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

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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.

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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

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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 (NeuGc), α1,3-linked galactose (Gal), α1,3-linked fucose, and β1,2-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′-ATTTGCGGCCGCCAT-GAAACACCTGTGGTTCTTC-3′ (forward primer for 5E4 H-chain), 5′-ATTTGCGGCCGCCATGGAGTTTGGGCTGAG-3′ (forward primer for 5A7 H-chain), 5′-ATACTCGAGGGTGCCAGGGGGAAGACCGATG-3′ (reverse primer for 5E4 or 5A7 H-chain), 5′-ATTTGCGGCCGC-CATGGCCTGGGTCTCATT-3′ (forward primer for 5E4 L-chain), 5′-ATTTGCGGCCGCCATGGCCTGGGCTCTGCT-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 *Not*I and *Xho*I, 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% CO_2 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 μL of 0.1 μg/mL influenza B antigen or 0.1 μg/mL H1N1 antigen or 10 μg/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, SIGMA*FAST*™ 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 μg/mL puromycin dihydrochloride, and 100 μg/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% CO_2 at 37 °C for 1 week. The medium was centrifuged at 1500g 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 μm, 0.3 mm × 5 mm) and an analytical column (3.5 mm, 75 μm × 150 mm) (both ZORBAX 300SB-C18, Agilent Technologies, Santa Clara, CA) were used for the