular weights of the intact H- and L-chains of 5E4 were calculated as 50644.1 and 22578.9 Da (Fig. 2C and 2D).

The mass spectra data from Fig. 2 were deconvoluted as shown in Fig. 3, in which the deconvoluted spectra of 5A7 and 5E4 are shown in panels A and C, respectively. The mass spectra derived from the H-chain of 5A7 are magnified in Fig. 3B. The multiple peaks mean that the H-chain of 5A7 is *N*-glycosylated. The spectra show that at least the glycan from the H-chain with 51320.2 Da contains two hexoses and one deoxyhexose, in addition to that with 50848.9 Da. In contrast, the L-chain was not *N*-glycosylated because of its single peak. The H-chain of 5E4 was also *N*-glycosylated containing one deoxyhexose or one hexose and deoxyhexose or two hexoses and one

deoxyhexose (Fig. 3D). The L-chain had no *N*-glycans.

3.4. *N*-Glycan analysis

No sialylated glycans were revealed by the HPLC analysis of a DEAE column (data not shown). Figure 4 shows RP-HPLC profiles of PA-glycans from antibodies produced in CHO cells and hybridomas. Each collected peak was further separated by an LC, and its molecular weight and glycan component were analyzed by MS. The compositions and relative amounts of the detected *N*-glycans are shown in Table 1.

The LC-MS analysis of the PA-glycans from 5A7 produced by CHO cells resulted in the detection of the following glycans: Man₈GlcNAc₂: M8 (0.6%), Man₇GlcNAc₂: M7 (1.0%), Man₆GlcNAc₂: M6 (0.2%), Man₅GlcNAc₂: M5 (3.8%), GlcNAcMan₃GlcNAc₂: GNM3 (9.7%), GlcNAc₂Man₃GlcNAc₂: GN2M3 (1.2%), GlcNAcMan₂FucGlcNAc₂: GNM2F (0.5%), GlcNAcMan₃FucGlcNAc₂: GNM3F (5.4%), GlcNAc₂Man₃FucGlcNAc₂: GN2M3F (10.3%), GalGlcNAcMan₃GlcNAc₂: GalGNM3 (0.5%), GalGlcNAcMan₃FucGlcNAc₂: GalGNM3F (1.7%), GalGlcNAc₂Man₃GlcNAc₂: GalGN2M3 (3.5%), GalGlcNAc₂Man₃FucGlcNAc₂: GalGN2M3F (52.1%), Gal₂GlcNAc₂Man₃GlcNAc₂: Gal2GN2M3 (0.6%), and Gal₂GlcNAc₂Man₃FucGlcNAc₂: Gal2GN2M3F (8.6%). The following glycans from 5E4 produced by CHO cells were also detected: M8 (0.5%), M7 (1.1%), M5 (5.4%), GN2M3 (7.4%), GNM3F (3.1%), GN2M3F (27.7%), GalGNM3 (0.3%), GalGNM3F (1.1%), GalGN2M3 (4.7%), GalGN2M3F (39.0%), and Gal2GN2M3F (6.2%). The distribution of *N*-glycan structures from antibodies produced by the hybridomas was slightly different from that of the CHO-produced antibodies. The compositions of the *N*-glycans from 5A7 and 5E4 were as follows: M8 (5.5%), M6 (2.0%), M5 (13.7%),

GNM3 (3.2%), GN2M3F (35.3%), GalGN2M3 (1.0%), GalGN2M3F (36.9%), and Gal2GN2M3F (5.5%); M8 (6.0%), M5 (12.8%), GNM3 (1.6%), GN2M3 (1.8%), GN2M3F (41.9%), GalGNM3F (0.6%), GalGN2M3 (0.8%), GalGN2M3F (34.6%), and Gal2GN2M3F (6.0%), respectively. These glycan structures are shown in Fig. 5.

3.5. Neutralizing activity assay

We evaluated the neutralizing activities of 5A7 and 5E4 produced by CHO cells by performing an *in vitro* virus neutralization assay on MDCK-7 cells infected with influenza virus, using purified antibodies from CHO cells and hybridomas. Influenza virus B/Florida/4/2006 for the Yamagata lineage and B/Malaysia/2506/2004 for the Victoria lineage were used against 5A7 antibodies. We observed effective neutralizing activity of recombinant 5A7 to the Yamagata and Victoria lineages compared to that of the hybridoma-produced 5A7 (Fig. 6A). Influenza virus A/Suita/1/2009 was also used against 5E4 antibodies, and the assay results demonstrated that 5E4 produced by CHO cells effectively neutralized this strain of influenza A virus with its serial fivefold dilutions (Fig. 6B).

4. Discussion

In previous studies, the promising human monoclonal antibodies 5E4 and 5A7 against pandemic A(H1N1)2009 influenza virus and influenza B virus were produced by establishing hybridomas using a novel human lymphocyte fusion partner, SPYMEG. It is expected that the use of 5E4 will increase our understanding of viral antigenic evolution because of its recognition of the antigenic site Sb of the HA protein in A(H1N1)pdm09. The use of 5A7 is also expected to contribute to the development of

future vaccines with its broad viral neutralizing activity against influenza B virus. However, hybridomas are not a useful tool for the industrial-scale antibody production. Recent biotechnological advances in CHO-production systems have made it possible to do the large-scale production of promising antibodies.

The identified variable region of H- and L-chains was produced in CHO-K1 cells as fusion proteins to the typical constant region of human IgG. Although there is no report of a recombinant antibody the gene of which was cloned from a hybridoma derived from SPYMEG, in the present study, purified recombinant 5A7 and 5E4 were demonstrated to be human antibodies with antigen recognition (Fig. 1). This result shows that a gene encoding human antibodies is stably integrated in hybridomas derived from SPYMEG without human chromosome deletion.

We subjected the purified antibodies to intact MS to rapidly analyze their molecular weight and the post-translational modification, *N*-glycosylation, on them. The molecular mass of the H-chain of 5A7 with the charge states (m/z 50995.3) was distinct from that calculated from its amino acid sequence without the signal peptide (m/z 49687.6). The deconvoluted multiple peaks in Fig. 3B mean that the H-chain of 5A7 was post-translationally modified and has one *N*-glycosylation site. The peak of m/z 51320.2 consists of the peak of m/z 50848.9, a deoxyhexose, and two hexoses. The difference between the measured mass and the calculated mass from the amino acid sequence shows that the peak of m/z 51320.2 has an *N*-glycan, Gal₂GlcNAc₂Man₃FucGlcNAc₂ the molecular weight of which is 1769.6. Similarly, the molecular mass of the H-chain of 5E4 with the charge states (m/z 50644.1) was distinct from that calculated from its amino acid sequence without the signal peptide (m/z 49334.1), and the deconvoluted multiple peaks in Fig. 3D show that the peak of m/z

50966.9 is from a polypeptide with an *N*-glycan, Gal₂GlcNAc₂Man₃FucGlcNAc₂. In contrast, 5A7 and 5E4 were easily detected with a single deconvoluted peak because their L-chains have no *N*-glycosylation (Fig. 3A and 3C). These glycan structures were also found in the detailed glycan analysis by HPLC and LC/MS (Fig. 5). Moreover, the glycans GalGlcNAc₂Man₃FucGlcNAc₂ and GlcNAc₂Man₃FucGlcNAc₂ occupying large amounts of the total *N*-glycans from recombinant 5A7 and 5E4 corresponded to those from the main peaks in Fig. 5. Therefore, it could be said that the intact MS analysis conveniently and quantitatively gives information about the *N*-glycan structures on purified proteins.

Information about the *N*-glycosylation pattern is essential for the production of biopharmaceutical proteins. Especially, insufficient *N*-glycan structures of proteins affect their biological half-life in blood and exogenous *N*-glycosylation patterns cause immunogenicity. In the present study, we analyzed and compared the detailed *N*-glycan structure of antibodies produced in hybridomas and CHO cells. Although similar *N*-glycan structures were found, the glycosylation pattern was slightly different between these antibodies (Table 1). The amount of high-mannose-type structures, Man₅₋₈GlcNAc₂ occupied approximately 20% of the total *N*-glycans of 5A7 and 5E4 secreted from SPYMEG. In contrast, that from CHO cells was under 10 % of the total *N*-glycans.

N-glycans with fucose residues that are related to antibody-dependent cellular cytotoxicity (ADCC) [18,19] occupied approximately 80% of the total *N*-glycans of IgGs secreted from both SPYMEG and CHO cells. Approximately 40% of the *N*-glycans of IgGs secreted from SPYMEG was galactosylated on the non-reducing end. The ratio of the galactosylation of total *N*-glycans was slightly increased in the IgGs

secreted from CHO cells. In contrast, no sialylated *N*-glycan was found in this glycan analysis. The amount of sialylated glycans on IgGs produced in mammals is significantly low and glycans with *N*-glycolylneuraminic acid (NeuGc) have been observed in almost all host mammals other than human and chicken [20]. It has also been reported that the ratio of sialylated glycans on IgGs produced in CHO cells is greatly changed according to the culture conditions such as the medium components, serum concentration, supplement combination, and more [21]. The reason why the CHO-produced 5A7 and 5E4 were not sialylated is unclear. Their three-dimensional structure may affect the catalysis of sialyltransferase. The sialylation to glycan of antibodies at the non-reducing end has been reported to reduce antibody-binding to Fc γ receptor (Fc γ R) [22]. It has been shown that the galactosylation gives no influence against ADCC activity [19]. However, contribution of the sialylation and the galactosylation to antibody function has been in controversy. Recently, antibody-binding to Fc γ R and ADCC activity were analyzed using antibodies with almost or completely homogenized glycosylation pattern [23,24]. In these studies, the authors demonstrated that the galactosylation but not the sialylation has significant effect on both antibody-Fc γ R, especially Fc γ RIIa and IIIa, binding affinity and increase of ADCC activity. Moreover, the sialylation was reported to reduce complement-dependent cytotoxicity (CDC) activity though the galactosylation increased antibody-complement binding affinity [25]. Considering these studies, the glycan residue of antibody *N*-glycan at the non-reducing end should be galactose residue. In this study, the ratio of galactosylated glycans to the total glycans of antibodies produced in CHO cells (5A7: 67.1%, 5E4: 51.3%) was higher than that in hybridoma (5A7: 43.4%, 5E4: 42.0%). Some anti-influenza virus antibodies showed

natural killer cells-mediated ADCC activity and complement-dependent lysis against influenza virus-infected cells [26,27]. Intensity of these activities may have been affected by antibody-glycosylation pattern. Therefore, it is expected that the recombinant 5A7 and 5E4 with high-galactosylated but not sialylated glycans show increasing ADCC and CDC activities with binding well to the Fc receptor and complement, respectively.

The neutralizing activities of 5A7 and 5E4 produced in the CHO cells were quite similar to that in the hybridomas (Fig. 6). Recombinant 5A7 well recognized influenza virus B/Florida/4/2006 of the Yamagata lineage and B/Malaysia/2506/2004 of the Victoria lineage. This means that the broad recognition of 5A7 against influenza B virus is conserved even if it is recombinantly produced in other mammalian cells. Similarly, the recognition of recombinant 5E4 against influenza virus A/Suita/1/2009 compares favorably with that from hybridomas. Recognition of the classical antigenic site Sb on HA protein must be conserved in recombinant 5E4. Moreover, it is thought that non-sialylated glycans on the recombinant IgGs did not affect their neutralizing activities.

This is the first report of the recombinant production of human antibodies identified from hybridoma fused with the promising fusion partner SPYMEG. We demonstrated that the quality of recombinant antibodies is quite similar to that of the native antibodies. Even if the recombinant anti-influenza antibodies produced in CHO cells were not sialylated on their *N*-glycans, the broad recognition against influenza B virus and a specific recognition against pandemic A(H1N1)2009 influenza virus were well conserved. The recombinant antibodies would be promising candidates for humanized biopharmaceutical proteins. The recognition and amino acid sequence of

5A7 against a conserved domain of HA is particularly valuable information for the selection and construction of useful antibodies that broadly recognize antigens.

Acknowledgements

This work was supported by the Japan Science and Technology Agency/Japan International Cooperation Agency, Science and Technology Research Partnership for Sustainable Development (JST/JICA, SATREPS).

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Figure legends

Fig. 1. The productivity and purification of CHO-produced antibodies against influenza virus. A) ELISA of CHO-cultured medium using plates coated with influenza B antigen: B, H1N1pdm antigen: H1N1, and anti-human IgG(H+L) antibody: Anti-Human Ab. B) CBB staining of purified antibodies produced in CHO-K1 cells and hybridomas. Arrowheads show H- and L-chains, respectively.

Fig. 2. Intact mass spectra of CHO-produced antibodies. A) H-chain of 5A7 eluted from 8.1 to 11.6 min on LC, B) L-chain of 5A7 eluted from 8.1 to 11.6 min on LC, C) H-chain of 5E4 eluted from 12.5 to 13.0 min on LC, and D) L-chain of 5E4 eluted from 12.5 to 13.0 min on LC. Detected ions are labeled with their charge state in each spectrum.

Fig. 3. Deconvolution of intact mass spectra detected in Fig. 2. A) H- and L-chains of 5A7, B) Magnified spectra of H-chain of 5A7, C) H- and L-chains of 5E4, and D) Magnified spectra of H-chain of 5E4. Opened and closed arrows mark the molecular weights of a hexose and a deoxyhexose, respectively.

Fig. 4. RP-HPLC profiles of PA-derivatives from antibodies. A) 5A7 from CHO cells, B) 5A7 from hybridomas, C) 5E4 from CHO cells, and D) 5E4 from hybridomas. The glucose units indicate the retention times of the PA-oligomers with the indicated number of glucose residues.

Fig. 5. List of the *N*-glycan structures prepared from antibodies in this study.

Fig. 6. Influenza virus-neutralizing activity assay of 5A7 and 5E4. Closed squares: CHO-produced antibodies. Open squares: Hybridoma-produced antibodies. Horizontal axis: the concentration of antibodies ($\mu\text{g/mL}$). Each infectivity value is the average of duplicate assay results.

Table 1

Composition of glycan structures of the IgG produced by CHO cells and hybridomas.

Structure	Abbreviation	5A7- CHO	5A7- Hybridoma	5E4- CHO	5E4- Hybridoma
Man ₈ GlcNAc ₂	M8	0.6	5.5	0.5	6.0
Man ₇ GlcNAc ₂	M7	1.0	-	1.1	-
Man ₆ GlcNAc ₂	M6	0.2	2.0	-	-
Man ₅ GlcNAc ₂	M5	3.8	13.7	5.4	12.8
GlcNAcMan ₃ GlcNAc ₂	GNM3	9.7	3.2	-	1.6
GlcNAc ₂ Man ₃ GlcNAc ₂	GN2M3	1.2	-	7.4	1.8
GlcNAcMan ₂ FucGlcNAc ₂	GNM2F	0.5	-	-	-
GlcNAcMan ₃ FucGlcNAc ₂	GNM3F	5.4	-	3.1	-
GlcNAc ₂ Man ₃ FucGlcNAc ₂	GN2M3F	10.3	35.3	27.7	41.9
GalGlcNAcMan ₃ GlcNAc ₂	GalGNM3	0.5	-	0.3	-
GalGlcNAcMan ₃ FucGlcNAc ₂	GalGNM3F	1.7	-	1.1	0.6
GalGlcNAc ₂ Man ₃ GlcNAc ₂	GalGN2M3	3.5	1.0	4.7	0.8
GalGlcNAc ₂ Man ₃ FucGlcNAc ₂	GalGN2M3F	52.1	36.9	39.0	34.6
Gal ₂ GlcNAc ₂ Man ₃ GlcNAc ₂	Gal2GN2M3	0.6	-	-	-
Gal ₂ GlcNAc ₂ Man ₃ FucGlcNAc ₂	Gal2GN2M3F	8.6	5.5	6.2	6.0
Total high-mannose-type <i>N</i> -glycans		5.6	21.2	7.0	18.8
Total fucosylated <i>N</i> -glycans		78.6	77.7	77.1	83.1
Total galactosylated <i>N</i> -glycans		67.1	43.4	51.3	42.0

Figure 1

A

B

Figure 2

Figure 3

Figure 4

A

B

C

D

Figure 5

Abbreviation	Structure
M8	<p>Chemical structure of M8: A branched N-glycan with a core fucose. The structure consists of a GlcNAcβ1-2Manα1 branch attached to a Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA backbone. The branch has a 2Manα1 at the top, a Manα1-2Manα1 in the middle, and a 2Manα1-2Manα1 at the bottom. The linkage between the branch and the backbone is a 6Manα1-3 linkage.</p>
M7	<p>Chemical structure of M7: A branched N-glycan with a core fucose. The structure consists of a GlcNAcβ1-2Manα1 branch attached to a Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA backbone. The branch has a 2Manα1 at the top, a 2Manα1 in the middle, and a 2Manα1-2Manα1 at the bottom. The linkage between the branch and the backbone is a 6Manα1-3 linkage.</p>
M6	<p>Chemical structure of M6: A branched N-glycan with a core fucose. The structure consists of a GlcNAcβ1-2Manα1 branch attached to a Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA backbone. The branch has a 2Manα1 at the top, a 2Manα1 in the middle, and a 2Manα1-2Manα1 at the bottom. The linkage between the branch and the backbone is a 6Manα1-3 linkage.</p>
M5	<p>Chemical structure of M5: A branched N-glycan with a core fucose. The structure consists of a GlcNAcβ1-2Manα1 branch attached to a Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA backbone. The branch has a 2Manα1 at the top, a 2Manα1 in the middle, and a Manα1 at the bottom. The linkage between the branch and the backbone is a 6Manα1-3 linkage.</p>
GNM3	<p>Chemical structure of GNM3: A branched N-glycan with a core fucose. The structure consists of a GlcNAcβ1-2Manα1 branch attached to a Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA backbone. The branch has a Manα1 at the top. The linkage between the branch and the backbone is a 6Manα1-3 linkage.</p>
GN2M3	<p>Chemical structure of GN2M3: A branched N-glycan with a core fucose. The structure consists of a GlcNAcβ1-2Manα1 branch attached to a Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA backbone. The branch has a GlcNAcβ1-2Manα1 at the top. The linkage between the branch and the backbone is a 6Manα1-3 linkage.</p>
GNM2F	<p>Chemical structure of GNM2F: A branched N-glycan with a core fucose. The structure consists of a GlcNAcβ1-2Manα1 branch attached to a Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA backbone. The branch has a GlcNAcβ1-2Manα1 at the top. The linkage between the branch and the backbone is a 6Manα1-3 linkage. A Fucα1 is attached to the backbone at the 6 position.</p>
GNM3F	<p>Chemical structure of GNM3F: A branched N-glycan with a core fucose. The structure consists of a GlcNAcβ1-2Manα1 branch attached to a Manβ1-4GlcNAcβ1-4GlcNAcβ1-PA backbone. The branch has a Manα1 at the top. The linkage between the branch and the backbone is a 6Manα1-3 linkage. A Fucα1 is attached to the backbone at the 6 position.</p>

Figure 5

Abbreviation	Structure
GN2M3F
GalGNM3
GalGNM3F
GalGN2M3
GalGN2M3F
Gal2GN2M3
Gal2GN2M3F

Figure 6

A

B