Biochemical and Biophysical Research Communications 450 (2014) 42–48

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Human monoclonal antibodies derived from a patient infected with 2009 pandemic influenza A virus broadly cross-neutralize group 1 influenza viruses

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ARTICLE INFO

Article history:
Received 2 May 2014
Available online 22 May 2014

Keywords:
Influenza A virus
Human monoclonal antibody
Cross-neutralizing antibody
Global epitope

ABSTRACT

Influenza viruses are a continuous threat to human public health because of their ability to evolve rapidly through genetic drift and reassortment. Three human monoclonal antibodies (HuMAbs) were generated in this study, 1H11, 2H5 and 5G2, and they cross-neutralize a diverse range of group 1 influenza A viruses, including seasonal H1N1, 2009 pandemic H1N1 (H1N1pdm) and avian H5N1 and H9N2. The three HuMAbs were prepared by fusing peripheral blood lymphocytes from an H1N1pdm-infected patient with a newly developed fusion partner cell line, SPYMEG. All the HuMAbs had little hemagglutination inhibition activity but had strong membrane-fusion inhibition activity against influenza viruses. A protease digestion assay showed the HuMAbs targeted commonly a short α-helix region in the stalk of the hemagglutinin. Furthermore, Ile45Phe and Glu47Gly double substitutions in the α-helix region made the HA unrecognizable by the HuMAbs. These two amino acid residues are highly conserved in the HAs of H1N1, H5N1 and H9N2 viruses. The HuMAbs reported here may be potential candidates for the development of therapeutic antibodies against group 1 influenza viruses.

1. Introduction

Vaccination is currently considered the best option to control influenza. However, this approach has several limitations, mainly because of viral antigenic changes, a phenomenon known as antigenic drift [1]. Seasonal influenza vaccines normally induce a panel of anti-hemagglutinin (HA) antibodies. HA is synthesized as a precursor, HA0, and proteolytic cleavage into disulfide-linked HA1 and HA2 is required for the infectivity of progeny virions [2]. HA is divided into two distinct structural regions, the globular head responsible for virus binding to the cell receptor and the stem region containing the fusion domain. Influenza infection and vaccination produces antibodies that target predominantly antigenic sites in the globular head domain, thereby blocking virus-cell interaction [3]. Vaccine-induced antibody responses usually are strain-specific. In addition, the rapid acquisition of amino acids substitutions, predominantly in the globular head, allows influenza viruses to escape from neutralizing immune responses. Consequently, the WHO updates annually the influenza virus strains included in the current vaccine, on the basis of those currently in circulation and those predicted to circulate the next influenza

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http://dx.doi.org/10.1016/j.bbrc.2014.05.060
0006-291X/

season [4,5]. A major limitation of current vaccine approach against a new pandemic is the time required to produce an appropriate quantity of antigenically matched vaccine [4,5]. In addition, the emergence of resistance to antiviral drugs in recent years further limits the options available for the control of influenza [6]. Therefore, many researchers are now examining the prospect of new prophylactic and therapeutic approaches, including antibody therapy.

Human monoclonal antibodies (HuMAbs) have advantageous properties as prophylactic and therapeutic reagents, including a long half-life in the serum and high specificity [7]. Several studies have reported cross-neutralizing HuMAbs against group 1 influenza A viruses (H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16) [7–12], group 2 influenza A viruses (H3, H4, H7, H10, H14 and H15) [13], both group 1 and 2 influenza A viruses [14,15], and influenza B virus [16,17]. Most of these HuMAbs were prepared from human display libraries and bind to a conserved epitope in the stem region of HA. However, the diversity of combinational phage display libraries is sometimes many orders of magnitude greater than the diversity of the human variable region repertoire [18]. In addition, HuMAbs generated by this method are the products of random combination between immunoglobulin variable region of the heavy (VH) chain and light (VL) chain genes.

On the other hand, we have reported a cell-to-cell fusion method using a new fusion partner cell line, SPYMEG, and have generated naturally functioning HuMAbs against influenza viruses [17,19,20] and dengue viruses [21,22]. This method has been optimized to fuse SPYMEG with patient-derived peripheral blood mononuclear cells (PBMCs) [23]. Here, we report the characterization of three anti-influenza HuMAbs generated from the PBMCs of an influenza-infected patient.

2. Materials and methods

2.1. Ethics statement

Human materials were collected using protocols approved by the Institutional Review Boards of Osaka University (Approval number 19-8-6).

2.2. HuMAb preparation

Hybridomas producing anti-influenza HuMAbs were prepared using the fusion partner cell line, SPYMEG (Medical & Biological Laboratories), as described previously [17,19]. Briefly, 10 ml blood was drawn in February 2011 from a patient (35 years old, male, Japanese) infected with H1N1pdm. H1N1pdm infection was diagnosed by the Prime Check Flu (H1N1) 2009 kit (Alfresa Pharma), which specifically detects H1N1pdm. PBMCs were isolated from blood samples by density gradient centrifugation through Ficoll-Paque Plus (GE Healthcare). The PBMCs were fused with SPYMEG using polyethylene glycol 1500 (Roche) and the fused cells were selectively cultured in Dulbecco's modified Eagle medium (Life Technologies) supplemented with 15% fetal bovine serum and hypoxanthine–aminopterin–thymidine. Cell supernatants were subjected to screening for influenza virus specific antibodies by immunofluorescence assay (IFA). The cells in the positive wells were cloned by limiting dilution and screened again. The hybridoma cell clones were then cultured and expanded in Hybridoma-SFM (Life Technologies). MAbs were purified from 100 ml hybridoma culture supernatant by affinity chromatography using HiTrap Protein G HP Columns (GE Healthcare) and then dialyzed against PBS.

2.3. IgG isotyping

ELISA microplates (MaxiSorp; ThermoFisher Scientific Nunc) were coated with goat anti-human IgG (Jackson ImmunoResearch Laboratories) in 0.05 M sodium bicarbonate buffer (pH 8.6) overnight at 4 °C. After washing with PBS containing 0.1% Tween-20 (PBS-T), the wells were blocked with 0.5% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. The wells were then washed with PBS-T and incubated with hybridoma supernatants or control serum for 2 h at 37 °C. After washing with PBS-T, the wells were incubated with HRP-conjugated anti-human IgG_1, IgG_2, IgG_3 or IgG_4 antibodies (SouthernBiotech) for 1 h at 37 °C. The wells were then washed five times with PBS-T and incubated with 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories) at room temperature (RT) in the dark. After 20 min, the reaction was stopped with 2 N H_2SO_4. Color development was measured at 450 nm in ELISA Photometer (BioTek Instruments).

2.4. Sequencing of the HuMAb variable regions

Total RNA was extracted from the hybridoma cells using an RNeasy Mini kit (Qiagen) and subjected to RT-PCR using PrimeScript RT reagent kit (Takara Bio) and oligo (dT) primer. The regions encoding the HuMAb H- and L-chains were amplified by PCR using the following primer pairs: 5′-ATGGAGTTTGGGCTGAGCTGGGTT-3′ (H-chain forward) and 5′-CTCCCGCGGCTTTGTCTTGGCATTA-3′ (H-chain reverse) or 5′-ATGGCCTGGRYCYCMYTCYWCCTM-3′ (L-chain forward) and 5′-TGGCAGCTGTAGCTTCTGTGGGACT-3′ (L-chain reverse). The PCR products were purified using a QIAquick PCR Purification kit (Qiagen). After electrophoresis, a discrete band was extracted using QIAquick Gel Extraction kit (Qiagen) and the nucleotide sequence was determined using a BigDye Terminator v3.1 Cycle Sequencing kit and ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The sequences were used to search the NCBI database using IgBLAST website (http://www.ncbi.nlm.nih.gov/igblast/).

2.5. Viruses

Six H1N1pdm virus strains (A/Suita/1/2009, A/Osaka/168/2009, A/California/07/2009, A/Suita/117/2011, A/Suita/104/2011 and A/Suita/105/2011), two seasonal H1N1 strains (A/Brisbane/59/2007 and A/PR8/1934), one H2N2 virus strain (A/Izumi/5/1965), two H3N2 virus strains (A/Aichi/2/1968 and A/Uruguay/716/2007), two H5N1 virus strains (A/Duck/Egypt/D1Br12/2007 and A/Chicken/Egypt/RIMD12-3/2008), one H7N7 virus strain (A/Tufted duck/Shimane/124R/1980), one H9N2 virus strain (A/Turkey/Wisconsin/1/1966) and two influenza B virus strains (B/Florida/4/2006 and B/Malaysia/2506/2004) were used in the study. Viruses were propagated in either Madin-Darby canine kidney (MDCK) cells or 9-day-old embryonated chicken eggs.

2.6. Construction of HA-expressing plasmids

Wild and truncated HA gene sequences from A/Suita/1/2009 were generated by one step RT-PCR and inserted into the pGEM-T Easy Vector (Promega). Mutant HA genes were generated by site-directed mutagenesis PCR (GeneTailor Site-Directed Mutagenesis System; Life technologies). Each gene was subcloned into the expression vector pCAGGS [17]. The expression plasmids were transfected into human embryonic kidney 293T cells using Lipofectamine 2000 (Life technologies) according to the manufacturer's instructions.

2.7. Western blotting

Baculovirus expressed HA protein (A/Suita/1/2009) was suspended in a loading buffer with or without β-mercaptoethanol, subjected to electrophoresis in 10% SDS–PAGE gels, followed by western blotting as previously described [21].

2.8. IFA

IFA was performed as previously described [20]. Briefly, infected MDCK cells or transfected 293T cells were fixed with 4% formaldehyde in PBS for 20 min at RT and incubated with hybridoma supernatant or primary antibody (20 μg/ml) for 1 h at 37 °C, followed by incubation with FITC-conjugated anti-human IgG (for HuMAbs) (Jackson ImmunoResearch Laboratories) or anti-mouse IgG (for mouse MAbs C179 and C43) (Jackson ImmunoResearch Laboratories) for 45 min at 37 °C. The cells were observed using a fluorescence microscope (Nikon).

2.9. Focus-forming assay

MDCK cells were cultured in a 96-well plate, followed by adsorption of serial 10-fold dilutions of influenza viruses for 1 h at 37 °C. The cells were then washed with PBS, incubated for 12 h at 37 °C and then fixed and subjected to IFA. Virus titers were determined as focus-forming units (FFU).

2.10. Virus neutralization (VN) assay

VN assays were performed as described previously [17]. Briefly, purified HuMAbs (100 μg/ml) were diluted serially 2-fold in minimum essential medium (MEM; Life Technologies) and mixed with 200 FFU of influenza viruses at 37 °C for 1 h. MDCK cells were then incubated with the mixtures for 1 h at 37 °C. After incubation for 16 h at 37 °C, the cells were fixed and subjected to IFA. The antibody concentration that suppressed viral infection by 50% (VN_{50}) was used as the VN titer.

2.11. Hemagglutination inhibition (HI) assay

HI assay was performed with A/Suita/1/2009 as described previously [20] and the reciprocal of the antibody dilution for complete inhibition of hemagglutination was designated as the HI titer.

2.12. Fusion inhibition assay

Cell-to-cell fusion was performed as described previously [17,24]. Briefly, monkey kidney CV-1 cells were infected with A/Suita/1/2009 at a multiplicity of infection of 1.6. After incubation for 24 h in MEM supplemented with 4% BSA and 2.5 μg/ml of acetylated trypsin (Sigma), the cells were washed with PBS and incubated with HuMAbs for 30 min. Thereafter, the cells were washed with PBS (pH 5.5) for 5 min at 37 °C. After replacing the medium, the cells were incubated for 3 h and then fixed with absolute methanol and stained with Giemsa solution (Wako).

2.13. Protease susceptibility assay

Protease susceptibility assays were performed as described previously [8], with minor modifications. Recombinant H1 HA protein (A/Suita/1/2009) was prepared using a baculovirus expression system. 100 μl of the samples (0.12 μg/ml) were pre-mixed with 6 μg HuMAbs and then incubated with trypsin (20 μg/ml, pH 5.3). The mixtures were then incubated for 1 h at 37 °C and subjected to western blotting using the 5E4 antibody that recognizes H1N1pdm HA [20].

2.14. Epitope analysis

The epitopes recognized by the HuMAbs were analyzed by protease digestion, followed by mass spectrometry, as described previously [25]. Briefly, MAb-immobilized columns were prepared by adding each of the HuMAbs or C179 to HiTrap NHS-activated HP columns (GE Healthcare), followed by addition of recombinant HA protein (A/California/07/2009, 2 μg/ml, Protein Sciences). After washing with PBS, modified trypsin (100 μg/ml; Promega) was added and the column was incubated for 2 h at 37 °C. After washing, initially with PBS and subsequently with ultrapure water, the undigested peptide fragments were eluted with 0.1% trifluoroacetic acid. The eluates were concentrated and the peptide fragments analyzed by MALDI-TOF-MS.

2.15. Molecular modeling

A model of the HA structure was constructed using PyMol, based on the crystal structures of A/California/07/2009 (PDBID code 3LZG).

2.16. Sequence population analysis of epitope sequences

All complete and partial influenza A HA sequences were downloaded from the NCBI Influenza Virus Resource (IVR) (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). At the time of this investigation (24th August, 2013), >10,000 sequences derived from human and avian influenza A viruses were collected. A BLASTP search [26] was performed using an *e*-value cutoff of 0.001. The amino acid numbering for the HA1 and HA2 regions in this study corresponds to that published by Nobusawa et al. [27].

3. Results

3.1. Preparation of HuMAbs against influenza A viruses

PBMCs were obtained from an H1N1pdm-infected patient at 3, 9, 16 and 23 days after the onset of influenza symptoms. The PBMCs were fused with SPYMEG cells to generate hybridomas. Ultimately, three hybridoma clones were selected: 1H11 and 5G2 were derived from PBMCs collected 9 days after onset of fever and 2H5 was derived from PBMCs collected 23 days after onset of fever. The cells secreted HuMAbs with a broad reactivity to group 1 influenza A viruses, including H1N1pdm, seasonal H1N1, H5N1 and H9N2 (Table 1). On the other hand, the HuMAbs did not react with the group 1 virus H2N2, group 2 influenza A viruses (H3N2 and H7N7) or influenza B virus.

The antibody isotype of all three HuMAbs was IgG_1. Sequence analysis of the VH and VL regions of the HuMAbs showed that they were derived from similar germline H-chain genes: VH1–69, DH3–10 and JH6 (Supplemental Table 1). IFA results of the cells transfected with HA-expressing plasmid showed that all the HuMAbs targeted HA (data not shown).

3.2. Neutralizing activity of the HuMAbs

We performed an *in vitro* VN assay to examine neutralizing ability of the HuMAbs against group 1 influenza A viruses (Table 1). A mouse MAb, C179, that is a representative with heterosubtypic neutralizing activity against group 1 influenza viruses [24] and an anti-dengue HuMAb D23-1G7C2 [21,22] were used as positive and negative controls, respectively. All the three HuMAbs showed cross-neutralizing activity against H1N1pdm, seasonal H1N1, and H5N1 virus strains, as did C179. In particular, the three HuMAbs showed strong neutralizing activity against the H1N1pdm 2009

Table 1
Summary of reactivity and neutralizing activity of MAbs.

Influenza virus				Reactivity (IFA)[a]						Neutralizing activity[b] (VN50 value μg/ml)			
				HuMAbs				Mouse MAbs		HuMAbs			Mouse MAb
Type	Lianage	Subtype	Strain	1H11	2H5	5G2	5A7[c]	C179[d]	C43[e]	1H11	2H5	5G2	C179
A	Group 1	H1N1pdm	A/Suita/1/2009	+	+	+	–	+	+	+(1.58)	+(0.76)	+(1.39)	+(5.65)
			A/Osaka/168/2009	+	+	+	–	+	+	+(0.78)	+(1.51)	+(2.00)	+(5.68)
			A/California/07/2009	+	+	+	–	+	+	+(1.52)	+(2.82)	+(3.02)	+(3.01)
			A/Suita/117/2011	+	+	+	–	+	+	+(11.60)	+(6.03)	+(11.61)	+(5.73)
			A/Suita/104/2011	+	+	+	–	+	+	+(11.35)	+(6.00)	+(22.32)	+(5.98)
			A/Suita/105/2011	+	+	+	–	+	+	+(11.38)	+(6.21)	+(12.05)	+(3.11)
		Seasonal H1N1	A/Brisbane/59/2007	+	+	+	–	+	+	+(2.89)	+(5.53)	+(5.69)	+(5.57)
			A/PR8/1934	+	+	+	–	+	+	+(2.87)	+(5.83)	+(5.78)	+(3.10)
		H5N1	A/Duck/Egypt/D1Br12/2007	+	+	+	–	+	+	+(6.08)	+(6.05)	+(6.03)	nd
			A/Chicken/Egypt/RIMD12-3/2008	+	+	+	–	+	+	+(6.12)	+(11.66)	+(11.27)	nd
		H9N2	A/Turkey/Wisconsin/1/1966	+	+	+	–	+	+	+(11.82)	–(>100)	+(11.08)	+(5.53)
		H2N2	A/Izumi/5/1965	–	–	–	–	+	+	nd[f]	nd	nd	nd
	Group 2	H3N2	A/Aichi/2/1968	–	–	–	–	–	+	nd	nd	nd	nd
			A/Uruguay/716/2007	–	–	–	–	–	+	nd	nd	nd	nd
		H7N7	A/Tufted duck/Shimane/124R/1980	–	–	–	–	–	+	nd	nd	nd	nd
B			B/Florida/4/2006	–	–	–	+	–	–	nd	nd	nd	nd
			B/Malaysia/2506/2004	–	–	–	+	–	–	nd	nd	nd	nd

[a] MDCK cells infected with the viruses indicated were used for IFA.
[b] MDCK cells infected with the viruses indicated were used for *in vitro* VN assay.
[c] 5A7 is a MAb that broadly cross-reacts with the HAs of influenza B viruses [17].
[d] C179 is a representative MAb with a broad spectrum against group 1 influenza A subtypes, including H1, H2, H5, H6 and H9 viruses [24].
[e] C43 is a MAb that broadly cross-reacts with the nucleoproteins of influenza A viruses.
[f] nd, not determined.

isolates, with VN_{50} values of 0.76–3.02 μg/ml, while the HuMAbs neutralized the 2011 isolates with VN_{50} values ranging from 6.03 to 22.32 μg/ml, approximately 8-fold higher than the VN_{50} for the 2009 isolates. Moreover, 1H11 and 5G2, but not 2H5, neutralized the H9N2 strain. D23-1G7C1 showed little neutralizing activity against the influenza viruses.

We then tried to investigate the mechanisms underlying the neutralization activity of the HuMAbs. An HI assay showed that the three HuMAbs had little HI activity, even at the highest concentration (100 μg/ml) tested (Table 2). However, a fusion inhibition assay showed that all the HuMAbs inhibited the viral membrane-fusion process (Table 2 and Fig. 1). The concentrations of the HuMAbs required for complete inhibition were comparable to that of C179, with a representative fusion inhibition activity [24]. D23-1G7C2 did not inhibit viral membrane-fusion even when used at a concentration of 200 μg/ml (Fig. 1).

3.3. Mapping the epitopes recognized by the HuMAbs

We next sought to elucidate the epitope regions recognized by the three HuMAbs. The reactivity of the HuMAbs to A/Suita/1/2009 HA was investigated by Western blotting. All the three HuMAbs recognized the HA under non-reducing conditions but lost the reactivity under reducing conditions (Table 2). In contrast, 5E4, which recognizes a linear HA epitope [20], detected the HA under both non-reducing and reducing conditions. This suggested that the epitopes recognized by the HuMAbs were conformational.

Table 2
Summary of characterization of MAbs.

Target		Clone	Reactivity (WB[a])		Reactivity (IFA[c])					Inhibition activity[e] for;		
			With β-ME[b]	Without β-ME	Wt[d]	I45F	E47K	E47G	I45F/E47G	Hemagglutination (HI assay)	Cell-to-cell fusion (Fusion inhibition assay)	HA conformational change (Protease susceptibility assay)
Anti-influenza	HuMAb	1H11	–	+	+	+	+	+	–	–	+	+
		2H5	–	+	+	+	+	+	–	–	+	+
		5G2	–	+	+	+	+	+	–	–	+	+
		5E4[f]	+	+	+	+	+	+	+	+	nd[h]	–
	Mouse MAb	C179[g]	nd	nd	nd	nd	nd	nd	nd	–	+	nd
Anti-dengue	HuMAb	D23-1G7C2	nd	nd	–	–	–	–	–	–	–	–

[a] Recombinant A/Suita/1/2009 HA was used for the assays.
[b] β-ME indicates β-mercaptoethanol.
[c] 293T cells transfected with expression plasmid encoding the indicated form of A/Suita/1/2009 HA was used for IFA.
[d] Wt indicates wild-type HA.
[e] A/Suita/1/2009 strain was used for the assays.
[f] 5E4 is a MAb that recognizes a linear epitope in HA1 of H1N1pdm [20].
[g] C179 is a MAb that recognizes an epitope in the α-helix stem region of group 1 influenza HAs [24].
[h] nd, not determined.

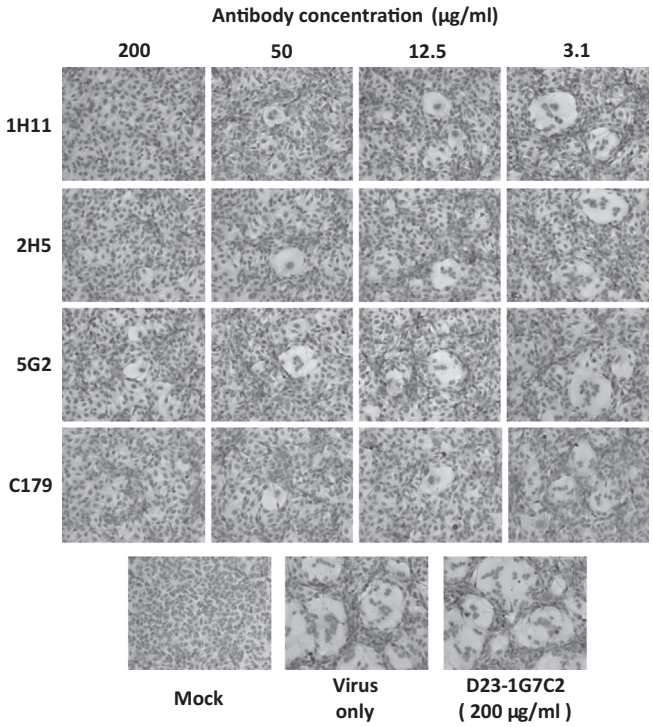


Fig. 1. Fusion inhibition assay. CV-1 cells infected with A/Suita/1/2009 were pre-treated with 1H11, 2H5, 5G2 or C179 at the concentrations indicated and exposed to low pH to induce polykaryon formation. Mock-infected cells (Mock), infected cells not treated with antibody (Virus only) and infected cells treated with 200 μg/ml of anti-dengue HuMAb D23-1G7C2 were also examined as controls.

We then performed a protease susceptibility assay. Exposure to low pH converts HA to a protease-susceptible, post-fusion state [12]. On the other hand, the result showed that most of the HA was retained in a protease resistant, pre-fusion form when the HA was pre-treated with the HuMAbs but not with 5E4, which targets an epitope in HA1 [20] (Table 2), indicating that pre-treatment with the HuAbs blocked the pH-induced conformational change. This suggests that the three HuAbs target the stalk region of HA.

Next, we performed a protease digestion assay and mass spectrometry. Mascot search results indicated that all the HuMAbs recognized similar peptides. A database search showed that the sequences of the peptides matched those of A/California/07/2009 HA. Based on the calculated threshold scores, we decided that scores greater than 67 were significant ($p < 0.05$). According to the scores, all the HuMAbs recognized the same peptide, amino acids 384–402, in A/Suita/1/200/2009 HA numbering (which corresponds to amino acids 40–58 in HA2), as indicated in red (score numbers 100-68) (Fig. 2A). In contrast, other peptides had lower score numbers, as indicated in purple (score numbers 67-33) or green (score numbers 32-10). The sequences largely corresponded to those from the column coupled with C179 that targets an epitope in the helical stem [24]. These results suggest that the epitope recognized by the HuMAbs is within the α-helix of HA2 (amino acid residues 40–58).

To investigate further the epitopes recognized by the three HuMAbs, we focused on the amino acid residues at positions 45 and 47 because the three HuMAbs distinctly neutralized the H1N1pdm 2009 strains, the 2011 strains and the H2N2 strain (Table 1) and these three virus groups differed in their α-helix residues at these two positions (Supplemental Table 2). We constructed four representative mutants of H1N1pdm 2009 HA, with Ile45Phe, Glu47Lys, Glu47Gly or Ile45Phe/Glu47Gly substitutions, and evaluated their reactivity with the HuMAbs. The three HuMAbs lost reactivity only when Ile45Phe/Glu47Gly double substitutions were introduced into the HA (Table 2). This suggests that amino acids at positions 45 and 47 are critical for reactivity with the three HuMAbs.

3.4. Sequence variations within the epitope region recognized by the three HuMAbs

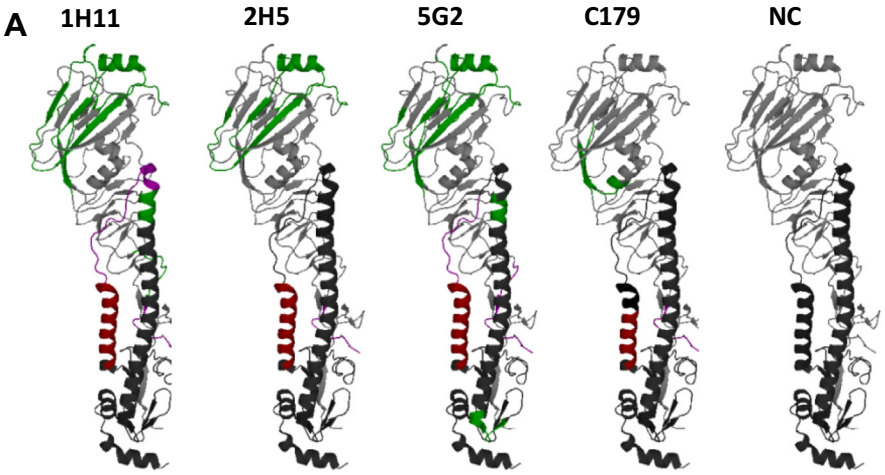
The sequences of the α-helix region of HA2 (at positions 40–58) recognized by the HuMAbs were retrieved from the NCBI IVR database and the sequences of several subtypes of influenza A and B viruses were compared. We calculated amino acid conservation of the α-helix region of HA2 from all sequences registered in the NCBI IVR database. Here, we show most of the sequences were detected at >1% population among the sequences of individual influenza A virus subtypes (Fig. 2B). The result showed that, in general, only H2N2 viruses have simultaneous substitutions at positions 45 and 47.

4. Discussion

Several heterosubtypic neutralizing HuMAbs with a broad spectrum against group 1 influenza A viruses have been reported recently [7–12]. Most of the HuMAbs utilized the VH1-69 germline and recognized epitopes located in the most conserved region of HA, the α-helix region of the stalk. The reactivity of the anti-stem HuMAbs also was verified *in vitro* and *in vivo*. However, while anti-influenza heterosubtypic neutralizing HuMAbs have been isolated mainly from phage display libraries [9,10] and, occasionally, from vaccinated volunteers [28], it is not known whether such antibodies are produced in the course of the immune response to influenza virus in natural infections. The main finding of this study is that influenza infection can elicit heterosubtypic IgG antibodies that broadly neutralize group 1 influenza A viruses. Although we cannot exclude priming of naïve B cells, the PBMCs were collected from an infected adult patient with a history of repeated influenza infections but without exposure to seasonal influenza vaccines. There finding suggest that the three heterosubtypic antibodies were produced by memory B cells. A previous study suggested that less than 10% of the human naïve B cell repertoire is capable of responding to this conserved influenza epitope [28].

Crystallization studies revealed that two potent HuMAbs, CR6261 and F10, which were derived from phage libraries, bind to a highly conserved helical region in the membrane proximal stem [8,9]. The binding is mediated only by the HCDR1 and HCDR2 encoded by the VH1-69 germline sequence, while, unprecedentedly, HCDR3 and the L-chain do not contribute to the binding. In the present study, all the three HuMAbs used the VH1-69 genes and also recognized the α-helical stem, in accordance with to these studies [8,9]. Interestingly, the three HuMAbs showed slight variation in their reactivity to a spectrum of group 1 influenza viruses: 1H11 and 5G2 broadly cross-neutralized H1N1, H5N1 and H9N2 viruses, while 2H5 neutralized only H1N1 and N5N1 viruses. 1H11 and 5G2 were characterized by their usage of VL2-14, which differed from VL1-51 carried by 2H5 (Supplemental Table 1). These findings imply that the distinct spectrum of reactivity of the HuMAbs might be related to the different configuration of their VH and VL genes. Further investigation of naturally functioning HuMAbs, including 1H11, 2H5 and 5G2, should elucidate this point.

The amino acid residues at positions 45 and 47 of HA2 are critical for the three HuMAbs binding to the HA stem (Table 2). A database search showed that H2N2 viruses generally have unique residues, Phe^{45} and Gly^{47}, which have not been observed so far in the H1N1pdm, seasonal H1N1, H5N1 and H9N2 virus populations,



B

		40 45 47 58	
		S T Q N A I D E I T N K V N S V I E K	
H1N1pdm	I	. . . . . K	n= 5535 (66.2%)
	II	. . . H	n= 2484 (29.7%)
	III	. . . . .	n= 100 (1.2%)
Seasonal H1N1	I	. . . . . N G	n= 1786 (92.3%)
	II	. . . . . G	n= 49 (2.5%)
	III	. . . . . N G . . . . . I . . .	n= 31 (1.6%)
	VI	. . . . . N G . . . . . T	n= 22 (1.1%)
H5N1	I	. . . K . . . G V . . . . . I . D .	n= 205 (91.9%)
	II	. . . K . . . G V . . . . . I . N .	n= 11 (4.9%)
	III	. . . R . . . G V . . . . . I . D .	n= 5 (2.2%)
H9N2	I	. . . K . . . K . . S . . . N I V D .	n= 3 (100.0%)
H2N2	I	. . . K . F . G	n= 60 (74.1%)
	II	. . . K . F . R	n= 12 (14.8%)
	III	. . . R . F . G	n= 3 (3.7%)
	IV	. . . K . F . G . . . . . A	n= 1 (1.2%)
	V	. . . K . F . G . . . . . M . . .	n= 1 (1.2%)
	VI	. . . K . F	n= 1 (1.2%)
	VII	. . . K . F . G . . . . E	n= 1 (1.2%)
	VIII	. . . K . F N G	n= 1 (1.2%)
	IX	. . . K . F . V	n= 1 (1.2%)
H3N2	I	. . . A . . . Q . N G . L . R L . G .	n= 3021 (51.9%)
	II	. . . A . . N Q . N G . L . R L . G .	n= 1191 (20.5%)
	III	. . . A . . N Q . N G . L . R L . . .	n= 826 (14.2%)
	IV	. . . A . . . Q . N G . L . R L . . .	n= 346 (5.9%)
	V	. . . A . . . Q . N G . L . R	n= 275 (4.7%)
H7N2	I	. . . S . . . Q . . G . L . R L . . .	n= 1 (100.0%)
H7N7	I	. . . S . . . Q . . G . L . R L . . .	n= 2 (100.0%)
H7N3	I	. . . S . . . Q . . G . L . R L . . .	n= 4 (100.0%)
H7N3	I	. . . S . . . O . . G . L . R L . . .	n= 15 (100.0%)

Fig. 2. Epitope mapping. (A) A MAb-coupled column was pre-treated with HA protein (A/California/07/2009) and incubated with a protease. Undigested peptide fragments were then eluted and applied to mass spectrometry. Sequences at positions 40–58 in the HA2 with high score numbers (100-68) are indicated in red. Other sequences with lower score numbers 67-33 and 32-10 are shown in purple and green, respectively. The HA1 and HA2 regions are shown in gray and black, respectively. The sequences from the Ab-uncoupled column are also shown. (B) Amino acid variations of the epitope recognized by the HuMAbs among several subtypes of influenza A viruses. Sequences of amino acid residues at positions 40–58 in the HA2 were downloaded from the NCBI IVR database. Sequences at >1% population among the database-derived variable sequences in individual influenza A virus subtypes are shown. The virus subtypes and the amino acid conservation are shown on the left and right, respectively. Amino acid position of HA2 is also shown on the top. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

suggesting that the two amino acid residues do not mutate easily. Indeed, despite several attempts, we were unable to isolate influenza virus escape mutants using the three HuMAbs. Thus, the HuMAbs reported here may be potential candidates for the future development of effective anti-influenza prophylactic and therapeutic antibodies.

Acknowledgments

We thank Dr Pratap Singhasivanon for encouragement and valuable discussions about the JST/JICA, SATREPS projects which supported this work; Dr Pathom Sawanpanyalert and Dr Jotika Boon-Long for coordinating the projects; and Dr. Kazunobu Saito for mass spectrometry analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.060.

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