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Inhibitory effects of an M2-specific monoclonal antibody on different strains of influenza A virus

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Abstract
New approaches to the treatment of influenza have been designed based on the highly conserved antigenicity of the M2 envelope protein among influenza A virus strains. The present study examined the anti-viral activities of an anti-M2 ectodomain (M2e) monoclonal antibody (clone rM2ss23), which binds to the M2 proteins of the influenza A virus strains A/Aichi/2/68 (H3N2) (Aichi) and A/PR/8/34 (H1N1) (PR8). The results showed that rM2ss23 bound to both Aichi and PR8 M2 proteins expressed on the cell surface. While the antibody did not prevent virus entry into cells, it significantly inhibited plaque formation by the Aichi strain in a dose-dependent manner when infected cells were cultured in the presence of the antibody. By contrast, the growth of PR8 (H1N1) was not affected by the antibody. A reverse genetics approach revealed that the inhibitory effect of rM2ss23 on the Aichi virus was abolished by replacing the genes encoding the HA and/or M proteins with those of the PR8 strain. These results suggest that rM2ss23 prevents virus release from infected cells and further suggest that the mechanisms underlying the virus budding mediates by HA and M2 proteins might differ between the Aichi and PR8 strains.

Key words: HA, influenza A virus, inhibitory activity, M2e antibody.

Introduction
Since the pandemic spread of the influenza virus, new strategies have been developed to prevent transmission or diminish the severity of this disease. Currently, it is estimated that
250,000–500,000 people die from influenza every year\textsuperscript{18}. Vaccination is the key to preventing severe illness and death from pandemic influenza\textsuperscript{24}. Following infection with influenza A, antibodies directed against viral hemagglutinin (HA) and neuraminidase (NA), both of which are envelope glycoproteins, act as important components of the mechanism of protective immunity against the influenza virus\textsuperscript{29}. In addition to HA and NA, a third viral envelope protein, M2, which is present at low levels on the virus particle surface but abundantly expressed on the surface of influenza A virus-infected cells, has been identified as a promising target candidate for antiviral drug design\textsuperscript{3,13}. Two of the three currently available anti-flu drugs, amantadine and rimantadine, act via the M2 protein. However, many strains of seasonal influenza A viruses, including the 2009 H1N1 pandemic strains, have become resistant to these two drugs\textsuperscript{17}.

Despite the abundance of HA and M2 proteins on the plasma membrane of infected cells, only a small proportion of the M2 protein is incorporated into each virion, which contains approximately 16–67 M2 monomers for every 500 HA spikes\textsuperscript{22}. In contrast to HA and NA, whose antigenicity are continually changing, M2 is relatively conserved among influenza A viruses. The low degree of variation in M2 compared with HA and NA is attributed to its genetic relationship to M1, the most conserved influenza A protein virus\textsuperscript{2}. The ectodomain of M2 (M2e) has not experienced antigenic drift and has maintained its conserved sequence since influenza virus was first isolated from humans in 1933, despite numerous epidemics and pandemics\textsuperscript{3,30}. Although the M2e sequence is remarkably conserved, two positions with variable amino acids have been identified: glycine or glutamate at position 16 and aspartate or glycine at position 21. With regard to the latter, the presence of either amino acid appears to have no effect on the efficacy of a recombinant vaccine in which the M2 protein is fused to the hepatitis B virus core protein\textsuperscript{15}.

The M2 protein has been implicated in various aspects of viral infectivity as well as in virion formation and release. M2 is not only the prototype viral ion-channel protein but also a multifunctional protein involved in virus assembly\textsuperscript{22}. The M2 protein exists as a homotetramer formed by two disulfide-linked 97 amino-acid dimers held together by a non-covalent interaction\textsuperscript{6,13,25}. Residues 1–24 comprise the extracellular domain (i.e., ectodomain), residues 25–43 constitute the transmembrane segment, and the remaining 54 residues form the cytoplasmic tail. The tetrameric M2 protein forms a proton-selective ion channel and plays an important role in facilitating viral entry\textsuperscript{7,23,27}. During the entry process, initial viral attachment is followed by endocytosis. Within the endosomes, M2 mediates an influx of protons into the virions, which facilitates dissociation of the matrix protein from the viral ribonucleoprotein (RNP) complex and enables the transport of free RNP to the nucleus, where viral RNA replication takes place\textsuperscript{5}.

Influenza HA and NA undergo genetic and antigenic alteration to escape the host immune response\textsuperscript{1,19}. The presence of HA neutralizing antibodies at systemic or mucosal sites of infection provides immediate protection against infection by influenza viruses, whereas clearance of the viruses partly depends on cell-mediated immunity\textsuperscript{4}. Although antibodies specific for NA do not neutralize infectivity, they restrict virus replication by preventing the release of new virus particles, a process that requires NA. Therefore, antibodies specific for NA can decrease the severity of the disease\textsuperscript{24}. Although antibodies specific for M2 are unable to bind efficiently to free virus particles and thus do not neutralize virus infectivity, they can bind to M2e expressed on the surface of virus-infected cells\textsuperscript{10}. Because the monoclonal antibody (MAb) against M2 is expected to have an effect similar to that of NA antibodies, it is a potential antiviral tool for preventing new virion release\textsuperscript{12,26}.

In the present study, we generated an M2e-specific MAb (rM2ss23) that inhibits replication of the influenza A/Aichi/2/68 (H3N2)
(Aichi) strain in MDCK cells. Interestingly, rM2ss23 did not inhibit replication of the A/PR/8/34 (H1N1)(PR8) strain, despite having a similar binding affinity for both Aichi and PR8 M2 proteins. We then constructed eight different reassortant viruses in which genomic RNA segments 7 and 4, which encode respectively the M1, M2 and HA proteins of Aichi and PR8, were exchanged. Viral plaque forming assays using these reassortant viruses showed that the inhibitory effect of rM2ss23 on Aichi replication was dependent on the products of the M and HA genes of the Aichi strain.

Materials and Methods

Cells:

The human embryonic kidney cell line, HEK293T, and Madin-Darby canine kidney (MDCK) cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, USA). All cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Plasmids for the A/Aichi/2/1968 (H3N2) reverse genetic system:

A reverse genetic system was used to generate the A/Aichi/2/68 (H3N2) strain. Briefly, RNA was extracted from the Aichi virus and cDNA was synthesized by reverse transcription using the universal primer, Uni 12 (5'-AGCAAAGCAGG-3'), and SuperScript® III reverse transcriptase (Invitrogen). To amplify the viral genes, cDNA was subjected to PCR using the KOD plus DNA polymerase kit (Toyobo, Tokyo, Japan) as directed by the manufacturer. The cDNAs encoding the 8 influenza segments (PB2, PB1, PA, HA, NP, NA, M, NS) were generated by gene-specific primer sets used for the Aichi genome segments (Table 1) and the PB2, PB1, PA and NP viral polymerases, which are sufficient for the plasmid-driven generation of influenza viruses (Table 2) were purchased from Sigma Genosys (Ishikari, Japan). The purified products (MonoFas, GL Science, Torrance, USA) were inserted into the pCXSN vector²¹ and

<table>
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<th>Segment</th>
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The underline indicates additional recognition sequence (5′-CGTCTCN₅₋₃') of BsmB I restriction endonuclease.
ligated by T4 DNA ligase (Takara, Tokyo, Japan). Transformed DH5α competent cells (Nippon gene, Tokyo, Japan) were plated on ampicillin-agar plates, and single colonies were amplified in liquid culture and extracted and purified using a DNA extraction kit (Qiagen, Valencia, USA). The sequences of the cloned cDNAs were confirmed using the BigDye terminator cycle sequencing ready reaction kit and analyzed on a 3130 Genetic analyzer (Applied Biosystems, Foster City, USA) according to the protocol recommended by the manufacturer.

**Generation of recombinant influenza viruses:**

To generate recombinant influenza virus,107, HEK293T cells (1 × 10⁶) were transfected with plasmids encoding the structural proteins of the wild-type (WT) PR8 (H1N1) (kindly provided by Prof. Kawaoka, University of Tokyo, Japan) or Aichi (H3N2) strains flanked by the human RNA polymerase I promoter and the mouse RNA polymerase terminator together with plasmids (pCAGGS) encoding viral nucleoprotein (NP) and PB2, PB1, PA viral polymerases. The cells were transfected with 1 µg of each plasmid using Trans IT LT-1 (Mirus, Madison, USA) according to the manufacturer's instructions. Briefly, the plasmids and transfection reagent were mixed (10 µl of Trans IT LT-1 for 12 µg of total DNA), incubated at room temperature for 30 min, and added to the cells. At 6–20 hr after transfection, the DNA-transfection reagent mixture was replaced with Opti-MEM (Gibco-BRL, Gaithersberg, USA). At 48 hr post-transfection, the supernatant was transferred to MDCK cells in the presence of TPCK-trypsin (final concentration, 1 µg/ml; Gibco-BRL) at 60–90% confluency in 6-well plates. Viruses were recovered and quantified by plaque assay. This strategy yielded > 5 × 10⁶ plaque forming units (pfu) per ml of supernatant at 48 hr post-transfection.

**Expression of M2e fragment and MAb production:**

The M2e fragment of A/chicken/Yamaguchi/7/2004 (H5N1) was cloned into a pET30a plasmid vector (Novagen, Darmstadt, Germany) and expressed in E. coli, BL21 (DE3) following the manufacturer's instructions. Briefly, the M2e region was amplified by PCR with the primer sets Ck-Ym-M1F-NcoI (5'-AACATGGGTCTTCAACCGAGG-3') and M2ssR2-SalI (5'-TTAGTCGACTTAGATCCAAATGATAACTTGC-3') using viral cDNA as the template. The PCR fragment was then inserted into a pET30a vector using the NcoI and SalI digestion sites, and the resultant plasmid was designated as pET30a/CkYm M2e. BL21 (DE3) E. coli, cells were transformed with pET30a/CkYm M2e and cultured in LB medium. Protein expression was induced by the addition of 0.4 mM IPTG, and recombinant M2e with a 6 × His tag was purified by Talon metal affinity resins (Clontech, Mountain View, USA) following the manufacturer's instructions. Anti-M2e MAb was produced according to the method described by

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**Table 2. List of primers for the Aichi viral polymerases**

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<td>PB1</td>
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<td>PB1</td>
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<td>PA-Not-R</td>
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<td>NP</td>
<td>NP-XhoI-F</td>
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<td>NP-NotI-R</td>
<td>AATGCAGACCCGCTTTAATGTGGACATTGAGAAG</td>
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</table>

The underline indicates additional recognition sequence (5'-GC/GGCCGC-3') of Not I, and (5'-C/TCGAG-3') of Xho I restriction endonuclease.
Kida et al. After cloning of the monoclonal antibodies, one clone (rM2ss23) was selected for use. The specificity of the rM2ss23 antibody for M2 was confirmed by immunoblot analysis of A/chicken/Yamaguchi/7/2004 (H5N1)-infected MDCK cells with the antibody purified from mouse ascites using protein A agarose columns (Bio-Rad, Hercules, USA).

**Immunofluorescence microscopy for cross-reactivity of rM2ss23:**

MDCK cells were transfected with expression plasmids for the M2 protein of the PR8 or Aichi virus strains using lipofectamine LTX reagent (2 μl/μg; Invitrogen) or inoculated with recombinant Aichi and PR8 viruses at 0.01 multiplicity of infection (MOI). At 12 hr after transfection or inoculation, cells were blocked with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 20 min at 25°C and incubated for 1 hr with rM2ss23. After three washes with PBS, cells were incubated for 1 hr with goat anti-mouse IgG (H + L) labeled with Alexa-Fluor 488 (1:1000 dilution; Invitrogen) and then fixed with 4% paraformaldehyde (PFA) in PBS. Images were acquired using an IX70 Fluorescence microscope (Olympus, Tokyo, Japan).

**Flow cytometry:**

HEK293T cells were transfected with a combination of plasmids expressing GFP alone, GFP + Aichi-M2 or GFP + PR8-M2 using Lipofectamine 2000 (Invitrogen). At 48 hr after transfection, the cells were stained with Alexa 647-conjugated rM2ss23. The labeling of rM2ss23 was performed following the manufacturer’s instructions of Apex Alexa Fluor 647 antibody labeling Kit (Invitrogen). The fluorescence activity of the cells was analyzed by flow cytometry (FACS Canto, BD Biosciences, San Jose, USA). GFP expression was used to identify gene-transfected cells, and the GFP (+) population was gated to construct histograms of rM2ss23-stained cells.

**Polyclonal antibodies against the Aichi and PR8 strains:**

To detect viral antigens in infected cells, chicken anti-influenza A virus antiserum against PR8 and Aichi was used as the source of primary antibodies, followed by secondary peroxidase-conjugated affinipure rabbit anti-chicken IgY ++ (IgG) (H + L) (Jackson ImmunoResearch, West Grove, USA). Purified mouse IgG2a was used as an isotype negative control (BioLegend, San Diego, USA).

**Neutralization by rM2ss23:**

To examine the neutralization activity of rM2ss23, Aichi or PR8 viruses (100–200 pfu) were incubated with serial dilutions of rM2ss23 (0, 0.01, 0.1, 1.0, 10, 100 μg/ml) prior to inoculation. Unbound viruses were removed by washing three times with PBS. Plaques were visualized by immunostaining with polyclonal antibodies. Briefly, cells were fixed with methanol and incubated in blocking solution (1% BSA in PBS) for 30 min at 25°C. Cells were incubated with anti-Aichi or anti-PR8 chicken antiserum for 1 hr at room temperature (RT), washed with PBS containing 1% Tween 20, and then incubated with peroxidase-conjugated affinipure rabbit anti-chicken IgY ++ (IgG) (H + L) (Jackson ImmunoResearch) that was same antibody described in the previous subsection, “Polyclonal antibodies against the Aichi and PR8 strains”. Immunoreactivity was detected using a 3,3’-diaminobenzidine (DAB) substrate. The plaque forming units per ml (pfu/ml) were determined using the formula: number of plaques × dilution factor/0.1 ml

**Plaque-formation inhibition in the presence of rM2ss23:**

To examine the inhibitory activity of rM2ss23 after virus entry, cells were infected with viruses and then overlaid with MEM containing serial dilutions of rM2ss23 (0, 0.01, 0.1, 1.0, 10 μg/ml) or mouse control IgG2a antibody (10 μg/ml, clone MG2a-53; BioLegend) and 2 μg/ml trypsin and
incubated for 48 hr (at 37°C, 5% CO₂). Plaques were visualized by immunostaining with anti-PR8 or anti-Aichi chicken antiserum as described above.1013

Statistical analyses:
Data from the quantitative analyses are expressed as the mean ± SE for samples without or with anti-M2e antibody. Statistical analyses were performed using Student’s t-test and the Mann-Whitney U test for plaque number and plaque size, respectively. p < 0.05 and p < 0.01 were considered to be statistically significant.

Results

Cross-reactivity of rM2ss23 with PR8 and Aichi M2 proteins:
Indirect immunofluorescence was used to determine whether the rM2ss23 antibody recognized the M2 proteins of Aichi and PR8 strains. MDCK cells were transfected with plasmids expressing the M2 protein of the Aichi or PR8 strains, as well as inoculated with each PR8 or Aichi strain (0.01 MOI). Twelve hours after transfection or inoculation, the cells were incubated with rM2ss23 followed by secondary anti-mouse IgG labeled with Alexa-Fluor 488, and then fixed with 4% PFA in PBS. Cells expressing PR8- or Aichi-M2 or inoculated with PR8 or Aichi strain exhibited similar patterns of immunostaining with rM2ss23 (Fig. 1A). In addition, rM2ss23 bound to the M2 proteins of both strains, as indicated by similar levels of fluorescence intensity detected by flow cytometric analysis of HEK293T cells transfected with the M2-expressing plasmids (Fig. 1B).

Neutralization activity of rM2ss23:
To determine whether rM2ss23 neutralizes infection by the PR8 or Aichi strains at the point of viral attachment or entry, 100–200 pfu of each strain were incubated with serial dilutions (0, 0.01, 0.1, 1, 10, 100 μg/ml) of rM2ss23, and then used to inoculate MDCK cells. rM2ss23 did not show any neutralizing activity in this assay and no significant differences were observed between the PR8 and Aichi strains (Figs. 2A and B).

Inhibition of plaque formation:
Next, the effect of rM2ss23 on viral replication or release was investigated. After virus adsorption of 100–200 pfu, cells were incubated with overlay medium containing serially diluted concentrations of rM2ss23 (0, 0.01, 0.1, 1, 10 μg/ml) for 48 hr. In the presence of rM2ss23, the number of visible plaques in Aichi-infected cells decreased in a dose-dependent manner, with a significant reduction in plaque size (Fig. 3B). By contrast, no visible change in the number or size of the plaques was observed in PR8-infected cells upon treatment with rM2ss23, even at a concentration of 10 μg/ml (Fig. 3A).

Identification of the viral proteins responsible for the inhibitory effects of rM2ss23:
To identify the viral protein(s) involved in the inhibitory activity of rM2ss23, a reverse genetic system was used to generate eight distinct influenza virus reassortants from the PR8 and Aichi strains by reciprocal replacement of the HA and/or M genome segments. MDCK cells were inoculated at a low multiplicity of infection (MOI = 0.0001) with PR8, Aichi (WT), or reassortant viruses with reciprocal HA, M, or HA and M and incubated in the absence or presence of either 1 or 10 μg/ml of rM2ss23 for 48 hr.

The relative numbers of visible plaques counted in cells infected with PR8 or single-segment reassortants (PR8/WT, PR8/Aichi-HA, or PR8/Aichi-M) were unchanged, regardless of the presence of rM2ss23. By contrast, a reduced number of plaques was observed in cells infected with PR8, in which both the HA and M genes had been replaced with those of the Aichi strain (PR8/Aichi-HA-M), in a dose-dependent manner (1 and 10 μg/ml; Fig. 4A). In Aichi/WT-infected cells, the relative number of plaques decreased after treatment with rM2ss23; however, replacement
Fig. 1. Immunofluorescence analysis of M2e expression. (A) The same field as imaged by immunofluorescence analysis staining with Alexa-fluor 488 and differential interference contrast (DIC) is presented to show the cross-reactivity of rM2ss23 to PR8- and Aichi-M2 protein. Cells were transfected with plasmids encoding M2 protein of PR8 or Aichi strains, or inoculated with PR8 or Aichi viruses. The cells were incubated with rM2ss23 (αM2e) followed by secondary mouse anti-IgG (H+L) Alexa-488 antibody and fixed with 4% PFA in PBS. (B) HEK293T cells were transfected with a combination of plasmids expressing GFP alone, GFP + Aichi-M2, or GFP + PR8-M2, and analyzed as described in Materials and Methods. Cells gated according to GFP expression were used to construct histograms of rM2ss23-stained cells.
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of the HA and/or M genome segments with those of PR8 reduced the inhibitory effect of rM2ss23 (Fig. 4B).

**Effect of rM2ss23 on plaque size:**

Next, the effect of rM2ss23 on plaque size was assessed in 40–60 plaques of virus-infected cells (Figs. 5 and 6). In cells infected with the Aichi/WT strain, rM2ss23 at 1 and 10 μg/ml significantly diminished the average size of plaques compared with control IgG antibody or no antibody (Fig. 6A). Replacement of the HA, M, or HA + M segments in the Aichi-background viruses with those of PR8 made the Aichi strain insensitive to rM2ss23, resulting in no significant change in average plaque size in either the presence or absence of rM2ss23 (Figs. 6B, C, D), similar to cells infected with wild-type PR8 strain (Fig. 6A). On the other hand, replacement of the M or M and HA segments in the
PR8-background viruses resulted in significantly reduced average plaque sizes in the presence of rM2ss23 (Figs. 5C and D). Interestingly, although the number of visible plaques in cells infected with single-segment reassortant PR8/Aichi-M was not decreased in the presence of rM2ss23 (Fig. 4A), the size of the plaques diminished.

Exchange of the HA segment between viruses reduced plaque size compared with the respective wild-type in both viruses (Figs. 5B and 6B), and this was not altered by the presence of rM2ss23.

Fig. 4. Effects of rM2ss23 on the replication of recombinant viruses. PR8 and PR8-based reassortant (A) and Aichi and Aichi-based reassortant (B) viruses were generated using a reverse genetic system and assayed for plaque formation in MDCK cells. After virus adsorption, cells were incubated for 48 hr in the presence or absence of rM2ss23 (1 and 10 µg/ml), and the resulting plaques were counted after immunostaining. Statistical significance was determined by Student’s t-test (*p < 0.05, **p < 0.01).
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Discussion

It was reported previously that the anti-M2e MAb clone 14C2 decreased the growth of multiple influenza A virus strains: A/ Udorn/72 (H3N2), A/ HK/8/68 (H3N2), A/Singapore/1/57 (H2N2), A/ FW/1/50 (H1N1) and A/USSR/90/70 (H1N1)\(^8,14\). In the present study, neutralization assays showed that the number of plaques formed in PR8- and Aichi-infected cells was unaffected by preincubation of the viruses with the M2-specific antibody, rM2ss23. This finding is consistent with a previous report in which anti-M2e antibody was unable to bind to free virus particles or neutralize the influenza virus prior to infection\(^10\). However, when the cells were incubated with rM2ss23 after virus adsorption, the number and size of the plaques formed by the Aichi strain were progressively reduced. By contrast, cells infected with the PR8 strain were insensitive to rM2ss23 regardless of antibody concentration. These findings suggest that anti-M2e antibodies can be effective for preventing virus growth, possibly by interfering with virus particle formation or budding\(^14\).

Interestingly, rM2ss23 showed a more pronounced inhibitory effect on plaque formation in cells infected by PR8 recombinant variants expressing Aichi-HA-M than by virus expressing only the Aichi M segment. In addition, the inhibitory effect of rM2ss23 on the Aichi strain was compromised after replacement of the HA and/or M segments with those of the PR8 strain. These findings suggest that the Aichi-HA and -M proteins are involved in the mechanism of inhibition by rM2ss23. The difference in the ability of rM2ss23 to affect the relative number and size of

![Fig. 5. Average plaque size and morphology of PR8 and PR8-based reassortant plaques in the presence of rM2ss23.](image-url)
plaques in PR8- and Aichi-infected cells might be due to differences in the interaction between the HA and M2 proteins during assembly on the cell surface.

The M2 protein of influenza A viruses is highly conserved antigenically, but still contains variability in some amino acid positions depending on the virus strain. In the present study, the Aichi and PR8 strains differed with respect to the amino acid at position 21; A/PR/8 contains a glycine while Aichi contains an aspartate. However, since the binding affinity of rM2ss23 for M2e was similar for both strains, it remains unclear how these amino acids affect the inhibitory activity of the anti-M2e antibody. In addition, the rM2ss23 epitope binding site has not been determined yet. A possible correlation with the HA-M2 interaction during the morphogenesis of budding virions is suggested by the frequent association and co-localization of the HA and M2 proteins in virus-infected cells. Further studies on the interaction between HA and M2 proteins in the Aichi strain will be necessary to fully elucidate the mechanism of viral growth inhibition by anti-M2e antibody and to delineate this novel mechanism of antibody-mediated neutralization, which may potentially reduce cell damage caused by influenza virus infection.
Acknowledgements

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