Broad neutralizing human monoclonal antibodies against influenza virus from vaccinated healthy donors

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Introduction

The technology of monoclonal antibody (MAbs) preparation is important to provide material for the characterization of unique epitopes and conformational structures as well as the development of rapid diagnostic kits. Further, acute infections could be more effectively prevented with a passive immune strategy, as evidenced by the successful treatment of healthcare workers infected with severe acute respiratory syndrome (SARS)-coronavirus by transfusion with convalescent phase plasma obtained from SARS patients [1] and successful protection against viral infection in a mouse model of SARS-coronavirus infection with human MAB (HuMABs) obtained from a patient’s memory cell repertoire [2]. Also, studies of Spanish flu that evaluated the effects of transfusion with influenza-convalescent blood products may offer insights regarding potential treatments for H5N1 infection [3]. Furthermore, it has been reported that an avian flu patient recovered following treatment with convalescent plasma from a donor [4]. Recently, there have been several trials using MAbs with neutralizing activity for the prevention of acute severe symptoms of influenza virus infection including H5N1 [5–8]. Further, protective properties of MAbs directed at the stem region of hemagglutinin (HA) of influenza A virus H1N1 [9] and H5N1 [6–8] were demonstrated in mice. Based on these data, preparation of HuMABs against viruses causing severe acute diseases that have potential therapeutic applications becomes of great importance.

Several factors affect the development of HuMABs: the source of immune cells, such as human-derived memory B or plasma cells or murine cells; and the procedure to immortalize the immune cells, such as fusion with an appropriate partner cell line, EB virus-based transformation, molecular cloning of the immunoglobulin gene using phage display, and humanization of murine MAbs. Recently, a rapid cloning method for high-affinity HuMABs against influenza virus was established using single-cell reverse transcriptase-polymerase chain reaction for the immunoglobulin variable regions. For that, single-sorted plasma cells secreting influenza-specific IgG+ derived from influenza-vaccinated healthy volunteers were used [10]. In this study, we employed a novel approach for the development of HuMABs against influenza viruses using a newly prepared cell line, named SPYMEG, that was generated by fusion of SP2 myeloma cells of murine origin and MEG-01 human megakaryoblastic leukemia cells [11] as partner cells with high fusion efficiency with human lymphocytes (Kuhara, Personal Communication). Using peripheral blood mononuclear cells (PBMCs) from influenza-vaccinated volunteers, we developed several individual antibodies...
HuMabs that showed broad neutralizing activity against human influenza A (H3N2) and B viruses.

**Materials and methods**

**Volunteers.** A total of five healthy volunteers who received influenza vaccination were selected (A, 28 year-old male; B, 35 year-old female; C, 28 year-old male; D, 57 year-old male; and E, 29 year-old female). Ten milliliters of blood were obtained from individual volunteer and the PBMCs were prepared by centrifugation through Ficoll Pack Plus (GE Healthcare, Uppsala, Sweden) for 40 min at 520g.

**Vaccine.** The 2006/2007 influenza vaccine containing A/New Caledonia/20/99, A/Hiroshima/52/05, and B/Malaysia/2506/04 was kindly provided by the Research Foundation for Microbial Diseases of Osaka University, Kagawa, Japan.

**Viruses.** One influenza A vaccine strain of H1N1 subtype (A/New Caledonia/20/99), five influenza A vaccine strains of H3N2 subtype (A/Aichi/2/68, A/Guizhou/54/89, A/Wyoming/2/03, A/New York/55/04 and A/Hiroshima/52/05) and four influenza B vaccine strains (B/Victoria/2/87, B/Mie/1993, B/Shanghai/261/02 and B/Malaysia/2506/04) were used in this study. The A/Hiroshima/52/05 and B/Malaysia/2506/04 strains were kindly provided by the National Institute of Infectious Diseases, Tokyo, Japan. Viruses were propagated in Madin–Darby canine kidney (MDCK) cells and the culture fluids were stored at −80 °C. Infectivity was titrated on MDCK cells and expressed as infectious focus-forming units (FFU) per milliliter.

**Fusion partner cell line, SPYMEG.** For establishment of the SPYMEG cell line, cultured cells of mouse myeloma cell line, SP2/O-Ag14 (Riken Cell Bank: RCB0209) were fused with cells of human megakaryoblastic cell line, MEG-01 (JCRB Cell Bank: IPO050151) using polyethylene glycol #1500 (Roche Diagnostics, Mannheim, Germany). The fused cells were cultured in RPMI medium containing 10% fetal calf serum (FCS) in the presence of hypoxanthine–aminopterin–thymidine (HAT) for 5 days and further cultured in FCS-free RPMI medium for 3 days. The remaining cells were subsequently cultured with 10 μg/mL of 8-azaguanine for 10 days, after which limiting dilution was performed, generating the cell line designated SPYMEG. SPYMEG is non-secretor of human or murine immunoglobulin, 8-azaguanine-resistant and HAT-sensitive.

**Cell fusion.** The PBMCs were fused with SPYMEG cells at a ratio of 10:1 with polyethylene glycol #1500. Fused cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 15% FCS in 96-well microplates for 10–14 days in the presence of HAT. The first screening of the culture medium for antibodies specific to influenza viruses was performed by enzyme-linked immunosorbent assay (ELISA), as described below. Specific antibody-positive wells were next subjected to cell cloning by limiting dilution. The second screening was also performed by ELISA.

**ELISA.** The 96-well microplates coated with viral antigens were reacted with the culture medium of fused cells for 30 min at room temperature, followed by peroxidase-conjugated rabbit anti-human IgG (Medical and Biological Laboratories, Aichi, Japan).

**Immunofluorescence assay.** Monolayers of MDCK cells in 8-well chamber slides were mock-infected or infected with influenza A and B viruses. After 8 h of incubation, the infected cells were fixed with ethanol then reacted with the culture medium of individual hybridoma cell clones. As control antibodies, we used several murine MAb clones, i.e., C43 to nucleoprotein (NP) of influenza A H3N2 virus [12], C179 to HA of H1N1 [12], F49 to HA of H3N2 (Okuno, unpublished), 9F3 to NP of influenza B virus [13], and 9E10 to HA of influenza B virus [14]. The cells were then reacted with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human or anti-mouse antibodies (Jackson ImmunoResearch, Cambridgeshire, UK).

**Western blotting.** Purified HA vaccine antigens in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer were subjected to electrophoresis in a 10% polyacrylamide gel. Proteins in the gel were blotted onto polyvinylidene difluoride membranes then incubated with the culture medium of individual hybridoma clones. After incubation with peroxidase-conjugated goat anti-human IgG (H + L) antibody (Jackson ImmunoResearch), the peroxidase reaction on the membrane was visualized using the ECL Western Blotting Detection System (GE Healthcare, Uppsala, Sweden).

**Peroxidase and anti-peroxidase (PAP) staining.** PAP staining was carried out as described previously [15]. Briefly, MDCK cells were infected at a multiplicity of infection of 0.1 with influenza virus and cultured for 6 h in FCS-free DMEM. The infected cells were fixed with ethanol and reacted with the culture medium of individual hybridoma clones. As controls, we used several murine MAb clones as mentioned above. The cells were further incubated with rabbit anti-human or anti-mouse IgG antibody (Cappel), then with goat anti-rabbit IgG antibody (Cappel), and finally with PAP complex (Cappel).

**Hemagglutinin-inhibition (HI) test.** HI test was carried out as described previously [12]. The culture medium of individual hybridoma clones was treated with receptor destroying enzyme (RDE). The results were expressed as the reciprocal of the highest dilution of the culture medium to show inhibition.

**Virus neutralization (VN) test.** The VN test was carried out as described previously [15]. Briefly, the culture media of individual hybridoma clones that were treated with RDE were diluted 1:8 with serum-free medium. The diluted culture medium of individual hybridoma clones (50 μL) was mixed with 100 FFU of influenza virus (50 μL), then applied to MDCK cells in a 96-well microplate. After culturing for 6 h, the cells were fixed with ethanol and stained with PAP as above. The data were expressed as percentage of infectivity.

**Sequence analysis of HuMAb variable regions.** Total RNA was extracted from 1 × 10^7 hybridoma cells and reverse transcription (RT) was performed at 42 °C for 90 min, using Human IgG RT-primer’ (5′-TGGAGGGCCACGTACACCCG-3′) and IgG1 RT-Primer (5′-TGTGACGGGCGAGCTCAGGC-3′) for kappa chain, and also using dT Primer for lambda chain. Next, we performed 5′ RACE PCR with the SMARTTM RACE cDNA Amplification Kit (Clontech Laboratories, Mountain View, CA), using Human IgG1 RACE-primer (5′-AAGGTTGTCACGCGGAGTCGC-3′), Human IgG1(kappa) RACE-primer (5′-GGTGCCTAGGGCTGTAGTG-3′) or Human IgG1(lambda) RACE-primer (5′-CCAYTGTCTTCCCACRGRCTCYYC-3′ and 5′-TCAGAGGGCRYGGACACAGAGTG-3′) as the reverse primer and Universal primer mix as the forward primer, and then cloned the PCR products into the cloning plasmid pMD20-T (Takara Bio, Kyoto, Japan). Sequence analysis was performed with an ABI3730 Sequencer (Perkin Elmer, Waltham, MA), using the M13 primer RV or M13 primer M4 and the BigDye Terminators v3.1 Cycle Sequencing Kit (Perkin Elmer).

**Epitope mapping.** A total of 158 sets of 15-residue peptides (overlapping by 13 amino acids) spanning amino acid (aa) residues 1–329 of the HA1 region of A/Hiroshima/52/05 (JPT Peptide Technologies GmbH, Berlin, Germany) were subjected to binding activity assay using peptide microarrays [16,17] with the HuMABs obtained.

**Ethics.** All human materials were collected using protocols approved by the Institutional Review Boards of the Research Institute for Microbial Diseases, Osaka University.
**Results**

**Development of HuMAbs**

Ten milliliters of blood from five volunteers was obtained at various time points after influenza vaccination for fusion with SPY-MEG cells. In 14 experiments, a total of 122 wells were positive for influenza virus-specific HuMAbs in the first screening. However, during subsequent cell cloning and expansion to a large-scale culture, most of the hybridoma cell clones lost the ability to produce HuMAbs. Eventually, 10 hybridoma clones were found to stably produce specific HuMAbs for at least 3 months (Table 1). The efficacy to detect the specific antibody-producing wells was higher with the PBMCs derived from vaccines at 2–3 weeks post-vaccination.

We next determined the influenza virus type recognized by individual HuMAbs. First, an immunofluorescence analysis was carried out and the results were as follows: E-1 recognized H1N1; A-2, B-1, B-2 and D-1 recognized H3N2; and A-1, B-3, C-1, D-2 and E-2 recognized B viruses. Representative results of B-1 and D-1 to influenza A virus (H3N2) and B-3 and E-2 to influenza B virus are shown in Fig. 1A. As control antibodies, we used several anti-influenza HA and NP murine MAbs. The staining pattern of infected MDCK cells obtained with our HuMAbs was very similar to that obtained with the anti-HA, but not anti-NP murine MAbs. PAP staining and ELISA of these HuMAbs gave the same results (Table 1). Five MAbs (E-1 to H1N1; B-1 and D-1 to H3N2; and A-1 and C-1 to B) showed distinct bands of HA protein on Western blotting, 1). Five MAbs (E-1 to H1N1; B-1 and D-1 to H3N2; and A-1 and C-1 to B) showed distinct bands of HA protein on Western blotting, but the other MAbs showed little or no reactivity with any viral proteins including HA by this method, indicating their recognition of conformational epitope(s) (Fig. 1B). As a control antibody, we used 2000-fold dilution of donor B-derived plasma.

**Table 1**

| Volunteer (age, sex) | Sampling (weeks post-vaccination) | ELISA+ wells | Stable clone | Recognizing HA virus | HI activity (%) | VN activity (VL) |
|----------------------|----------------------------------|--------------|-------------|----------------------|----------------|-----------------|
| A (28, male)         | 2                                | 44           | A-1         | B                    | <2             | 0               |
|                      |                                  |              | A-2         | H3                   | <2             | 4.3             |
| B (35, female)       | 1                                | 1            | 1           | B                    | <2             | 98.9            |
|                      |                                  |              | B-2         | H3                   | <2             | 0               |
|                      |                                  |              | B-3         | B                    | <2             | 31.7            |
| C (28, male)         | 1                                | 3            | 1           |                      |                |                 |
|                      |                                  |              | 3           |                      |                |                 |
|                      |                                  |              | 4           |                      |                |                 |
|                      |                                  |              | 4           |                      |                |                 |
| D (57, male)         | 3.5                              | 23           | D-1         | H3                   | <2             | 80.5            |
|                      |                                  |              | D-2         | B                    | <2             | 16.7            |
| E (29, female)       | 3                                | 9            | E-1         | H1                   | <2             | 0               |
|                      |                                  |              | E-2         | B                    | <2             | 37.8            |

* The fused cells from all volunteers were seeded in four 96-well microwell plates (384 wells).

**Table 2**

| Volunteer (age, sex) | ELISA+ wells | Stable clone | Recognizing HI activity | VN activity (VL) |
|----------------------|--------------|-------------|-------------------------|-----------------|
| A (28, male)         | 2            | 44          | A-1                     | B               |
|                      |              | A-2         | H3                      | <2             |
| B (35, female)       | 1            | 1           | B-1                     | H3              |
|                      |              | B-2         | H3                      | <2             |
| C (28, male)         | 1            | 3           | C-1                     | B               |
|                      |              | 3           |                         | <2             |
|                      |              | 4           |                         |                 |
|                      |              | 4           |                         |                 |
| D (57, male)         | 3.5          | 23          | D-1                     | H3              |
|                      |              | D-2         | B                       | <2             |
| E (29, female)       | 3            | 9           | E-1                     | H1              |
|                      |              | E-2         | B                       | <2             |

* The HI test was performed with A/New Caledonia/20/99, A/Hiroshima/52/05, and B/Malaysia/2506/04 as H1, H3, and B viruses, respectively.

**Discussion**

Two serotypes of influenza virus, influenza A (H1N1 and H3N2) and influenza B viruses are circulating predominantly with the H3N2 serotype and B viruses highly prevalent during the last 2 years in Japan [18,19]. In fact, HuMAbs obtained from five Japanese volunteers given the 2007 vaccine mostly recognized H3N2 and B viruses. Those HuMAbs are broadly reactive with the past strains of H3N2 and B viruses, as revealed by PAP staining as well as the VN assay.

Nine of 10 HuMAbs obtained in this study showed cross-reactivity with a wide-range of past strains of influenza A H3N2 or B virus. In addition, cross-reactivity was also observed for most of them in the VN test. This result may suggest that since humans have history of exposure to influenza virus antigens by natural infection as well as influenza vaccination, the immune cells recognizing global epitopes in the influenza virus seem to quickly respond by vaccination and therefore the use of PBMCs few weeks post-vaccination for cell fusion may be advantageous for the preparation of a wide-range subtype-reactive MAbs like B-1 and D-1.
Both HuMAbs recognized two separate regions (aa 173–181 and aa 227–239), located close to each other in the three dimensional structure of HA1, a novel human epitope. This result suggested that B-1 and D-1 recognized a conformational epitope. There have been several studies of HA epitopes of influenza A H3N2, using murine MAbs [20–25], or rabbit [26,27] and human [28] polyclonal antisera. Moreover, a mouse MAb recognizing a conformational epitope containing aa 186, 220, 229 and 230 was reported [23]. However, this epitope overlapped only at aa 229 and 230 with the epitope of B-1 and D-1. It is noteworthy to mention that the above two HuMAbs recognizing the same epitope with a highly conserved sequence were independently prepared from two volunteers, as confirmed from the different sequences of their CDRs.

In conclusion, the development of HuMAbs using PBMCs from volunteers who have been naturally exposed to influenza viruses or vaccinated, as well as from patients with influenza infection at a convalescent phase, would provide potential therapeutic application(s).
Acknowledgments

We are grateful to Drs. Takaaki Nakaya, Madiha S. Ibrahim, and Yohei Watanabe in our institute for valuable discussion and critical reading of the manuscript. This study was partly supported by the budget from Benesis Corporation as a collaboration with Osaka University.

Table 2
Cross-reactivity of HuMAbs to various isolates of influenza A and B viruses by PAP staining.

| HuMAb | Influenza A virus (H3N2) | Influenza B virus<sup>a</sup> |  |
|-------|--------------------------|-----------------------------|---|
|       | Ai/68                    | Gz/89                       | Wy/03 | NY/04 | Hi/05 |       | Vl/87 | Ma/04 | Mi/93 | Sh/02 |
| A-2   | >128                     | >128                        | >128  | >128  | >128  |       |       |       |       |       |
| B-1   | 128                      | >128                        | >128  | >128  | >128  |       |       |       |       |       |
| B-2   | <2                       | >128                        | 128   | >128  | >128  |       |       |       |       |       |
| D-1   | <2                       | >128                        | >128  | >128  |       |       |       |       |       |       |
| A-1   | <2                       | <2                          | <2    | >128  | >128  |       |       |       |       |       |
| B-3   | <2                       | <2                          | <2    | 32    | 32    |       |       |       |       |       |
| C-1   | <2                       | <2                          | <2    | 128   | 128   | >128  | >128  | >128  | >128  | >128  |
| D-2   | <2                       | <2                          | <2    | <2    | <2    | <2    | <2    | <2    | <2    | <2    |
| E-2   | <2                       | <2                          | <2    | <2    | <2    | <2    | <2    | <2    | <2    | <2    |

<sup>a</sup> Ai/68 for A/Aichi/2/68; Gz/89 for A/Guizhou/54/89; Wy/03 for A/Wyoming/2/03; NY/04 for A/New York/55/04; and Hi/05 for A/Hiroshima/52/05.

<sup>b</sup> Vl/87 for B/Victoria/2/87; B/Malaysia/2506/04; Mi/93 for B/Mie/1/93; and Sh/02 for B/Shanghai/261/02.

<sup>c</sup> Not done.

Fig. 2. The antigenic regions of broadly neutralizing HuMAbs. (A) Based on the reactivity of B-1 and D-1 HuMAbs with a series of overlapping peptides covering sequences of the HA1 region, amino acid sequences from aa 167–187 and from aa 225–241 are shown. Common amino acid sequences reactive with HA1 peptides are aa 173–181 and from aa 227–239 that are boxed by red and green, respectively. (B) The monomeric structure of H3 HA is shown. The image was created with RasMol 2.6.4, and the HA structure was obtained from the Protein Data Bank (PDB Accession No. 2VIU). The positions of the binding sites are shown as red dots (aa 173–181) and green dots (aa 227–239). The receptor-binding sites are shown as black ribbons. (C) The deduced amino acid sequences of CDRs in the VH and VL of HuMAbs B-1 and D-1 are shown.

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