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Short communication

Characterization of a fully human monoclonal antibody against extracellular domain of matrix protein 2 of influenza A virus

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ABSTRACT

The extra-cellular domain of the influenza virus matrix protein 2 (M2e) is highly conserved between influenza A virus strains compared to hemagglutinin and neuraminidase, and has long been viewed as a potential and universal vaccine target. M2e induces no or only weak and transient immune responses following infection, making it difficult to detect M2e-specific antibodies producing B-cells in human peripheral blood lymphocytes. Recently, using a single-cell manipulation method, immunospot array assay on a chip (ISAAC), we obtained an M2e-specific human antibody (Ab1-10) from the peripheral blood of a healthy volunteer. In this report, we have demonstrate that Ab1-10 reacted not only to seasonal influenza A viruses, but also to pandemic (H1N1) 2009 virus (2009 H1N1) and highly pathogenic avian influenza A virus, and that the antibody-bound M2e of 2009 H1N1 inactivated the virus with high affinity ($\sim 10^{-10}$ M). More importantly, it inhibited 2009 H1N1 viral propagation *in vitro*. These results suggest that Ab1-10 might be a potential candidate for antibody therapeutics for a wide range of influenza A viruses.

Matrix protein 2 (M2) is a 97 amino acid-long transmembrane protein in influenza A viruses, and forms homotetramers (Holsinger and Lamb, 1991; Lamb et al., 1985; Sugrue and Hay, 1991). The M2 is a good candidate for a universal vaccine against a much wider range of influenza A viruses than does the current vaccine based on the following reasons: (1) The extracellular domain of M2 (M2e) is remarkably conserved among human influenza A virus strains (Feng et al., 2006; Ito et al., 1991; Zebedee and Lamb, 1989), (2) A murine M2e-specific monoclonal antibody (mAb) was proven to reduce viral replication *in vitro* (Gabbard et al., 2009; Hughey et al., 1995; Zebedee and Lamb, 1988), (3) Immunization with M2e peptide or administration of M2e-specific mAbs provided protection against influenza A virus challenges in mice, ferrets, and rhesus monkeys models (Beerli et al., 2009; De Filette et al., 2008; Fan et al., 2004; Frace et al., 1999; Fu et al., 2009; Liu and Chen, 2005; Mozdzanowska et al., 2003; Neirynck et al., 1999; Okuda et al., 2001). However, it is difficult to identify M2e-specific antibody-producing B-cells in human peripheral blood lymphocytes since the M2e-specific humoral immune response is generally very low or absent (Black et al., 1993; Feng et al., 2006).

Previously, we reported a novel screening method, immunospot array assay on a chip (ISAAC), which detects human antigen (Ag)-specific B-cells in peripheral blood using a microwell array chip (Jin et al., 2009). We applied this method to obtain many human mAbs reacting to influenza viruses from vaccinated volunteers and characterized their functions. Interestingly, one of them (Ab1-10) specifically reacted to a 23 amino acid-long M2e peptide (Jin et al., 2009). In this study, we characterized the neutralization activity of Abl-10 M2e-specific mAb. To this end, we co-transfected chinese hamster ovary (CHO) cells with both the heavy and light chain expression vectors encoding the whole antibody molecules using the FreeStyle™ MAX CHO Expression System (Invitrogen), collected the supernatant of cultured cells, and then purified the antibody using a protein G column, as previously described (Jin et al., 2009).

We first performed Western blot analysis to examine the specificity of Ab1-10 against M2 of various influenza virus strains including inactivated influenza virus antigen A/NewCaledonia/20/99(H1N1), A/Panama/2007/99 (H3N2), and B/Tokio/53/99 strains (purchased from HyTest Ltd.) and A/Vietnam/1194/04 (H5N1 avian influenza virus) and A/California/7/2009 (2009 H1N1 pandemic influenza virus) strains (purchased from the National Institute for

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Biological Standards and Control). Proteins of approximately 16 and 26 kDa were detected not only from seasonal influenza A viruses (H1N1 and H3N2) but also from H5N1 avian influenza virus strain and 2009 H1N1 pandemic influenza virus strain (Fig. 1A). They were not detected from influenza B virus. To confirm the result in detail, we used M1- and M2-specific mAbs (purchased from Funakoshi and Affinity BioReagents, respectively) for Western blot analysis. M1 and M2-specific mAbs bound to the proteins of approximately 26 kDa and 16 kDa, respectively (Fig. 1B). Since M1 and M2 proteins share the first eight amino acids, excluding methionine (Lamb et al., 1981), we examined the binding activity of Ab1-10 for the first eight amino acid peptides (M1M2e common peptide; SLLTEVETK-biotin, Operon Biotechnologies) as well as M2e peptide (SLLTEVETPIRNEWGCRCNDSSDK-biotin, Operon Biotechnologies) by ELISA using streptavidin-precoated plates (Nunc). Ab1-10 specifically bound to the M1M2e common peptide as well as M2e peptide in a dose-dependent manner (Fig. 1C). These results showed that Ab1-10 bound to the first eight amino acids of M2e that are shared with the M1 protein and are conserved in various virus strains.

In order to analyze the epitope of Ab1-10 in detail, we constructed M2 variants with substitution of amino acid residues with alanine in M2e portion (Fig. 2A) and transfected their expression vectors to CHO cells. Two days after transfection, the cells were stained with 1 μg/ml of Ab1-10, 14C2, or irrelevant IgG, and phycoerythrin-conjugated anti-IgG antibodies. Thereafter, the cells were analyzed with a FACSCanto flow cytometer. Fig. 2B showed that the Ab1-10 reacted to L4A, V7A, E8A, and T9A M2 variants as well as consensus M2 as strongly as control 14C2 antibody. Ab1-10 also reacted to S2A, L3A variants, but more weakly than 14C2 control antibody. In contrast, Ab1-10 did not react to T5A variant, but 14C2 did strongly. Interestingly, Ab1-10 reacted to E6A variant, and M2 of H5N1 or 2009 H1N1 more strongly than14C2 (Fig. 2B). Consequently, in consensus M2e (SLLTEVET), SL*T**** is a pre-requisite for the binding of Ab1-10 to M2e. Feng et al. compared the M2e sequences of 1505 human isolates (records in public data banks on August 17, 2006) and showed that first eight amino acids were conserved approximately 100% among recent viruses (Feng et al., 2006), including A/Vietnam/1194/04(H5N1) and A/California/7/2009(H1N1). Since Ab1-10 bound to this consensus sequence, Ab1-10 has the potential to protect against a wide range of influenza A viruses. In contrast, the epitope of 14C2 has been shown as EVETPIRNEW in M2e (Wang et al., 2008), which does not correspond to the M2e of the recent viruses including H5N1 and 2009 H1N1, resulting in the weak binding of 14C2 to H5N1 and 2009 H1N1 (Fig. 2).

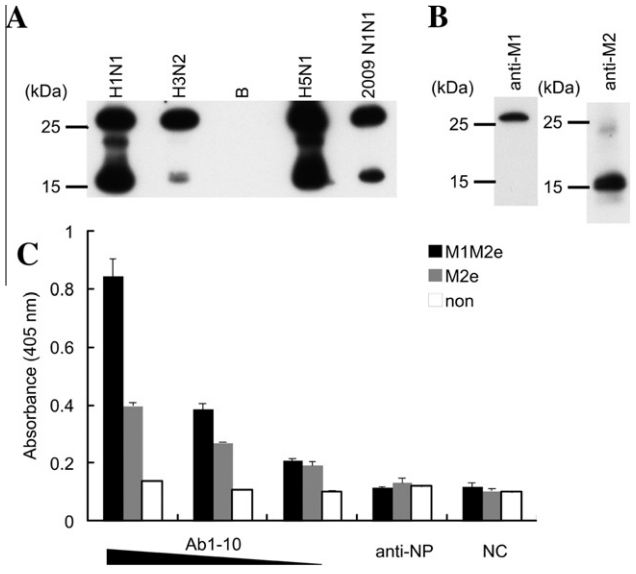

Fig. 1. Characterization of antigen for Ab1-10. (A) Examination of antigenic peptides for Ab1-10 in various inactivated viruses. Approximately 0.3–0.6 μg of inactivated viruses (H1N1, A/NewCaledonia/20/99; H3N2, A/Panama/2007/99; B, B/Tokio/53/99; H5N1, A/Vietnam/1194/04; and 2009 H1N1, A/California/7/2009(H1N1)) were separated on SDS–PAGE under reducing conditions and the binding of antigenic proteins with Ab1-10 was detected with Western blotting. (B) Detection of 26 and 16 kDa proteins with M1- and M2-specific mAb, respectively. Approximately 0.3 μg of inactivated virus (H1N1, A/NewCaledonia/20/99) was separated on SDS–PAGE under reducing conditions and the binding of antigenic proteins with M1 or M2-specific mAb was examined by Western blotting. (C) Reactivity of Ab1-10 to M1M2e common peptide or full-length M2e peptide. Streptavidin-precoated plates were coated with the peptides. ELISA was used to analyze the binding of Ab1-10 to the plates coated with M1M2e common peptide (closed column), M2e peptide (gray column), or without peptide (open column). Various doses (12.5, 6.3, and 3.1 μg/ml) of Ab1-10 were added. Anti-NP indicates influenza nucleoprotein-specific antibody. NC indicates negative control. Data represent means ± SD and are a representative of two independent experiments with similar results.

Next, to analyze the affinity of Ab1-10, we measured the binding of Ab1-10 to human influenza A virus and M2e peptide by surface plasmon resonance (SPR) using a BIAcore 2000 instrument (BIAcore AB). To this end, we coated CM5 sensor chips (GE Healthcare) with virus antigen (2009 H1N1 pandemic and seasonal influenza virus strains) inactivated by β-propiolactone and SA sensor chips (GE Healthcare) coated with M2e peptide according to the Manufacturer's instructions. We then injected 300, 150, 72.5, 37.25, 18.75, or 0 nM Ab1-10 antibody and measured the K_D (Table 1). The K_D of Ab1-10 to 2009 H1N1 pandemic and seasonal strains were calculated at approximately 5.8×10^{-10} M and 2.7×10^{-10} M, respectively. In contrast, the K_D of M2e peptide was calculated at approximately 2.3×10^{-7} M. An irrelevant human monoclonal antibody was not bound to these antigens ($K_D > 10^{-6}$ M). Why was the affinity of Ab1-10 to whole viruses approximately 400- to 1000-fold higher than that for M2e peptides? One possibility is that the β-propiolactone that was used for the inactivation of virus antigens modified the protein structure and affected the binding of antibodies. β-propiolactone is highly reactive to a wide variety of substances and can severely affect the protein structure. However, we used these antigens in ELISA, Western blotting, and ISAAC analyses, and detected good signals. Thus we think that these inactivated antigens keep the native structure of Ab-binding epitopes. Another and more probable possibility is that the conformational difference of antibody epitopes between M2e peptide and that on virus surface affected the binding of Ab1-10. In this context, Fu et al. showed that protective M2e-specific monoclonal antibodies preferentially bind to M2 multimers composed of two or more M2 peptides in parallel orientation (Fu et al., 2009). De Filette et al. showed the importance of the tetrameric configuration of M2 for inducing protection (De Filette et al., 2008). It has been reported that the M2 is expressed as a tetramer on the virus surface (Holsinger and Lamb, 1991). Taken together, Ab1-10 might preferentially bind to M2e epitopes formed by tetrameric M2 expressed on the virus surface compared to monomeric M2e peptides.

M2e-specific mAbs have previously been demonstrated to inhibit influenza virus replication in an *in vitro* plaque-reduction assay (Gabbard et al., 2009; Hughey et al., 1995; Zebedee and Lamb, 1988). We therefore investigated whether Ab1-10 inhibited viral replication in an *in vitro* plaque reduction assay. Madin-Darby canine kidney (MDCK) cells were infected with approximately 150 pfu of A/PuertoRico/8/1934(H1N1) (PR8) virus that were pretreated without or with 5 μg/ml Ab1-10 or influenza nucleoprotein-specific antibody (anti-NP). Then, the MDCK cells were overlaid with MEM medium containing 0.8% agarose, 0.2% BSA, 0.1% glucose, 0.01% DEAE-dextran, 1.2 μg/ml trypsin, and 25 μg/ml antibodies (Ab1-10, anti-NP antibody, or without antibody).

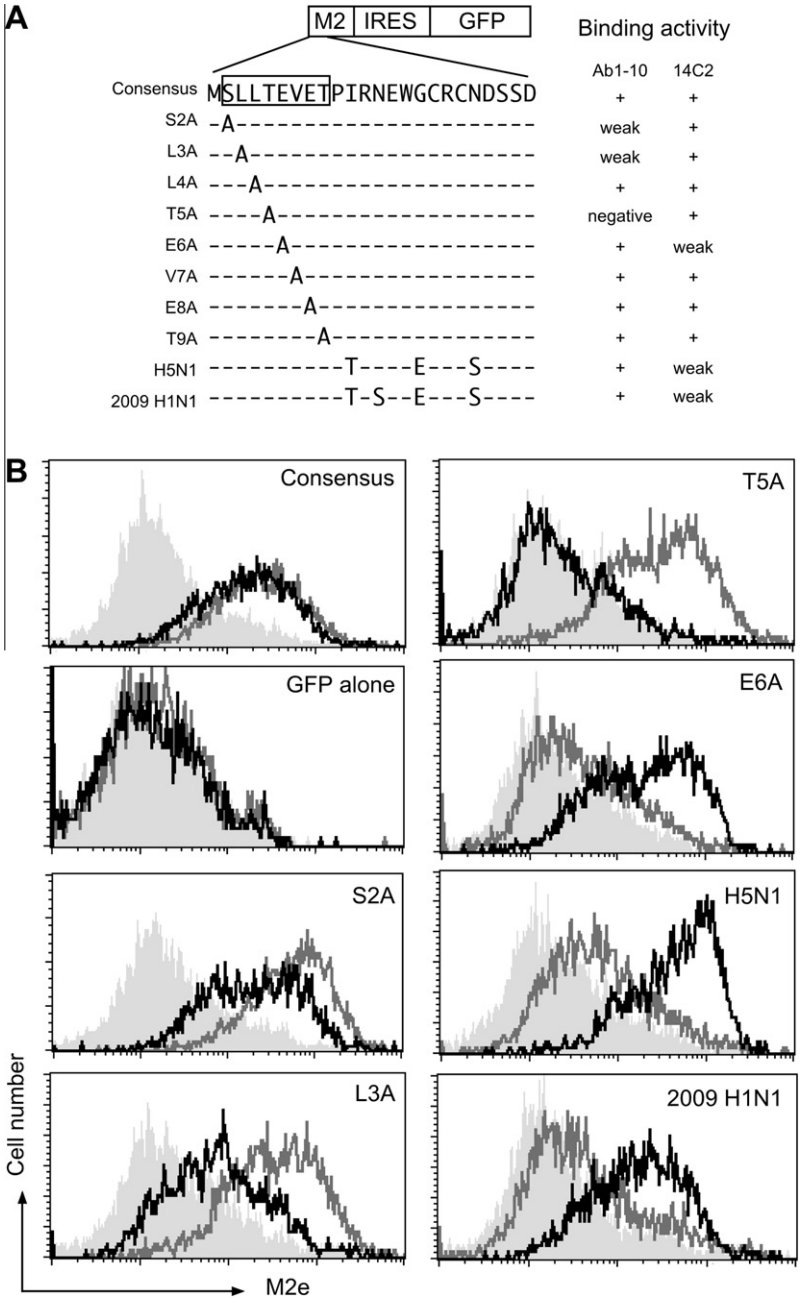

Fig. 2. Binding of Ab1-10 to M2e variants. (A) Scheme of the constructs for the expression of M2e variants. The IRES is used to direct translation of GFP. Box in the amino acid sequence indicates M1M2 common peptide. Binding activity of either Ab1-10 or 14C2 to cells that express M2e variants is indicated in the right. "+" indicates the binding to GFP^+ cells strongly; "weak" indicates the binding to GFP^+ cells weakly than "+", "negative" indicates no binding to GFP^+ cells. (B) Histograms show the binding of Ab1-10 (black line), 14C2 (gray line), or irrelevant IgG (light gray shade) to GFP^+ cells. Horizontal axis indicates the log intensities of fluorescence; vertical axis indicates cell number. Representative histograms in two independent experiments are shown.

Table 1
Determination of affinity of Ab1-10.

Antigen	K_D (M)
2009 H1N1 pandemic influenza virus strain inactivated antigen[a]	5.3×10^{-10}
Seasonal influenza virus strain inactivated antigen[b]	2.7×10^{-10}
M2e peptide[c]	2.3×10^{-7}

Data are a representative of two independent experiments with similar results.
[a] Virus strain: A/California/7/2009(H1N1).
[b] Virus strain: A/NewCaledonia/20/99(H1N1).
[c] Peptide sequence: SLLTEVETPIRNEWGCRCNDSSDK-biotin.

After culture, the plaques were fixed with 5% formaldehyde and visualized with 0.03% methylene blue. The size of the plaques was measured with a micrometer caliper. The result showed that Ab1-10 significantly reduced plaque size compared with NP-specific antibody or antibody-negative control ($p < 0.0001$, t-test) (Fig. 3A). In contrast, the number of plaques was not affected (data not shown). Furthermore, we investigated whether Ab1-10 inhibits the human influenza A pandemic virus (A/Osaka/132/2009(H1N1)) propagation. As shown in Fig. 3B, Ab1-10 reduced plaque size significantly when compared with NP-specific antibody or antibody-negative control ($p < 0.001$, t-test), but did not affect the number of plaques. These results demonstrated that Ab1-10 inhibited the propagation of 2009 H1N1 pandemic influenza virus as well as sea-

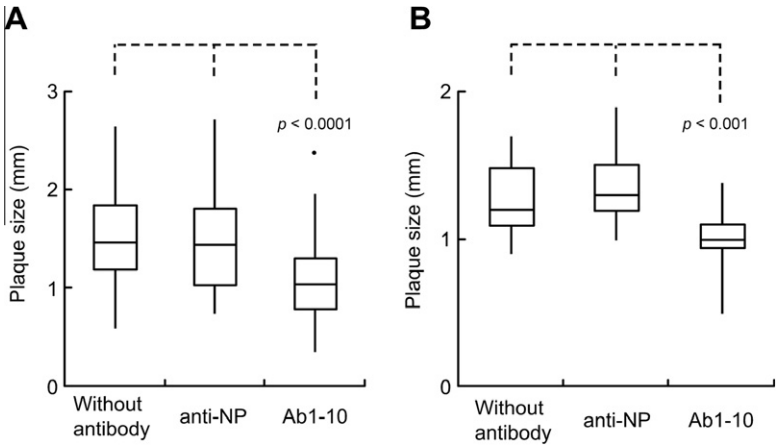

Fig. 3. In vitro plaque reduction assay. MDCK cells were infected with PR8 virus (A) or influenza A pandemics (B) and overlaid without antibody, with 25 μg/ml anti-NP antibody, or 25 μg/ml Ab1-10. After 2 days incubation at 37 °C, plaques were fixed with formaldehyde and visualized by methylene blue staining. The size of the plaque was measured. Distribution of plaques size is shown using box-and-whisker plots. Data are a representative of two independent experiments with similar results.

sonal influenza virus *in vitro*. Thus, Ab1-10 might be a good candidate for antibody therapeutics for newly emerging influenza viruses such as the highly pathogenic avian influenza virus. The effect of anti-M2e antibody on influenza virus replication is still controversial. Zebedee and Lamb showed that 14C2 M2e-specific monoclonal antibody reduced plaque size but not the number of plaques (Zebedee and Lamb, 1988). In contrast, Gabbard et al. demonstrated that the scFv 14C2 antibody reduced not only plaque size but also the number of plaques (Gabbard et al., 2009). Our Ab1-10 reduced the size of plaques, but did not reduce the number. The result indicated that Ab1-10 anti-M2e antibody suppressed the spread of infection within developing plaques. In this regard, Hughey et al. showed previously that anti-M2e antibody affected virus-particle formation by reducing the cell surface expression of M2 protein on virus-infected cells (Hughey et al., 1995), which may result in blocking the release of the virus from the cells and the spread of infection from cell to cell.

14C2 is the most prominent among the anti-M2e antibodies. Thus, we used the commercially available-14C2 antibody by the same method. Interestingly enough, the result showed that plaques of PR8 viruses were completely inhibited with either 5 or 25 μg/ml of 14C2 in overlay medium condition (data not shown). Our results obviously differed from the previous study (Zebedee and Lamb, 1988). There exists three possibilities; the first is the differences of experimental conditions such as medium contents we used. It is likely that different medium contents such as trypsin and *N*-acetyl trypsin, BSA, glucose, or DEAE-dextran may change osmotic pressure or pH, causing the different effects on the reduction assay. Secondary, the difference of the MDCK cell lines may cause the discrepancy: the cell lines are generally labile and easily change the *in vitro* responses. Thirdly, the difference of PR8 virus may be the reason: the conversion of the virus easily occurs in culture, purification process, or preservation conditions. At any rate, our data show that 14C2 antibody in concern displays significant reduction activity on PR8 reduction assay in certain conditions.

It has been reported that individuals show no or only a weak and transient immune response to the M2e epitope following influenza virus infection (Black et al., 1993; Feng et al., 2006) and it has been very difficult to detect M2e-specific mAbs production in human peripheral blood. This is because M2 is expressed at low-level on the surface of influenza A virus particles (Zebedee and Lamb, 1988), which results in the weak immunogenicity of M2e. In this study, we obtained more than 50 clones of the influenza-specific antibodies from peripheral blood lymphocytes prepared from vaccinated subjects (unpublished data), but identified only a single clone of M2e-specific mAbs with an inhibitory effect on virus replication using the ISAAC method (Jin et al., 2009). We believe that this is the first report of obtaining M2e mAb directly from vaccinated volunteers. Thus, ISAAC seems a powerful method for obtaining human mAbs against weak antigenic epitopes in pathogenic microorganisms directly from infected patients or vaccinated volunteers.

Regarding the advantages and disadvantages of Ab1-10 in therapeutic application, the advantages are (1) that epitope of Ab1-10, the first eight amino acids of M2e, is highly conserved within influenza A viruses (Feng et al., 2006) including recent viruses of A/Vietnam/1194/04(H5N1) and A/California/7/2009(H1N1), and (2) that Ab1-10 suppressed the spread of virus infection from cell to cell (Fig. 3). In contrast, the disadvantage is that Ab1-10 could not neutralize the virus infection (Fig. 3) as demonstrated for other M2e-specific antibodies (Wang et al., 2008). Hence, in order to show the maximum effect of Ab1-10 in the therapy, combination with other anti-viral agents should be necessary, as indicated for other anti-viral drugs (Ilyushina et al., 2007; Masihi et al., 2007).

In conclusion, the Ab1-10 binds to and has the potential to protect against a wide range of influenza A viruses, as compared to other anti-M2e antibody. Further studies on this effect in the combination of other therapeutic reagents might provide more effective therapy against a wide range of influenza A viruses.

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