Journal of Microbiology (2015) Vol. 53, No. 2, pp. 166–175
DOI 10.1007/s12275-015-4642-2

Immunological characterization of monoclonal antibodies used in rapid influenza diagnostic test for detection of the 2009 pandemic influenza A(H1N1)pdm09 infection

Hwajung Yi[1]*, Mi-Seon Lee[1], Joo-Yeon Lee[1], Hae Kyung Lee[1], and Chun Kang[1,2]

[1]Division of Influenza Virus and [2]Division of AIDS, Center for Infectious Diseases, Korea National Institute of Health, Centers for Disease Control and Prevention, Cheongju 363-951, Republic of Korea

(Received Nov 5, 2014 / Revised Jan 12, 2015 / Accepted Jan 12, 2015)

Since the 2009 pandemic, monoclonal antibodies (mAbs) for rapid influenza diagnostic tests (RIDT) have been developed for specific diagnostics of pandemic viral infection. Most of the mAbs were poorly characterized because of urgency during the pandemic. Further characterization of the mAbs for RIDTs would be beneficial for understanding the immunological properties of the pandemic virus and utilizing the mAbs for other research purposes. In this study, it was confirmed that two mAbs (I38 and D383) in an RIDT for H1N1pdm09 diagnostics were able to detect H1N1pdm09 virus through enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA). Also, the two mAbs exhibited reactivity to hemagglutinins (HAs) of both the H1N1pdm09 and 1918 H1N1 viruses; therefore, the RIDT using the mAbs could detect HAs of H1N1pdm09 and also HAs of 1918 H1N1-like strains. In an extension to our previous study, the epitopes (Sa antigenic site and the interface area of F' and vestigial esterase subdomains on the HA1 domain of HA of H1N1pdm09) recognized by the mAbs were corroborated in depth by IFA with escape-mutants from the mAbs and mapping of the epitopes on the crystal structure of human H1N1 viral HAs. Collectively, these results imply that the mAbs for the RIDT may be suitable for use in studying the immunological properties of H1N1pdm09 viruses and that the Sa antigenic site and the interface area between F' and vestigial esterase subdomains on influenza viral HA recognized by the mAbs are immunologically conserved regions between H1N1pdm09 and 1918 H1N1.

Keywords: 2009 influenza pandemic, monoclonal antibody, RIDT, H1N1pdm09, 1918 H1N1

Introduction

A novel influenza virus (H1N1pdm09) emerged in April of 2009 and rapidly spread worldwide (Brockwell-Staats *et al.*, 2009; Cutler *et al.*, 2009). The influenza viral infection resulted in nearly 18,000 laboratory-confirmed deaths and it was estimated that death cases of respiratory and cardiovascular mortality associated with the pandemic were perhaps 15 times higher than the reported number of laboratory confirmed cases from April of 2009 to August of 2010 (Dawood *et al.*, 2012), causing a pandemic that raised serious public health issues (Patel *et al.*, 2010). Controlling pandemic influenza requires efficient antiviral therapeutic regimens and vaccination strategies (Germann *et al.*, 2006; Morris, 2009). In addition, rapid and accurate diagnosis is a critical component of control and preparedness for pandemic influenza (Amano and Cheng, 2005; Landry, 2011).

The current standard procedure for influenza virus detection and classification entails conventional virus isolation in embryonated eggs or MDCK cells, followed by HA and neuraminidase (NA) subtyping, using serological or PCR-based methods (Amano and Cheng, 2005). The most commonly used method for detecting and subtyping influenza viruses is real-time reverse transcription-PCR (rRT-PCR), in which subtype specificity is achieved through the amplification of a region of the HA or M gene, using primers specific to the individual influenza subtypes (Peacey *et al.*, 2009; Dhiman *et al.*, 2010; Landry, 2011). However, the PCR-based method for pandemic influenza diagnosis may not be available in every local clinic. Thus, the development of a new RIDT for the pandemic strains was urgently required. With an RIDT, the reliability of immunoassays for pandemic influenza detection depends on the quality of the immune reagents used such as anti-sera or antibodies against the pandemic influenza virus.

All of the influenza pandemics have been caused by influenza A strains carrying an antigenically novel HA segment in populations immunologically naïve to that particular HA (Taubenberger and Morens, 2006; Patel *et al.*, 2010). It is known that HA and NA are important targets for neutralizing antibodies against influenza viral infections (Okuno *et al.*, 1993; Corti *et al.*, 2010; Grandea *et al.*, 2010). Especially, HA glycoproteins form spikes on the surface of influenza virion, thereby mediating influenza viral attachment to sialoside receptors on host cells and subsequent entry via membrane fusion (Eisen *et al.*, 1997; Skehel and Wiley, 2000). Thus, neutralizing antibodies directed against the HA is the primary mediator of protection against influenza virus infection (Dormitzer *et al.*, 2011). On the other hand, the most immunogenic and hyper-variable region on HA is the head domain (HA1 or globular domain) which contains five anti-

*For correspondence. E-mail: pobee@korea.kr; Tel.: +82-43-719-8196; Fax: +82-43-719-8219

genic regions (Ca1, Ca2, Cb, Sa, and Sb) involved in the antigenic drift amongst influenza viral strains (Sriwilaijaroen and Suzuki, 2012), thereby, most of the mAbs against the HA1 of an influenza viral subtype or strain possess reactivity of a narrow range against influenza viral strains, in other words, strain or subtype specificity (Carrat and Flahault, 2007). Collectively, these indicate that the HA1 of a novel influenza virus such as the H1N1pdm09 of the 2009 pandemic can be suitable targets for generating specific antibodies which can be used in RIDTs for specific diagnosis of novel or pandemic influenza viruses.

Under this milieu, mAb-D383 and -I38 specific to the H1N1pdm09 virus were generated by the immunization of an H1N1pdm09 strain (A/Korea/01/2009) and the consequential hybridoma technique. With the two mAbs as essential components for the capturing and detecting of antibodies, an RIDT (SDpdm RIDT) which could differentially detect the pandemic viral infection was developed and evaluated with clinical samples in previous studies (Choi *et al.*, 2010; Kwon *et al.*, 2011).

Initially, the mAbs in the SDpdm RIDT were not characterized sufficiently because of the urgent need for them during the pandemic and only the specific hemagglutination inhibition (HAI) titer of the mAbs to the H1N1pdm09 virus was confirmed (Choi *et al.*, 2010; Kwon *et al.*, 2011). In this study, further immunological characterization with the mAbs used in the SDpdm RIDT was performed.

Here, we show clearly that mAb-D383 and -I38 are suitable for use in detecting H1N1pdm09 viruses in a specific manner by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA). Also we corroborate that the two mAbs (D383 and I38) recognize epitopes located in the Sa antigenic site and in the interface area of F' and vestigial esterase (VE) subdomains on the HA1 domain of the H1N1-pdm09 virus. In addition, it was elucidated that the two mAbs (-D383 and -I38) and the SDpdm RIDT using the mAbs may also recognize 1918 H1N1 viral strains as well as H1N1pdm09.

Collectively, these findings suggest that the characterization in depth of mAbs in an RIDT for the diagnosis of the 2009 pandemic influenza virus will provide useful information for studying the immunological characteristics of H1N1-pdm09 viral strains, which have replaced the pre-pandemic seasonal H1N1 viral strains to become the main seasonal viral strains (Pica *et al.*, 2012).

Materials and Methods

Virus strains and cell cultures

The influenza viral strains used in this study are A/Solomon Islands/3/2006 (H1N1), A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), B/Brisbane/60/2008, and A/Korea/01/2009 (H1N1pdm09). These viruses were propagated in 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs or in Madin-Darby canine kidney (MDCK) cells maintained in modified Eagle's medium containing 10% fetal bovine serum.

Monoclonal antibodies

For the rapid and convenient diagnosis of H1N1pdm09 viral infection in appropriate clinical settings, a new Rapid Influenza Diagnostic Test (SDpdm RIDT) was developed in previous studies (Choi *et al.*, 2010; Kwon *et al.*, 2011). In the RIDT kit, mAb-D383 and -I38 specific to H1N1pdm09 were used as detecting and capturing antibodies, respectively. The antibodies are generated and produced by conventional hybridoma techniques under screening though the HAI assay with various influenza subtypes.

Recombinant influenza viral HA proteins

Recombinant HA proteins of influenza virus used for the ELISA, RIDT, and surface plasmon resonance (SPR) were purchased from Sino Biological Inc. (Table 4). All recombinant HA proteins were expressed in insect cells infected with baculoviral vector encoding HA genes of indicated influenza viral strains (Table 4).

Generation of escape mutants

The escape-mutant viruses used in this study were generated in our previous study (Yi *et al.*, 2013). Briefly explaining the generation of escape mutants, an H1N1pdm09 strain (A/Korea/01/2009) was incubated with an excess amount (more than 1 mg/ml in which both mAb-I38 and mAb-D383 were able to neutralize A/Korea/01/2009, data not shown) of the indicated mAbs (mAb-D383 and -I38) for 1 h at 20°C, and the mixture was inoculated into 10-day-old embryonated chicken eggs and incubated for 48 h at 37°C. Viruses were harvested and used for the limiting-dilution cloning of escape-mutant viruses (which have defective binding to the mAb-I38 because they have escape-mutations on their HAs) in embryonated chicken eggs. After confirming HA mutations of individual escape mutants by sequencing analysis, the escape mutants were stored at -80°C prior to their use.

Hemagglutination inhibition (HAI) assays

An amount of 25 μl of inactivated A/Korea/01/2009 or another influenza viral strain (4 HA units) was mixed with 25 μl of serially diluted (two fold) receptor-destroying enzyme (RDE)-treated serum or mAbs (D383 and I38, 3 mg/ml) in V-bottom 96-well plates. After 30 min of incubation at room temperature (RT), 50 μl of 0.5% turkey RBC was added to each well and the mixture incubated for 30 min at RT. The HAI titer was defined as the highest dilution of the serum able to completely inhibit the hemagglutination of the indicated viruses.

Microneutralization assays

Sequential two-fold dilutions of the indicated heat-inactivated sera were tested in a microneutralization assay to determine the highest dilutions (neutralizing titers) of antibodies that are able to neutralize the infectivity of 100$TCID_{50}$ (50% tissue culture infectious dose) of the indicated H1N1 viral strains on MDCK cells in a 96-well plate as described in previous study (Grund *et al.*, 2011). After 10–16 h of incubation, the cells were fixed. Then ELISA was performed to detect the presence of viral nucleoprotein (NP) and determine the neu-

Table 1. **HAI titer of mAbs (D383 and I38) specific to the H1N1pdm09 virus**

viruses	Antibodies or anti-sera							
	mAb-I38[a]	mAb-D383[b]	anti-pdmH1N1[c]	anti-pdmH1N1[d]	anti-H1N1	anti-H3N2	anti-Flu-B[e]	anti-Flu-B[f]
H1N1pdm	40	>4000	1280	2560	<10	<10	<10	<10
H1N1	<10	<10	<10	<10	640	<10	<10	<10
H3N2	<10	<10	<10	<10	<10	2560	<10	<10
B (Brisbane)	<10	<10	<10	<10	<10	40	2560	80
B (Florida)	<10	<10	<10	<10	<10	80	40	2560

For the HAI assay, anti-sera samples against influenza A(H1N1)pdm09, A(H1N1), A(H3N2), and B were used, as well as the two mAbs (-I38[a] and -D383[b] which were isotyped as IgG2b and Kappa by IgG isotyping) of 3 μg/ml. Ani-pdmH1N1[c] and -H1N1[d] respectively denote the ferret antiserum against A/Korea/01/2009 and A(H1N1)pdm09 control sheep antiserum from the National Institute of Infectious Diseases. Anti-Flu-B[e] and Anti-Flu-B[f] indicate influenza B reference sheep antisera against B/Brisbane/60/2008 and B/Florida/4/2006 respectively. HAI titers are the GMT of 3 individual assays.

tralizing antibody titers (Table 2).

ELISA

ELISA plates of 96 wells coated with the indicated viral strains of 16 HA units or influenza viral HA proteins (2 μg/ml) were used to measure strain-specific reactivity for the two mAbs. One hundred microliters of the mAbs (indicated dilution from 3 μg/ml) in 1% (w/v) milk in phosphate-buffered saline with 0.05% Tween 20 (PBST) were added to the wells of the coated ELISA plates and incubated for 1 h at RT. After aspiration of the antibody solution, the wells were washed three times with PBST. The wells were incubated with 100 μl of rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP) diluted 1:5,000 in PBST and washed five times with PBST. The HRP conjugates were detected by adding 100 μl of a TMB substrate solution for the ELISA, stopping the reaction after color development with 100 μl 0.5 M HCl, and reading the optical density (OD) at 450nm.

Binding affinity measurement of mAbs by using SPR

Binding affinity of the mAb-D383 and -I38 to the recombinant HAs of A/California/07/2009 (an H1N1pdm09 strain) and A/Brevig Mission/1/1918 (a 1918 H1N1 strain) were determined by SPR experiments performed with the SR7500DC system (Reichert) at 25°C. For the SPR experiment, the recombinant HA proteins in immobilization buffer were immobilized on gold slide PEG chips (Reichert) by the amine coupling method. The two mAbs (D383 and I38) in PBS were injected to be trapped on the chip through the immobilized HA proteins. Evaluation and calculation of the binding parameters were carried out according to the Scrubber 2 software.

Immunochromatography

A test strip for immunochromatography is composed of a pad assembly (a gold conjugated pad that is able to detect antibodies conjugated with gold particles and an absorbent pad) and a nitrocellulose membrane with immobilized capturing antibodies. An indicated mAb (0.64 μg of mAb-I38 or -D383) as a capturing antibody was immobilized to a spot on a strip of nitrocellulose membrane. Meanwhile, an indicated mAb (mAb-I38 or -D383) as a detecting antibody was conjugated with colloidal gold particles and the gold conjugated mAb were dried on a glass fiber for a gold conjugated pad. The indicated viral samples were diluted with assay buffer (0.1 M NaCl, 0.4 M Tricine, and 1% Triton X-100) in test tubes, and the test strips were put into the test tubes.

Immunofluorescence assay (IFA)

MDCK cells were grown on collagen-coated slides and infected with 1.0 MOI of the indicated viral strains. After 24 h, the cells were washed with PBS and fixed with 4% paraformaldehyde. The cells were then permeabilized with 0.2% Triton X-100 in PBS and washed with PBS. Nonspecific binding was blocked with 3% BSA in PBS for 1 h at RT. Next, the cells were incubated with 1 μg/ml of the indicated mAbs in PBS containing 1% BSA for 1.5 h at RT. After washing with PBS containing 1% BSA, the cells were incubated overnight at 4°C with anti-mouse IgG conjugated with FITC. Cells were further washed extensively with PBS containing 1% BSA and then the slides were mounted with mounting medium.

Results

Prior to further characterization of the two mAbs used in the SDpdm RIDT, we corroborated the H1N1pdm09 specificity of the two mAbs through the HAI assay. The HAI assay was performed with pre-pandemic strains of influenza virus, anti-serum against the strains, and the two mAbs (designated as D383 and I38 respectively) (Table 1). As expected, the two mAbs exhibited specific HAI reactivity to

Table 2. **Neutralizing titers of mAb-D383 and -I38 to H1N1pdm09 and H1N1**

Viruses	Antibodies or anti-sera			
	mAb-D383[a]	mAb-I38[b]	anti-pdmH1N1[c]	anti-H1N1[d]
H1N1pdm[e]	32,000	<10	8,000	<10
H1N1[f]	<10	<10	<10	16,000

For the neutralizing assay, mAb-D383[a] (30 ng/ml) and -I38[b] (3 μg/ml) were used. Ani-pdmH1N1[c] and -H1N1[d] denote the ferret antisera against H1N1pdm09 and A/Brisbane/59/2007(H1N1) respectively provided from the WHO. H1N1pdm[e] and H1N1[f] denote A/Korea/01/2009 and A/Brisbane/59/2007 strains, respectively. Neutralizing titers are the GMT of 3 individual assays by microneutralization assay.

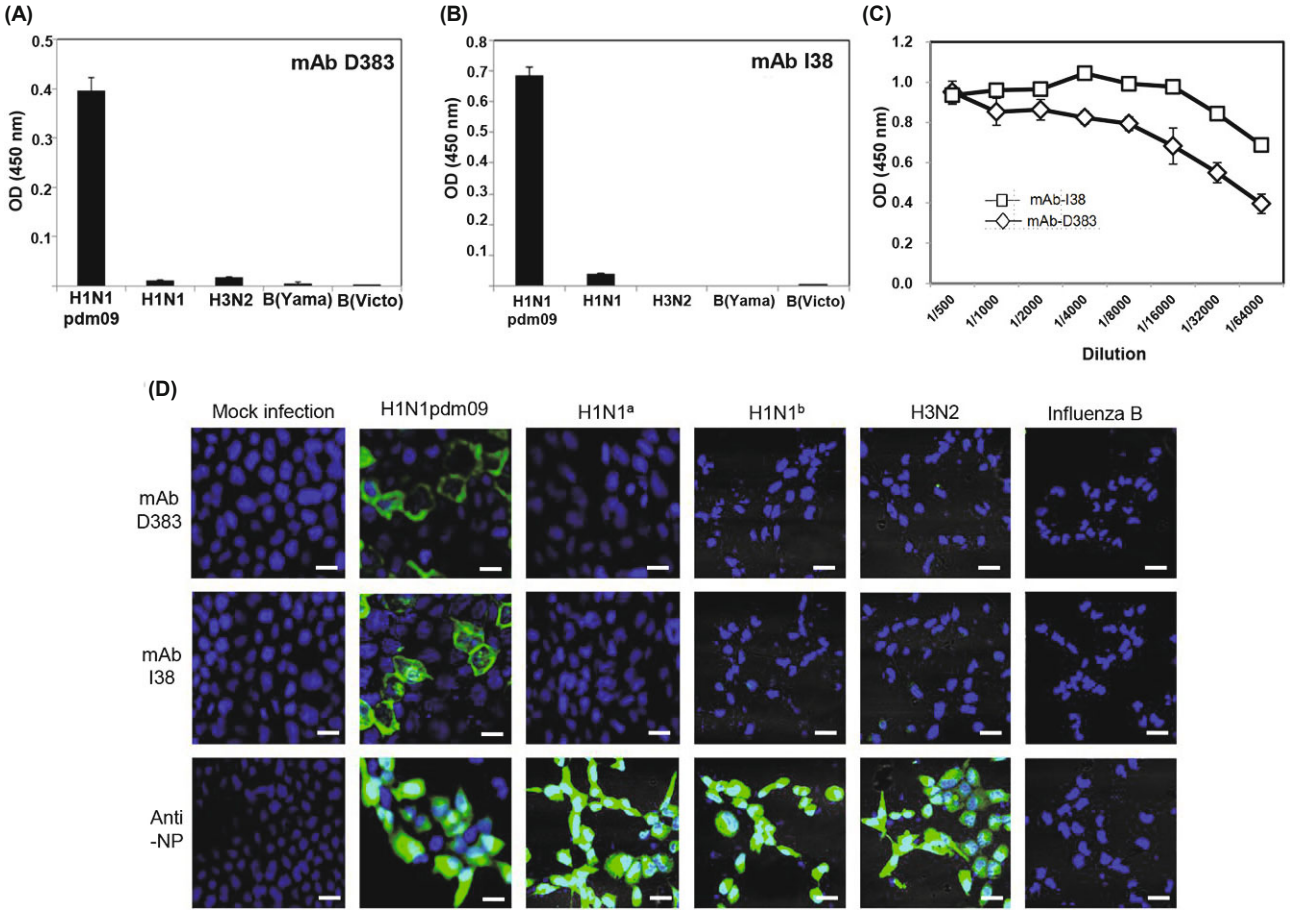

Fig. 1. The mAbs (D383 and I38) have specific reactivity to H1N1pdm09. For the ELISA assay in (A) and (B), inactivated viral antigens of H1N1pdm09, A/H1N1, A/H3N2, B/Wisconsin/1/2010-like (Yamagata lineage), and B/Brisbane/60/2008-like (Victoria lineage) were coated on the wells of 96 well ELISA plates and reacted with the two monoclonal antibodies with a 1:64,000 dilution from 3 μg/ml. For (C), inactivated H1N1pdm09 (A/Korea/01/2009) viral antigen was coated in ELISA plates and reacted with the two mAbs with indicated dilution from 3 μg/ml. (D) For the immunofluorescence assay (IFA), MDCK cells were infected with the indicated viruses and reacted with the two monoclonal antibodies and the anti-NP (nucleoprotein) antibody and then stained with FITC-labeled anti-mouse IgG. Blue-colored spots indicate the nucleus of MDCK cells stained by DAPI and green colored cells indicate A(H1N1)pdm09-infected cells stained by FITC-labeled anti-mouse IgG. A(H1N1)pdm09, H1N1[a], H1N1[b], H3N2, and influenza B designate A/Korea/01/2009, A/Brisbane/ 59/2007, A/Solomon Islands/3/2006, and B/Brisbane/60/2008 in that order in (D). White lines in the images of (D) indicate scale bars of 25 μm.

the H1N1pdm09 virus with a lack of cross-reactivity to other strains (Table 1). Also, mAb-D383 exhibited a high level of HAI activity, whereas mAb-I38 showed very weak HAI activity (Table 1). Similarly, mAb-D383 showed strong neutralizing activity against the H1N1pdm09 virus, while mAb-I38 did not neutralize the infection of H1N1pdm09 virus in MDCK cells (Table 2). These results imply that mAb-D383 interacts with a receptor binding domain or its vicinity on the influenza viral HA head, whereas mAb-I38 interacts with regions on HA irrelevant to the host receptor binding of influenza viral HA.

It was also observed with ELISA that the H1N1pdm09-specificity of mAb-D383 and -I38 is valid by showing that the two mAbs have specific reactivity to the H1N1pdm09 virus with a lack of reactivity to other strains (Fig. 1A and B). In addition, with IFA the two mAbs showed specific binding to MDCK cells infected with H1N1pdm09 virus and no cross-reactions with other influenza strains (Fig. 1D).

In the IFA, the mAbs (D383 and I38) were detected in the cell-membrane region of the MDCK cells infected with H1N1pdm09 virus, whereas the anti-NP antibodies were detected in the cytosol, including the nucleus (Fig. 1D). This implies that mAb-D383 and -I38 have HAI activity to the H1N1pdm09 virus and also interact with the transmembrane surface proteins (HA or NA) of the H1N1pdm09 virion (Table 1). In order to verify whether the two mAbs interact with the surface glycoprotein HAs of the influenza virion, an ELISA was performed with a coating of HA proteins of the H1N1pdm09 and 1918 H1N1 strains (Fig. 2A and C). The 1918 H1N1 strains are known to share common epitopes with H1N1pdm09 viral HA as an ancient strain of H1N1pdm09 (Igarashi *et al.*, 2010).

The results showed that mAb-D383 and -I38 exhibited reactivity to both the HAs of H1N1pdm09 (A/California/07/2009) and 1918 H1N1 (A/Brevig Mission/1/1918) (Fig. 2A and C). In detail, mAb-I38 showed stronger reactivity than mAb-D383 to the HA of H1N1pdm09 (A/California/07/2009) (Fig. 2A), which is consistent with mAb-I38 showing supe-

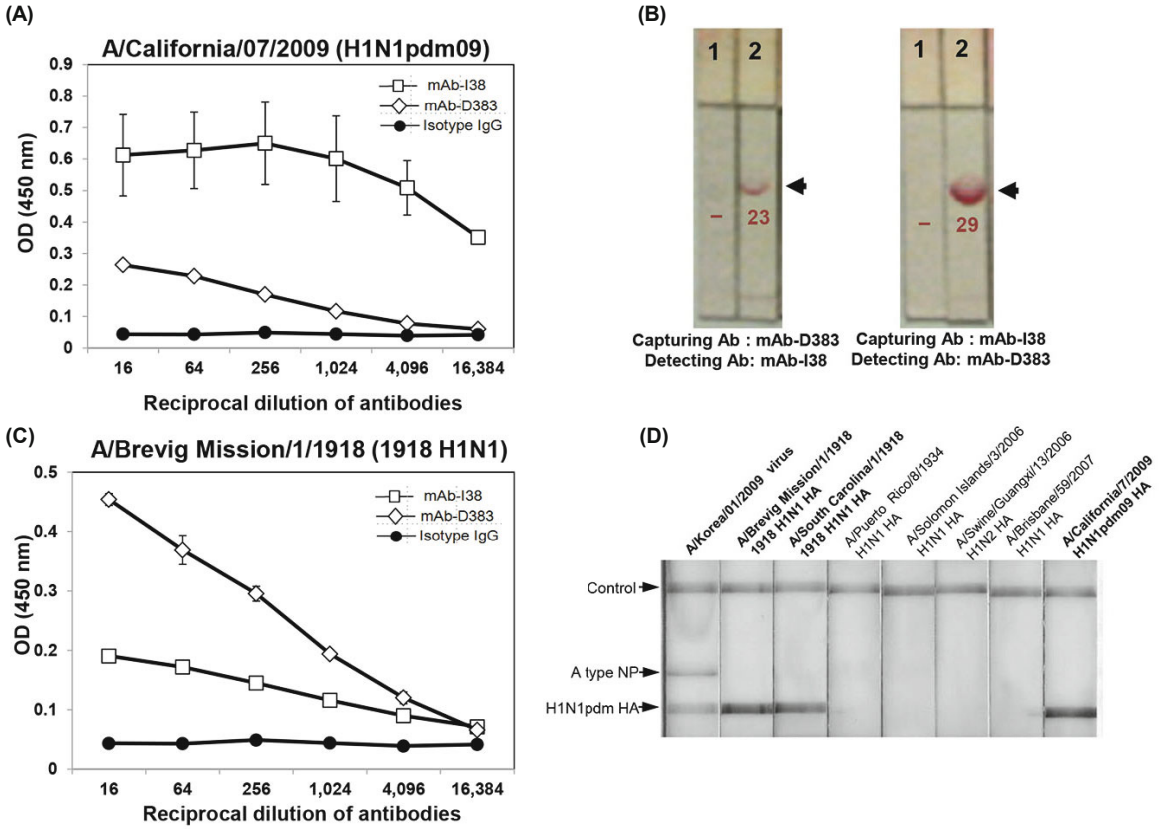

Fig. 2. The mAb-D383 and -I38 have reactivity to the HAs of the H1N1pdm09 and 1918 H1N1 viruses. For the ELISA assay in (A) and (C), recombinant HAs of A/California/07/2009 (H1N1pdm09) and A/Brevig Mission/1/1918 (1918 H1N1) were coated on the wells of 96 well ELISA plates and reacted with the two monoclonal antibodies. In (B), the immunochromatographic strips were tested with the indicated viruses (1: Newcastle disease, 2: H1N1pdm09 virus from the allantoic fluid of chicken embryos). In the left panel, mAb-D383 was immobilized on the test lines as a capturing antibody and mAb-I38 was used as a detecting antibody, conjugated with colloidal gold particles. In the right panel, mAb-I38 and mAb-D383 were used as the capturing and detecting antibodies respectively. Arrows indicate the test lines on the strip. The chroma of the bands in the test lines is proportional to the sensitivity to the indicated viruses. Under the test lines, purplish numbers are the evaluated values compared with the chroma index of the color. In (D), recombinant HAs (1.5 μg) of influenza viruses representing H1N1pdm09 and H1N1 were tested by the SDpdm RIDTs (SD Rapid Influenza Detection Tests), which use mAb-D383 and -I38 as the detecting and capturing antibodies respectively in their lines for detecting H1N1pdm HA. In addition, an H1N1pdm09 virus (A/Korea/01/2009) was applied to the first RIDT strip. The strains and influenza viral HAs shown in bold were detected by RIDT. Although none of the influenza viral HAs were detected in the NP (nucleoprotein) lines on the RIDT strips for detecting NP of all the influenza A subtypes, the A/Korea/01/2009 virus was detected in the NP line because NP exists in the influenza virus and not in the recombinant HA protein.

rior reactivity than mAb-D383 to the H1N1pdm09 viral particle in the ELISA (Fig. 1C). Although ELISAs may provide important information about the binding of mAbs to their antigens, ELISA titers may not reflect true binding constants (García-Ojeda *et al.*, 2004; Rich and Myszka, 2004).

SPR technology can assess real-time binding and allows kinetic as well as equilibrium and affinity measurements about interaction between antibodies and antigens (García-Ojeda *et al.*, 2004; Rich and Myszka, 2004); thus, SPR was used to determine the binding of the mAbs (D383 and I38) to recombinant HAs of H1N1pdm09 and 1918 H1N1 (Table 3). The two mAbs (D383 and I38) bound to recombinant HA of H1N1pdm09 with nanomolar affinity (3.80 ± 0.03 nM and 5.86 ± 0.03 nM, respectively) (Table 3). Meanwhile, the K_D of mAb-D383 binding affinity to HA of 1918 H1N1 was 0.568 ± 0.001 nM, about six times stronger than that of mAb-I38 (3.6 ± 0.1 nM) (Table 3). Taken together, the SPR results show that mAb-D383 and -I38 have similar affinity at the nanomolar level to the HA protein of H1N1pdm09, whereas mAb-D383 has notably stronger affinity than mAb-I38 to the HA of 1918 H1N1.

Immunochromatography was also used to investigate whether a combination of the two mAbs (D383 and I38) as detection and capture antibodies showed increased sensitivity to the H1N1pdm09 virus (Fig. 2B). The immunochromatographic strips show that the use of mAb-I38 and -D383 as capture and detection antibodies respectively (Fig. 2B, right panel) enhanced sensitivity to the H1N1pdm09 virus compared with the use of mAb-I38 and -D383 as detection and capture antibodies respectively (Fig. 2B, left panel).

In addition, mAb-D383 and -I38 exhibited reactivity to the HA of 1918 H1N1 as well as to the HA of H1N1pdm09 (Fig. 2C) even though mAb-D383 and -I38 were generated from mice immunized by an H1N1pdm09 viral strain (A/Korea/01/2009). This implies that the SDpdm RIDT using mAb-I38 and -D383 on the test strip may be able to detect 1918 H1N1 viral strains. To confirm whether or not the SDpdm RIDT is able to detect the 1918 H1N1 viruses, the

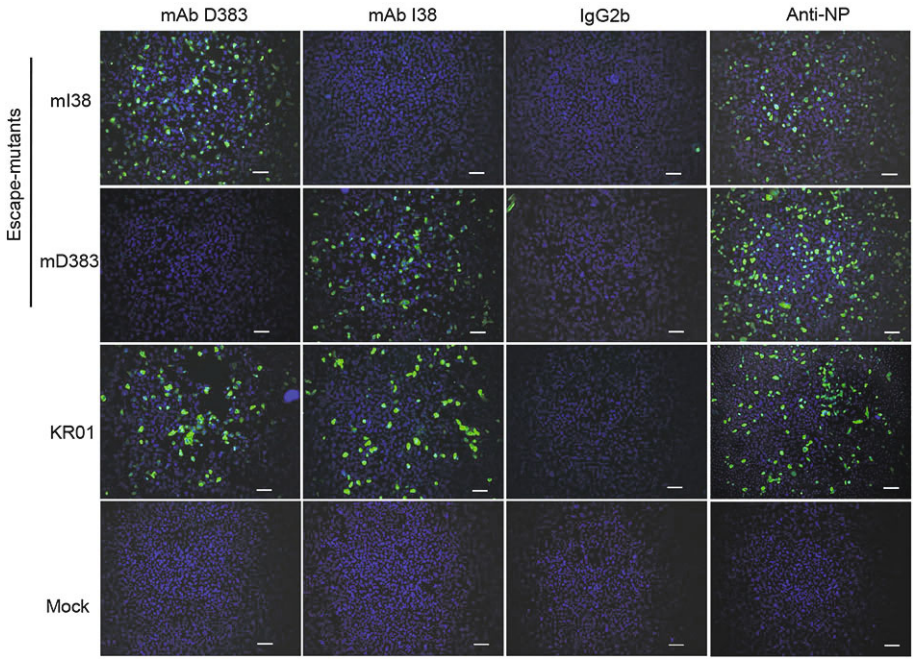

Fig. 3. Reactivity of the mAbs (I38 and D383) to escape-mutant viruses generated by the mAbs as measured by immunofluorescence staining. MDCK cells were mock infected or infected with the indicated escape-mutants or non-mutant viruses. The dilutes of mAb-I38 or -D383 or anti-NP Ab (as a control of influenza viral infection) were used for primary staining with consequent secondary anti-mouse IgG-FITC. All images of the panels in the figure were taken under identical exposure time, condition, and magnitude (100X) by fluorescent microscopes. Blue-colored spots in background indicate the nucleus of MDCK cells stained by DAPI and green colored cells indicate FITC-labeled anti-mouse IgG. The influenza strains depicted in the left side of the figure are escape-mutant viruses (mI38 and mD383) and a non-escape mutant virus (KR01) generated in our previous study (Yi *et al.*, 2013). The mI38 and mD383 contain G56E and G172E/P176Q escape-mutations from mAb-I38 and mAb-D383 respectively on their HAs. Mock means non-viral infection. White scale bars are 200 μm.

RIDT was tested with recombinant HA of 1918 H1N1 viral strains (A/Brevig Mission/1/1918 and A/South Carolina/1/1918) (Fig. 2D). The result showed that the RIDT is able to detect the HAs of 1918 H1N1 viral strains, while the HAs of other H1N1 strains were not detected by the RIDT (Fig. 2D).

In our previous study, escape mutant viruses were generated by cultivating H1N1pdm09 viruses under excess amounts of mAbs (D383 and I38) to investigate quasi-species poorly recognized by the RIDT using the two mAbs. In this study, MDCK cells were infected with the escape-mutant viruses or their mother strain to investigate whether mAb-D383 and -I38 are able to recognize in a more relevant system their escape-mutant viruses (which have escape mutations, G172E and P176Q for mAb-D383 and G56E for mAb-I38, on their HAs). IFA results indicate that mAb-D383 and -I38 failed to recognize MDCK cells infected with their escape-mutant viruses, whereas the mAbs were capable of binding to the mother strain KR01 (H1N1pdm09-like) virus (Fig. 3).

In addition, the amino acid residues [the escape-mutation sites: G172, P176, and K180 for mAb-D383; G56 for mAb-I38 (Yi *et al.*, 2013)] recognized by mAb-D383 and -I38 were also mapped directly to the HA structures of H1N1 viral strains (Fig. 4A and B). The results demonstrate that the residues recognized by mAb-D383 and -I38 are located respectively in the Sa antigenic site on the top of the HA1 globular structure (head domain) and in the F' subdomain on the sides of the H1N1pdm09 viral HA trimmer (Fig. 4A). Meanwhile, to investigate in depth the structures of these epitope sites interacting with mAb-D383 and -I38, close-up views in 3D structures of the HA molecules of human H1N1 viral strains [1918 H1N1, 1934 H1N1, 2007 H1N1, and 2009 H1N1 (H1N1-pdm09)] were compared by mapping the escape-mutations and all the amino acid residues in the vicinity of the escape-mutation sites as the epitopes for mAb-D383 and -I38 (Fig. 4B). It was shown (upper panels of Fig. 4B) that all the escape mutation sites (172, 176, and 180 amino acid residues) recognized by mAb-D383 belong completely to homologous Sa antigenic sites of 1918 H1N1, 1934 H1N1, 2007 H1N1 and 2009 H1N1(H1N1pdm09). On the other hand, the escape mutation site (G56 residue) interacting with mAb-I38 is surrounded by amino acid residues (54, 55, 57, and 300) of F' subdomains and by amino acid residues (277, 278, and 290) of the VE subdomain on the HAs of human H1N1 viral strains (Fig. 4B, lower panels). This result implies that the epitope recognized by mAb-I38 is not located entirely in the F' subdomain, but rather it is in the interface area (F'-VE) between the F' subdomain and VE subdomain (Figs. 4B and 5, lower panels).

In the linear schematic view of influenza viral HA (Fig. 4C), the expected epitope region (corresponding to the F'-VE area in the lower panels of Fig. 4B) recognized by mAb-I38 exists separately in two F' subdomains (N-terminal and C-terminal) and the VE subdomain, while the Sa antigenic site recognized by mAb-D383 is located within the receptor binding subdomain (RB). Also, both the residues of the Sa antigenic site and F'-VE area interacting with the two respective mAbs are not continuous on the HA linear structure (Fig. 4C), which means that the epitopes recognized by mAb-D383 and -I38 are not linear but rather discontinuous and conformation-dependent. This is supported by the amino acid residues of both the Sa antigenic site and the F'-VE area being placed closely on the surface of the HA 3D-structure (Fig. 4B).

In the close-up views, it was revealed that these sites (escape-mutations and their vicinity on 3D-structures of HAs as epitopes recognized by mAb-D383 and -I38) of 2009 H1N1 viral HA were highly conserved with those of 1918 H1N1 viral HA (Fig. 4B). By contrast, amino acid residues at these positions were predominantly altered in 1934 H1N1 and 2007 H1N1 viral HAs (Fig. 4B) compared with those of 1918 H1N1 and 2009 H1N1 viral HAs. Also, the amino acid sequences corresponding to the residues of the HA close-up views shown

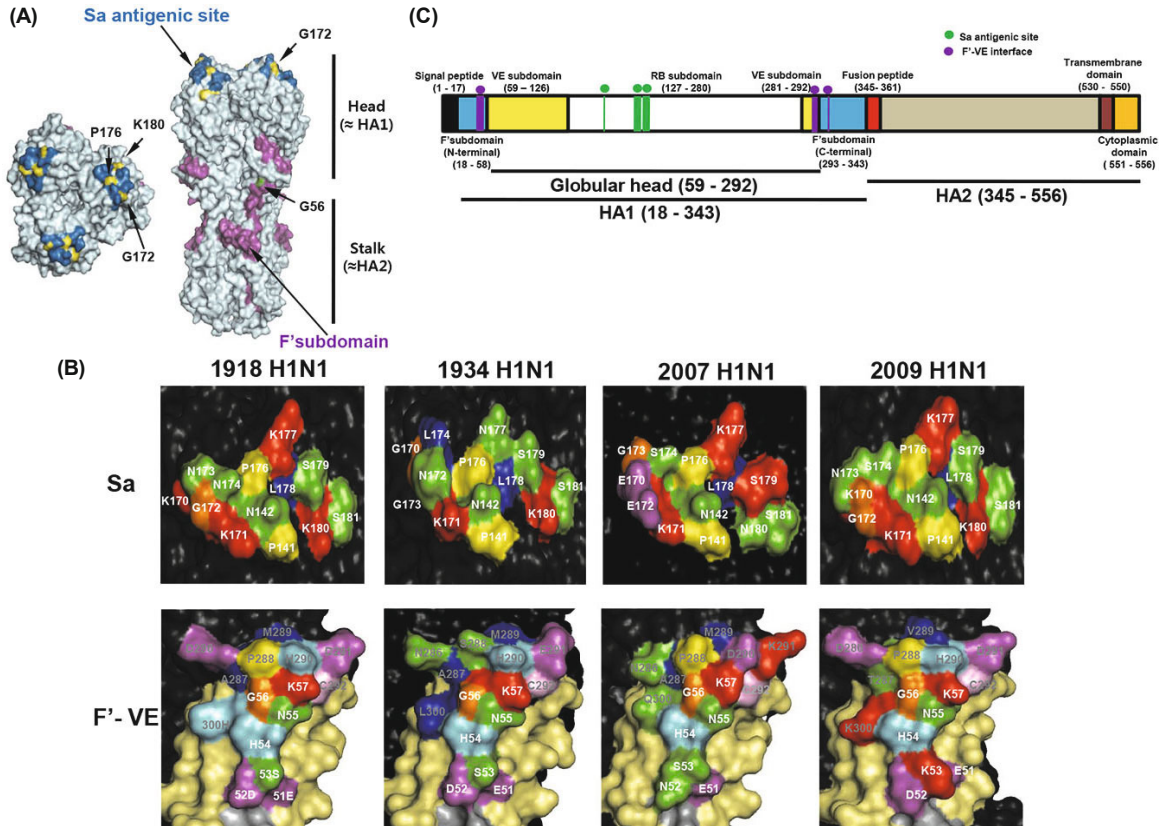

Fig. 4. Epitopes for mAb-I38 and -D383 are mapped on the HA structures of human influenza viral H1N1 strains. (A) The Sa antigenic sites and F' subdomains (N-terminal of HA1) in the tri-structure of H1N1pdm09 HA are colored blue and purple respectively. In the blue Sa antigenic sites, G172E, P176Q, and K180E mutation sites that were elicited as escape-mutation sites to the mAb-D383 in our previous study (Yi *et al.*, 2013) are highlighted in yellow. In the purple F' subdomains, the G56E mutation site that was elicited as an escape-mutation site from mAb-I38 is marked in green. The right and left figures of A are top- and side-views of the HA tri-structure. (B) Each amino acid residue of the Sa antigenic sites (which contain the escape-mutations from mAb-D383) and the interface area (F'-VE) between the F' and vestigial esterase (VE) subdomains (which contain the escape mutant from mAb-I38) is mapped on the close-up views of 1918 H1N1, 1934 H1N1, 2007 H1N1, and 2009 H1N1(H1N1pdm09) HAs. HA structures of 1918 H1N1(A/South Carolina/1/1918), 1934 H1N1(A/Puerto Rico/8/1934), and 2009 H1N1(A/California/4/2009) were obtained from the Protein Data Bank (accession numbers 1RUZ, IRU7, and 3LZG respectively). HA structure of 2007 H1N1 (A/Brisbane/59/2007) constructed by homology modeling based on H1 HA sequences was obtained from a previous report (Igarashi *et al.*, 2010). The solvent-accessible surface images of influenza viral HAs were created with PyMOL 0.99 with the HA structures. Amino acids are colored by the default ClustalX color scheme (Igarashi *et al.*, 2010): Trp, Leu, Val, Ile, Met, Phe, and Ala (blue); Thr, Ser, Asn, and Gln (green); Lys and Arg (red); Asp and Glu (magenta); His and Tyr (cyan); Cys (pink); Gly (orange); Pro (yellow). In the panels for F'-VE, the light yellow and gray colored in backgrounds denote VE and F' subdomains respectively. (C) Schematic view of the influenza viral HA protein to show the Sa antigenic site and the interface between the F' subdomain and VE subdomain recognized by mAb-D383 and -I38. The globular head consists of the receptor-binding (RB) subdomain (white) and the VE subdomain (yellow). The F' subdomain in HA1 is colored blue and the HA2 stalk domain is colored gray. The locations of the Sa antigenic site recognized by mAb-D383 are highlighted in green on the RB subdomain. The locations recognized by mAb-I38 are highlighted in magenta on the F' and VE subdomains.

in Fig. 4B are presented in Fig. 5 to show any similarities between the amino acid sequences of the residues of 1918 H1N1 and individual H1N1 viral HAs. This reveals that the residues of 2009 H1N1 (H1N1pdm09) have the highest similarity with the residues of 1918 H1N1 HA (Fig. 5). Collectively, these results suggest that the Sa antigenic site and the F'-VE region recognized by mAb-D383 and -I38 are conserved epitopes between 1918 H1N1 and 2009 H1H1 viral HAs.

Discussion

Since the 2009 pandemic there have been several other studies to develop mAbs specific to H1N1pdm09 as well as those studies for SDpdmRIDT. Mizuike et al. developed an RIDT containing two mAbs specific to the HA and NP of H1N1pdm09 for the detection of H1N1pdm09 (Mizuike *et al.*, 2011). Samuel *et al.* (2011) generated mAbs to the HA1 of the H1N1pdm09 viral HA for the serodiagnosis of H1N1-pdm09 infections. Using an anti-NP mAb generated by the hybridoma technique with the immunization of NP that originated from the H5N1 virus, an immunochromatographic assay specific to the H1N1pdm09 virus was generated by another group (Miyoshi-Akiyama *et al.*, 2010). Most of the mAbs in these studies are insufficiently characterized except for their specificities to the H1N1pdm09 virion by HAI, ELISA, or Western blot.

An SDpdm RIDT that used both mAb-D383 and -I38 exhibited 100% specificity to H1N1pdm09 viral infections compared with real-time reverse transcription-PCR (Choi *et al.*,

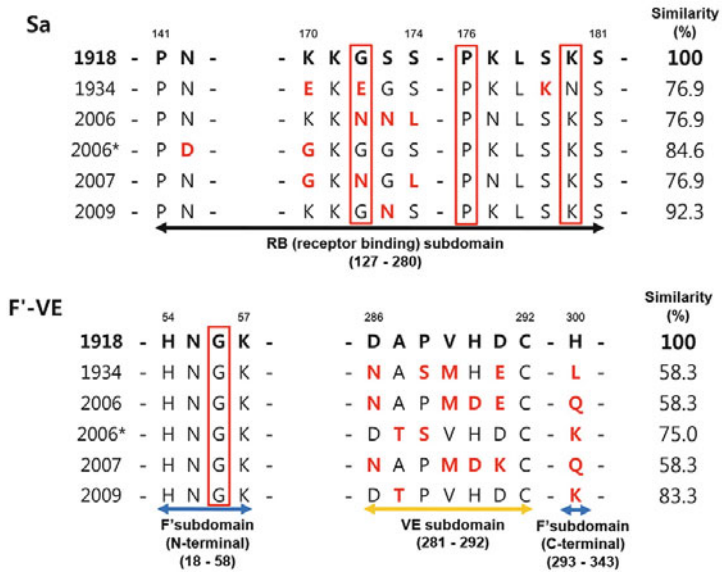

Fig. 5. Comparison of the amino acid residues on the Sa antigenic sites and the interface of the F'-VE subdomains of human H1N1 influenza viral HA. Amino acid sequences of the Sa antigenic site (Sa) and interface (F'-VE) between the F' subdomain and VE subdomain on HAs of human H1N1 viruses are shown. Sequence data correspond to those of virus strains shown in Figs. 2D and 4B. Amino acid residues different from 1918 are shown in red. The individual similarity indicates the matched percentage of amino acids on Sa and F'-VE between 1918 and indicated H1N1 viral strains. The escape-mutation sites (172, 176, and 180 residues) from mAb-D383 recognized in the Sa antigenic site are highlighted in red boxes. The escape-mutation site (56 residue) recognized by mAb-I38 is highlighted in the red box on the F' and VE subdomains.

2010). In another cohort, an RIDT with both mAbs showed specificities of 98.4% and 97.6% to specimens infected by the H1N1pdm09 virus compared with rRT-PCR and viral culture respectively (Kwon *et al.*, 2011). These results provide support to the idea that mAb-D383 and -I38 in an RIDT have reliable specificity and reactivity to H1N1pdm09 viral strains. Under this milieu, after confirming the specific HAI titer of the two mAbs to the H1N1pmd09 virus (Table 1), IgG-isotyping in this study identified the two mAbs (I38 and D383) as belonging to the IgG2b heavy and Kappa light chain classes (footnote in Table 1).

A human mAb (hmAb, EM4C04) highly specific to the H1N1pdm09 virus was isolated from a patient infected with the H1N1pdm09 virus in another study (O'Donnell *et al.*, 2012). One of the escape-mutants generated under immune pressure with the hmAb (EM4C04) has the residue G172E (G158E in H3 numbering) mutation on HA1 of H1N1pdm09. Also, escape-mutants by immunological pressure with mAb-31C2 generated from a mouse infected with a H1N1pdm09 strain (A/California/09/2009) have G172E substitutions on their Sa sites (O'Donnell *et al.*, 2012). Thus mAb31C2 was used as a site Sa HA-specific antibody, detecting the Sa antigenic site of HA (Medina *et al.*, 2010). Rudneva *et al.* (2012) also generated H1N1pdm09 specific mAbs (5F7 and 6A3), and it was demonstrated that escape-mutants generated by immune pressure of the mAbs have HA G172E substitution. As previously mentioned, immunological pressure by our mAb-D383 also generated HA G172E escape- mutation on the HA of the H1N1pdm09 virus (Yi *et al.*, 2013). Reverse-genetics experiments showed that a single G172E HA on the Sa site of HA was sufficient to escape from sera isolated from ferrets infected with an H1N1pdm09 strain (A/California/07/2009). This indicates that the Sa site on HA is immunodominant and an important element of the immunological properties of the H1N1pdm09 virus.

Meanwhile, the results of in this study (Figs. 3 and 4) corroborate the results of our previous study (Yi *et al.*, 2013) that the residue 56 and its vicinity on the F' subdomain and VE subdomain of HA is an epitope recognized by mAb-I38. Even though the F' subdomain, including residue 56 of HA, belongs to HA1 (Fig. 4C) (Gamblin *et al.*, 2004), it is geographically closer to the middle section of HA2 (which has the function of membrane-fusion) than to the membrane-distal globular domain of HA1 (Fig. 4A and B); and this is thought to be because the F' subdomain of HA originated evolutionary from a part having a membrane-fusion function in an ancient influenza virus (Gamblin *et al.*, 2004; Stevens *et al.*, 2004). This supports the finding that mAb-I38 showed trivial HAI and no neutralizing titers to the H1N1pdm09 virus in physiological concentrations. No other groups have reported residue 56 and its vicinity on the F' subdomain (18 to 58 residues of N-terminal and 300 residues of C-terminal on HA1) and the VE subdomain (281 to 292 residues on HA1) close to the F' subdomain (293 to 343 residues in HA1

Table 3. Binding affinity of mAb-D383 and -I38 to HAs of H1N1pdm09 and 1918 H1N1

HA protein[a]	Antibody	k_{on} ($M^{-1}\cdot s^{-1} \times 10^6$)	k_{off} ($s^{-1} \times 10^{-3}$)	K_D (nM)
H1N1pdm09 HA (A/California/07/2009)	mAb-D383	1.100 ± 0.008	4.18 ± 0.03	3.80 ± 0.03
	mAb-I38	0.588 ± 0.003	3.45 ± 0.02	5.86 ± 0.03
1918 H1N1 HA (A/Brevig Mission/1/1918)	mAb-D383	1.919 ± 0.005	1.089 ± 0.002	0.568 ± 0.001
	mAb-I38	0.85 ± 0.03	3.05 ± 0.02	3.6 ± 0.1

Kinetic parameters for binding of mAb-D383 and -I38 to recombinant HAs of H1N1pdm09 and H1N1 strains by SPR.
[a] The two HA proteins were covalently coupled with carboxyl groups on the gold surfaces of two separate sensor chips. Then, interaction kinetics of the two mAbs (D383 and I38) to the individual HA proteins were measured separately in the two chips; thus, the kinetic parameters and K_D values of the mAb-D383 and -I38 to the HA proteins should be compared separately in individual HA proteins. In other words, it may be inappropriate to compare the kinetic and K_D values of the mAb-D383 and -I38 to HA protein of H1N1pdm09 with the values of the two mAbs to HA protein of 1918 H1N1.

Table 4. Recombinant influenza hemagglutinin proteins used in ELISA, RIDT, and SPR

Strain (subtype) [a]	Assay(s)	Comment
A/Brevig Mission/1/1918 (H1N1)	ELISA[b], RIDT[c], SPR[d]	1918 H1N1 strain
A/South Carolina/1/1918 (H1N1)	ELISA, RIDT	1918 H1N1 strain
A/Puerto Rico/8/1934 (H1N1)	RIDT	-
A/Solomon Islands/3/2006 (H1N1)	RIDT	-
A/Swine/Guangxi/13/2006 (H1N2)	RIDT	-
A/Brisbane/59/2007 (H1N1)	RIDT	-
A/California/7/2009 (H1N1)	RIDT, SPR	H1N1pdm09 strain

[a] All recombinant HA proteins of the indicated influenza viral strains are expressed in baculovirus-insect cells.
[b] See Fig. 2A and C.
[c] See Fig. 2D.
[d] See Table 3.

N-terminal) (Fig. 4C) as an epitope of a mAb specific to the HA of influenza virions, and it would be useful to study the roles of the F' subdomain and its vicinity with the mAb-I38 targeting the interface area of the F' subdomain and VE subdomain on influenza HA.

It has been reported that the H1N1pdm09 virus exhibits a HA sequence and structural homology with the 1918 H1N1 virus, which caused the most devastating pandemic in the 20th century, killing approximately 50 million people worldwide in 1918–1919 (Taubenberger and Morens, 2006). Supportively, it was shown that mice vaccinated with 1918 H1N1-like strains elicited protective antibodies against H1N1pdm09 viral challenge and conversely the animals immunized with H1N1pdm09 virus generated cross protective antibodies against 1918 H1N1 or H1N1pdm09 viral infection (Manicassamy *et al.*, 2010; Medina *et al.*, 2010). Thus, the cross reactivity of our mAb-D383 and -I38 against 1918 H1N1 viral strains was investigated and the results of ELISA showed that both mAb-D383 and -I38 are able to interact with the HA of 1918 H1N1 as well as the HA of H1N1pdm09 (Fig. 2A and C). In addition, it was demonstrated that the Sa antigenic site and interface area (F'-VE) of F' and VE subdomains targeted by mAb-D383 and -I38 are relatively conserved between 1918 H1N1 and 2009 H1N1(H1N1pdm09) HAs compared with other human H1N1 influenza viral HAs (Figs. 4B and 5). Intriguingly, mAb-D383 exhibited stronger affinity than mAb-I38 to the HA of 1918 H1N1 (Fig. 2C and Table 3). Meanwhile, mAb-I38 showed similar or slightly less affinity than mAb-D383 to the HA of H1N1pdm09 (Table 3).

We considered why mAb-D383 has stronger affinity than mAb-I38 to the HA of 1918 H1N1 (Fig. 2C and Table 3). The sequence similarity of amino acids residues between the homologous Sa antigenic sites (as a common epitope recognized by mAb-D383) of both H1N1pdm09 and 1918 H1N1 is 92.3% (Fig. 5, upper panel) (Igarashi *et al.*, 2010). On the other hand, the sequence similarity between the F'-VE areas (as a common epitope recognized by mAb-I38) of both H1N1pdm09 and 1918 H1N1 is 83.1% (Fig. 5, lower phanel). This indicates that the similarity of homologous epitopes in the Sa antigenic site (between both 2009 H1N1 and 1918 H1N1 HAs) targeted by mAb-D383 is rather higher than the similarity of the epitope (F'-VE area) recognized by mAb-I38 from the aspect of sequence homologue of amino acid residues on the Sa site and F'-VE area. From these observations, it is speculated that the spatial tertiary structural similarity between the Sa antigenic sites (recognized by mAb-D383) of HAs of H1N1pdm09 and 1918 H1N1 is relatively higher than the similarity of the F'-VE areas (recognized by mAb-I38) between H1N1pdm09 and 1918 H1N1. Collectively, this might explain why the mAb-D383 targeting the Sa site showed stronger affinity and reactivity to the HA of 1918 H1N1 than that exhibited by mAb-I38 in the SPR and ELISA experiments (Fig. 2C and Table 3).

From the results it was observed also (Fig. 2D) that the SDpdm RIDT could detect HAs of both H1N1pdm09 and 1918 H1N1 strains (recombinant HAs of the H1N1s were tested instead of influenza viruses because the 1918 H1N1 viral strains are not available to us), but it could not detect the HAs of other H1N1 strains prevalent in 2006 and 2007. This result is coincident with the epitopes in the Sa site and F'-VE area recognized by mAb-D383 and -I38 being relatively well conserved between the 1918 H1N1 and 2009 H1H1 HAs compared with other H1N1 HAs (Fig. 4B). This is the first report that an RIDT specific to H1N1pdm09 also detects 1918 H1N1-like strains.

In summary, it was demonstrated that mAb-D383 and -I38 used in the SDpdm RIDT can be used for the specific detection of the H1N1pdm09 virus through ELISA and H1N1-pdm09 viral infected cells through IFA. In addition, it was found that an RIDT using mAbs (I38 and D383) generated for the specific detection of H1N1pdm09 possibly detects 1918 H1N1-like strains. Further characterization of the mAbs with their escape-mutants elucidated that mAb-D383 and -I38 recognize the conserved epitopes (the Sa antigenic site and the interface area of F' subdomain and VE subdomain, individually, on HAs of H1N1pdm09 and 1918 H1N1). This suggests that the immunological characterization of mAbs generated for the development of RIDTs would lead to useful and meaningful information for the study and characterization of influenza viral strains.

Acknowledgements

We thank Mi Jung Ji, Standard Diagnostics Inc., Yongin, Republic of Korea, for performing the immunochromatography and providing mAb-D383 and -I38. This work was supported by intramural funds (#2012-N43003-00 and #4834-303-210-13) from the Korea National Institute of Health and the Korea Center for Disease Control and Prevention.

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