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Factors responsible for plaque formation of A/duck/Siberia/272/1998 (H13N6) influenza virus on MDCK cells

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ABSTRACT

Many influenza A viruses form plaques on Madin-Darby canine kidney (MDCK) cells in the presence of trypsin. A/duck/Siberia/272/1998 (H13N6) (Sib272), however, does not form plaque on MDCK cells. After three blind passages of the strain on MDCK cells, plaque-forming variant was obtained and designated as A/duck/Siberia/272PF/1998 (H13N6) (Sib272PF). Genetic and functional analyses of Sib272 and Sib272PF revealed that amino acid substitutions, F3L of the HA2 subunit and T379K of the PB1, were responsible for plaque formation of Sib272PF by enhancing fusion and polymerase activities, respectively.

1. Introduction

Influenza A virus belongs to the family *Orthomyxoviridae* and has been isolated from birds and mammals, including humans. On the basis of the antigenic specificity of hemagglutinin (HA) and neuraminidase (NA), influenza A viruses is derived into subtypes H1–H16 and N1–N9, respectively (Fouchier et al., 2005; Hinshaw et al., 1982; Palese and Shaw, 2006; Webster et al., 1992). Influenza A virus contains eight segments of negative-stranded RNA genome, encoding eleven different proteins. Two glycoprotein spikes (HA and NA) and M2 protein are embedded in the lipid bilayer envelope underlain by the matrix protein (M1) (Palese and Shaw, 2006). In virus particles, genomic RNAs are associated with the RNA-dependent RNA polymerase complex consisting of polymerase basic proteins (PB1 and PB2) and acidic protein (PA), nucleoprotein (NP) and nonstructural protein 2 (NS2), together forming the ribonucleoprotein (RNP) complex (Akarsu et al., 2003; Kingsbury and Webster, 1969; Neumann et al., 2000). NS1 protein found in cells infected with influenza A viruses is multi-functional (Hatada and Fukuda, 1992; Lamb and Choppin, 1979). Recently, PB1-F2 which is encoded by a second open reading frame (+1) of PB1 gene was found as the protein that induces apoptosis (Chen et al., 2001).

Plaque assay is one of the most important procedures for the isolation and titration of viruses. Influenza A viruses form plaques on cell lines such as chicken embryo fibroblast cells, African green monkey kidney (Vero) cells and MDCK cells in the presence of trypsin (Babiker and Rott, 1968; Tobita et al., 1975). The role of influenza virus proteins in plaque formation has been studied. A/Udorn/72 (H3N2) formed plaques on MDCK cells, whereas the variant with mutant HA C562S and C565S failed to form plaque (Chen et al., 2005). Wagner et al. (2000) proposed that glycans flanking the receptor-binding pocket of the HA are potent regulators of virus growth in cell culture. Lack of oligosaccharides from Asn123 and Asn149 reduced virus growth and plaque size on MDCK cells. The M1 protein of A/WSN/33 was responsible for the determination of virus growth and plaque size in MDCK cells (Yasuda et al., 1994). The plaque size of a variant virus bearing mutant NS1 protein that loses binding activity to dsRNA was smaller than that of the original strain A/Udorn/72 (Min and Krug, 2006).

A/duck/Siberia/272/1998 (H13N6) (Sib272) does not form plaques on MDCK cells. After the blind passages of this strain in MDCK cells, a plaque-forming variant, Sib272PF was obtained. In the present study, the molecular basis of the plaque formation of Sib272PF was studied.

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2. Materials and methods

2.1. Cells and viruses

MDCK cells were maintained in Minimum Essential Medium (MEM) supplemented with 0.3 mg/ml L-glutamine, 10% calf serum, 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 8 μg/ml gentamicin. Human embryonic kidney (293T) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.3 mg/ml L-glutamine, 10% fetal calf serum and antibiotics. A/duck/Siberia/272/1998 (H13N6) (Sib272), which does not form plaque on MDCK cells, was isolated from the fecal sample of a feral duck in Siberia (Okazaki et al., 2000). After three blind passages of the strain in MDCK cells, the plaque-forming variant was obtained and designated as A/duck/Siberia/272PF/1998 (H13N6) (Sib272PF). Sib272 and Sib272PF were propagated in 10-day-old embryonated chicken eggs at 35 °C for 48 h.

2.2. Plaque assay

Ten-fold dilutions of viruses were inoculated onto confluent monolayers of MDCK cells and incubated at 35 °C for 1 h. Unbound viruses were removed, and the cells were washed with MEM. Cells were then overlaid with MEM containing 0.7% bact-agar (Gibco) and 5 μg/ml acetyl trypsin (Sigma). After 48 h of incubation at 35 °C, cells were stained with 0.005% neutral red. Blind passaging of the virus was as follows: cells showing cytopathic effects were picked up by a sterile capillary pipette under a microscope and suspended in MEM. The cells were diluted 10-fold and inoculated into MDCK cells. After three successive passages, visible plaques were found. To evaluate plaque morphology, the cells were stained with 0.1% crystal violet solution with 10% formaldehyde 72 h, post-inoculation.

2.3. Sequence analysis

Viral RNAs were isolated from virus-containing allantoic fluid using Trizol-LS (Sigma) and ethanol. Universal primer sets for influenza A virus were used for RT-PCR (Hoffmann et al., 2001). PCR products were reacted with gene-specific primers and the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter). Sequences of the DNA template were determined using CEQ 2000XL DNA analyzers (Beckman Coulter).

2.4. Preparation of mutant viruses derived from Sib272PF by reverse genetics system

Each of the PCR products of the eight gene segments of Sib272PF was cloned in TA vector. Mutations of Sib272, which are L3F of the HA2 and Q184H and K379T of the PB1, were introduced into cDNAs from Sib272PF by site-directed mutagenesis with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Eight segments of Sib272PF and mutated constructs were cloned into a dual-promoter plasmid, pHW2000 (Hoffmann et al., 2000b). MDCK and 293T cells were co-cultured and transfected with 1 μg of each of the eight plasmids and 16 μl of TransIT-293 (Mirus Bio) in 150 μl of OPTI-MEM (Gibco). After 30 h of incubation, 1 ml of OPTI-MEM containing 5 μg/ml acetyl trypsin was added to each well. After 48 h of incubation at 35 °C, the supernatant was collected, and 100 μl was injected into the allantoic cavity of 10-day-old embryonated chicken eggs. Using reverse genetics, rg-272, rg-272PF and other mutated variants were generated (Table 2). The infectivity of rg-viruses were determined as 50% egg infectious dose (EID_{50}) and 50% tissue culture infectious dose ($TCID_{50}$).

2.5. Growth curve of viruses

Mutant viruses generated by reverse genetics, rg-272, rg-HAF3L, rg-HAF3L/PB1T379K and rg-272PF, were inoculated onto the confluent monolayer of MDCK cells at a multiplicity of infection (MOI) of 0.01. After 1-h incubation at 35 °C, unbound viruses were washed away and MEM with 5 μg/ml acetyl trypsin was added. Cells were incubated at 35 °C, and the supernatant collected at 6, 9, 12, 24, 36 and 48 h after infection. EID_{50} of the supernatants were periodically monitored.

2.6. Hemolysis assay

Hemolysis assay was performed as described previously (Kida et al., 1983). Briefly, rg-272 and rg-272PF were centrifuged at 25,000 rpm for 1.5 h and the pellets were resuspended in phosphate-buffered saline, pH 7.2. Virus concentrates containing 200 HA units (50 μl) were added to 2.0 ml of 1% chicken erythrocytes in saline buffered with 0.1 M citric acid–sodium citrate, at 0 °C for 60 min, and then incubated at 37 °C for 60 min with mixing every 15 min. The cells were sedimented by centrifugation and the supernatants were measured for hemoglobin at 540 nm.

2.7. Polymerase assay

The luciferase assay was performed as described (Salomon et al., 2006). Subconfluent monolayers of 293T cells were transfected with 1 μg of luciferase reporter plasmid (EGFP open reading frame in pHW72-EGFP substituted with the luciferase gene (Hoffmann et al., 2000a)), renilla luciferase plasmid (Promega) and a mixture of PB2, PB1, PA, and NP plasmids in quantities of 1, 1, 1, and 2 μg, respectively. Forty-eight hours post-transfection, cell extracts were prepared in 250 μl of lysis buffer and luciferase levels were assayed with the Dual-Glo™ Luciferase Assay System (Promega) and read on LUMAT LB9507 (Berthold Technologies). Luciferase activities of the reporter gene were standardized by renilla luciferase activity. Polymerase activity was expressed as folds of luciferase activity.

3. Results

3.1. Comparison of Sib272 and plaque-forming variant, Sib272PF

In 1998, non-pathogenic influenza A virus strain Sib272 was isolated from the fecal sample of a feral duck in Siberia. This strain does not form plaques on MDCK cells (Fig. 1). After three blind passages of Sib272 in MDCK cells, the plaque-forming variant, Sib272PF was obtained. Each of the eight gene segments of the original virus and plaque-forming variant was analyzed and amino acid sequences were compared. An amino acid substitution of F to L at position 3 in the HA2 subunit and two amino acid substitutions of H to Q at position 184 and T to K at position 379 in the PB1 molecule were found (Table 1). Amino acid sequences of the other proteins including PB1-F2 of plaque-forming variant were the same as those of the original virus. It was confirmed that rg-272PF generated by reverse genetics formed plaque on MDCK cells but rg-272 did not (Table 2).

Table 1
Amino acid substitutions between Sib272 and Sib272PF.

Protein	Position	Amino acid	
		Sib272	Sib272PF
HA2	3	F	L
PB1	184	H	Q
	379	T	K

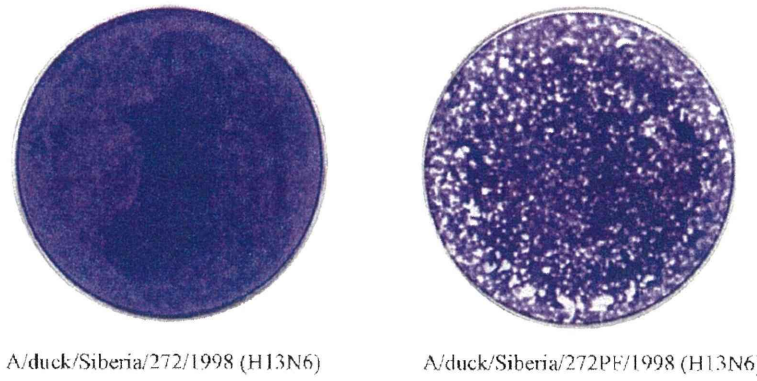

Fig. 1. Plaque formation of Sib272 and Sib272PF. Sib272 and Sib272PF were inoculated onto MDCK cells at 4.7 $\log_{10}$ EID_{50}/well, and incubated at 35 C for 3 days on 0.7% bact-agar in MEM. Cells were stained with crystal violet after removal of overlay agar.

3.2. Amino acid residues responsible for plaque formation

To determine the amino acid residues responsible for plaque formation, recombinant vituses with HA and/or PB1 mutant proteins were generated. Of these recombinants, only rg-HAF3L/PB1T379K formed plaques (Table 2). Infectivity titers on MDCK cells of the viruses with HA2 3L (5.0–6.6 $\log_{10}$ $TCID_{50}$/100 μl) were 100–1000 times higher than those of the viruses with HA2 3F (2.8–3.8 $\log_{10}$ $TCID_{50}$/100 μl), while infectivity titers in eggs were similar. The present results indicate that substitution of F3L of the HA2 subunit is a critical factor for virus growth on MDCK cells, and T379K of the PB1 is also responsible for plaque formation.

3.3. Growth of viruses in MDCK cells

To assess the effect of amino acid substitutions of HA2 F3L and PB1 T379K, growth kinetics of rg-272, rg-HAF3L, rg-HAF3L/PB1T379K or rg-272PF were analyzed. As shown in Fig. 2, rg-272 showed poor growth in MDCK cells. The plaque-forming variant, rg-272PF, grew to titers 100–10000 times higher than rg-272 and showed rapid growth. Rg-HAF3L/PB1T379K, which also forms plaques, reached a high titer. Single mutant virus rg-HAF3L showed slower growth than plaque-forming variant.

3.4. Comparison of fusion activity of rg-272 and rg-272PF

Amino acid residue at position 3 of the HA2 subunit is on the fusion peptide which leads to the fusion process for virus genome entry. Fusion activity of virus was analyzed by hemolysis assay. The hemolysis activity of rg-272 and rg-272PF was examined between pH 5.0 and 7.2. Maximal hemolysis with rg-272 was observed at pH 5.5 (Fig. 3). Hemolysis activity of rg-272PF showed extensive

Table 2
Plaque formation of viruses generated by reverse genetics.

Virus	Amino acid			Plaque formation	EID_{50}[a]	$TCID_{50}$[b]
	HA2	PB1				
	3	184	379			
rg-272	F	H	T	–	6.7	3.0
rg-272PF	L	Q	K	+	6.7	6.6
rg-PB1H184Q	F	Q	T	–	6.0	2.8
rg-PB1T379K	F	H	K	–	7.5	3.8
rg-HAF3L	L	H	T	–	6.3	5.0
rg-PB1H184Q/T379K	F	Q	K	–	7.3	3.3
rg-HAF3L/PB1H184Q	L	Q	T	–	6.5	5.0
rg-HAF3L/PB1T379K	L	H	K	+	6.3	5.3

[a] $\log_{10}$ EID_{50}/100 μl.
[b] $\log_{10}$ $TCID_{50}$/100 μl.

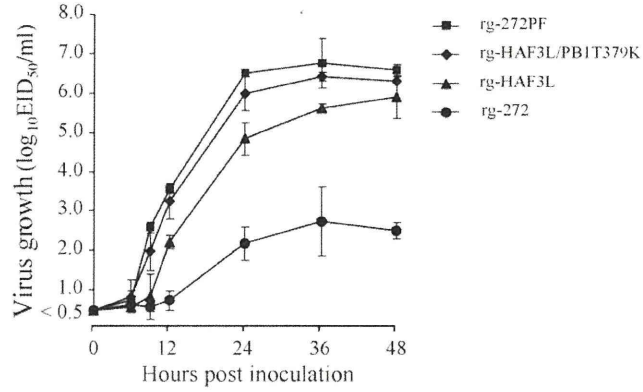

Fig. 2. Growth kinetics of viruses in MDCK cells. MDCK cells were inoculated with rg-272 (●), rg-HAF3L (▲), rg-HAF3L/PB1T379K (♦) and rg-272PF (■) at an MOI of 0.01. Infected cells were incubated at 35 C and supernatants were collected at the indicated times. Virus titer of supernatants was expressed as EID_{50}. The results are the mean ± S.D. of three independent experiments.

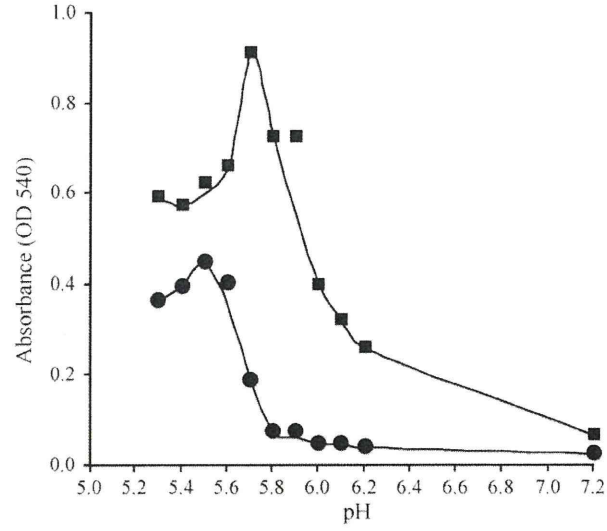

Fig. 3. Hemolysis assay of rg-272 and rg-272PF. Purified viruses rg-272 (●) and rg-272PF (■) were added to 2.0 ml of 1% chicken erythrocytes in saline buffer with 0.1 M citric acid–sodium citrate at various pHs, placed at 0 C for 60 min, and then incubated at 37 C for 60 min, mixing at every 15 min. The mixtures were then centrifuged and supernatants were measured at a wavelength of 540 nm.

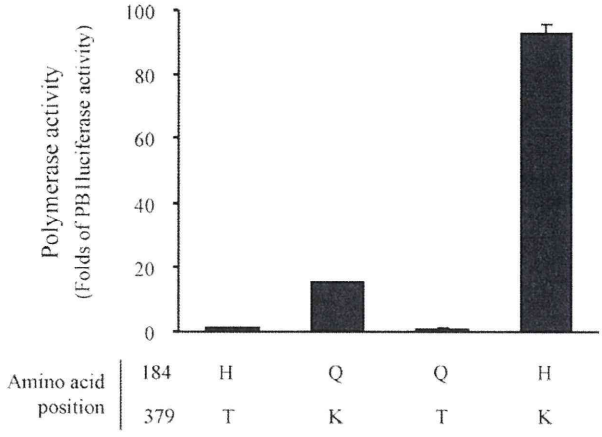

Fig. 4. Effects of PB1 mutations H184Q and T379K on polymerase activity. Polymerase activity was assayed by viral UTR-driven luciferase reporter genes. 293T cells were transfected with plasmids encoding PB1 or mutant PB1 gene with H184Q and/or T379K and PB2, PA and NP genes. After 48 h of incubation at 35 °C, cell extracts were tested for luciferase activity. Luciferase activity was standardized by renilla luciferase activity, and polymerase activity was expressed as folds of luciferase activity of PB1. The results are the mean + S.D. of triplicate samples in two different experiments.

enhancement than that of rg-272 and optimal pH for hemolysis was shifted to pH 5.7 from pH 5.5.

3.5. Polymerase activity of viruses with PB1 mutants

To compare the polymerase activity of variant viruses bearing mutant PB1 with that of the original virus, 293T cells were transfected with plasmids containing the PB1 or mutant PB1 gene with H184Q and/or T379K, and PB2, PA and NP genes. Polymerase complex with PB1 379K showed extensively high polymerase activity (Fig. 4).

4. Discussion

The plaque-forming variant, A/duck/Siberia/272PF/1998 (H13N6), was obtained by three successive passages from A/duck/Siberia/272/1998 (H13N6), which does not form plaque on MDCK cells. Genetic and functional analyses of Sib272 and Sib272PF revealed that amino acid substitutions of HA2 F3L and PB1 T379K were responsible for plaque formation of Sib272PF.

The major functions of HA are receptor binding and subsequent fusion of the virus envelope with the cellular endosomal membrane (Palese and Shaw, 2006). HA is synthesized as a single polypeptide (HA0) and cleaved into HA1 and HA2 subunits by trypsin-like endoprotease as an event in post-translational modification (Skehel and Wiley, 2000). The N-terminal region of HA2 polypeptide is highly conserved and is a critical component of the fusion process of virus entry (Cross et al., 2001). The substitution of glutamine for the glycine residue at the N-terminal of HA2 abolished fusion activity (Gething et al., 1986). Some mutants selected during passages in MDCK cells elevated the pH of membrane fusion (Lin et al., 1997; Rott et al., 1984). Amino acid substitution of G1V, W14A or F9A/I10A of HA2 of X31 (H3N2) influenza virus lost the stabilization of the boomerang structure of the fusion peptide (Lai et al., 2006; Lai and Tamm, 2007). In the present study, it was found that the amino acid substitution of HA2 F3L significantly enhanced fusion activity and induced effective entry of the virus genome into the cytoplasm of MDCK cells.

The RNA polymerase complex of the influenza virus consists of PA, PB1 and PB2 proteins, and PB1 is a key component of RNA polymerization. PB1 contains the polymerase motif in the amino acid region 298–484 and nuclear localization signal of the PB1 is identified between residues 180–195 and 202–252 (Kerry et al., 2008; Ohtsu et al., 2002; Poch et al., 1989). An amino acid substitution of PB1 T379K, which is located within the polymerase motif, increased polymerase activity. Polymerase proteins play important roles in virus replication, hence pathogenicity and host range (Gabriel et al., 2007; Hatta et al., 2001; Pappas et al., 2008; Salomon et al., 2006). On the other hand, increased polymerase activity does not necessarily correlate with the pathogenicity of viruses in vivo (Hulse-Post et al., 2007). Sib272 and even Sib272PF did not experimentally infect chickens (data not shown). The present results indicate that amino acid substitutions in Sib272PF affect the enhancement of replication in MDCK cells but not the infectiousness to chickens. Additionally, it is considered that the amino acid substitutions found in the HA and PB1 of Sib272PF may not constitute adaptation to mammalian hosts, since these amino acid residues are not typical in mammalian nor avian influenza viruses in data base.

It was concluded that two amino acid substitutions of HA2 F3L and PB1 T379K enhanced fusion and polymerase the activities of virus, respectively, resulting in plaque formation of Sib272PF on MDCK cells.

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Cross-Protective Potential of a Novel Monoclonal Antibody Directed against Antigenic Site B of the Hemagglutinin of Influenza A Viruses

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Abstract

The hemagglutinin (HA) of influenza A viruses has been classified into sixteen distinct subtypes (H1–H16) to date. The HA subtypes of influenza A viruses are principally defined as serotypes determined by neutralization or hemagglutination inhibition tests using polyclonal antisera to the respective HA subtypes, which have little cross-reactivity to the other HA subtypes. Thus, it is generally believed that the neutralizing antibodies are not broadly cross-reactive among HA subtypes. In this study, we generated a novel monoclonal antibody (MAb) specific to HA, designated MAb S139/1, which showed heterosubtypic cross-reactive neutralization and hemagglutination inhibition of influenza A viruses. This MAb was found to have broad reactivity to many other viruses (H1, H2, H3, H5, H9, and H13 subtypes) in enzyme-linked immunosorbent assays. We further found that MAb S139/1 showed neutralization and hemagglutination-inhibition activities against particular strains of H1, H2, H3, and H13 subtypes of influenza A viruses. Mutant viruses that escaped neutralization by MAb S139/1 were selected from the A/Aichi/2/68 (H3N2), A/Adachi/2/57 (H2N2), and A/WSN/33 (H1N1) strains, and sequence analysis of the HA genes of these escape mutants revealed amino acid substitutions at positions 156, 158, and 193 (H3 numbering). A molecular modeling study showed that these amino acids were located on the globular head of the HA and formed a novel conformational epitope adjacent to the receptor-binding domain of HA. Furthermore, passive immunization of mice with MAb S139/1 provided heterosubtypic protection. These results demonstrate that MAb S139/1 binds to a common antigenic site shared among a variety of HA subtypes and neutralizes viral infectivity in vitro and in vivo by affecting viral attachment to cells. The present study supports the notion that cross-reactive antibodies play some roles in heterosubtypic immunity against influenza A virus infection, and underscores the potential therapeutic utility of cross-reactive antibodies against influenza.

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Introduction

Neutralizing antibodies play a critical role in protection from influenza virus infection. Most neutralizing antibodies recognize hemagglutinin (HA), which is the major surface glycoprotein of influenza viruses. The HA of influenza A viruses has been classified into sixteen antigenically distinct subtypes (H1–H16) that are maintained in avian and mammalian species in nature [1,2].

HA is responsible for virus entry into target cells, virus binding to the host receptor, internalization of the virus, and subsequent membrane-fusion events. It is initially synthesized as a precursor polypeptide, HA0, that requires proteolytic cleavage into disulfide-linked HA1 and HA2 before it is functional and virus particles are infectious. The major part of HA1 forms the "globular head" region, which contains the necessary structure for binding to the sialic acid receptors. The "stem" region is mostly formed by HA2, which contains the fusion peptide and membrane anchor domain. It has been recognized that there is considerable amino acid variability (antigenic difference) in the globular head region among HA subtypes, whereas the structure of the stem region is relatively conserved.

The HA antigenic structure of the H3 subtype has been well characterized by using the sequence information on naturally occurring and laboratory-selected antigenic variants [3,4,5,6]. Five different antigenic sites have been identified and mapped mainly on the HA1 globular head region in the three-dimensional structure of the H3 HA molecule [3,4]. Antigenic sites of H1 [7] and H2 [8] subtypes were then characterized by the identification of amino acid substitutions found in the HA sequences of variants that escaped from neutralization by antibodies. Recently, it was suggested that the structures of antigenic sites of H5 [9,10] and H9 [11] subtypes were different from those of the H1, H2, and H3 subtypes.

Author Summary

Neutralizing antibodies play a critical role in protection from influenza A virus infection. Most neutralizing antibodies recognize hemagglutinin (HA), which is the major surface glycoprotein of influenza viruses. The HA has been classified into sixteen antigenically distinct subtypes. Since HA subtypes of influenza A viruses are principally defined as serotypes determined by neutralization or hemagglutination inhibition tests using polyclonal antisera to the respective HA subtypes, which have little cross-reactivity to the other HA subtypes, it is generally believed that the neutralizing antibodies are not broadly cross-reactive among HA subtypes. Herein we present a novel cross-neutralizing monoclonal antibody that reacts with a variety of HA subtypes in vitro and provides heterosubtypic protection against influenza A virus infections in mice. We demonstrate that this antibody recognizes a common epitope adjacent to the receptor binding region of HA and inhibits virus binding to the cells. The present study supports the notion that cross-reactive antibodies, as well as cytotoxic T lymphocytes, play some roles in heterosubtypic immunity against influenza A virus infection, and underscores the potential therapeutic utility of cross-reactive monoclonal antibodies for multivalent prophylaxis and treatment against infection with influenza A viruses, including the hypothetical new pandemic influenza viruses.

In general, HA subtypes of influenza A viruses are principally defined as serotypes determined by neutralization or hemagglutination inhibition (HI) tests using polyclonal antisera to the respective HA subtypes, which have little cross-reactivity to the other HA subtypes. Furthermore, since the structures of HA antigenic sites vary among not only different subtypes of viruses but also the same subtype, it is generally believed that the neutralizing antibodies are not broadly cross-reactive among HA subtypes. Therefore, studies on cross-reactive HA-specific antibodies to multiple HA subtypes have been limited [12,13,14].

Recently, it has been shown that intranasal immunization with inactivated viruses provided heterosubtypic protection in a mouse model, suggesting a role for cross-reactive antibodies in the heterosubtypic immunity against influenza viruses [15,16,17]. In this study, we generated a novel cross-neutralizing monoclonal antibody (MAb) that reacts with a variety of HA subtypes by intranasal immunization of mice. This antibody recognizes a common epitope on the globular head region of HA and inhibits virus binding to the sialic acid receptors. The present study suggests the further potential of antibodies in heterosubtypic immunity against influenza A virus infection.

Results

Characterization of MAb S139/1 in vitro

MAb S139/1 (IgG2a) was originally produced as an H3 HA-specific antibody. The cross-reactivity of MAb S139/1 to multiple subtypes of influenza A virus HAs was then tested by enzyme-linked immunosorbent assay (ELISA) using several H1, H2, H3, H5, H9, and H13 subtypes (Fig. 1). We found that MAb S139/1 reacted with all influenza A virus but not B virus strains tested, with higher binding activities to the A/WSN RG/33 (WSN) (H1), A/Adachi/2/57 (Adachi) (H2), A/Aichi/2/68 (Aichi) (H3), and A/gull/Maryland/704/77 (Maryland) (H13) strains than to the other strains. We confirmed the specificity of MAb S139/1 by Western blotting using purified viruses. MAb S139/1 bound to HA molecules under non-reducing conditions, whereas it bound very weakly to HA1 but not HA2 under reducing conditions (data not shown), suggesting that MAb S139/1 recognized a conformational epitope on the HA1 subunit of the HA molecule.

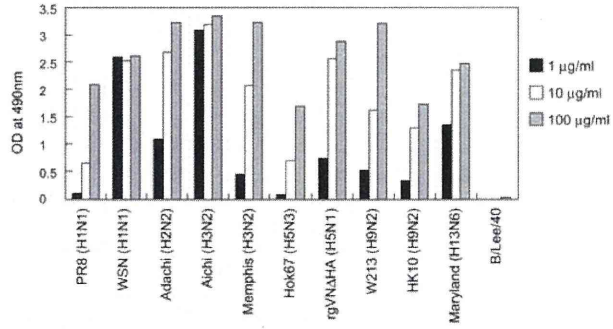

Figure 1. Reactivity of MAb S139/1 to various influenza virus strains. Binding activity of MAb S139/1 at the concentrations of 100 μg/ml (grey), 10 μg/ml (white), and 1 μg/ml (black) to the indicated virus strains was measured by ELISA as described in Materials and Methods.
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We next tested HI activity of MAb S139/1 to various influenza virus strains (Table 1). MAb S139/1 exhibited high HI titers to a particular H1 strain and most of the H3 strains tested, and moderate activity to H2 and H13 strains, but not to H5 and H9 or type B strains. Neutralizing activity of MAb S139/1 was then determined in vitro by a plaque reduction assay using Madin-Darby canine kidney (MDCK) cells (Fig. 2). Consistent with its reactivity profile in HI tests and ELISA, MAb S139/1 neutralized infectivity of WSN (H1), Adachi (H2), Aichi (H3), and Maryland (H13) strains. Relative neutralizing activities of MAb139/1 were also correlated with its reactivity to these viruses in the HI test and ELISA (i.e., MAb S139/1 neutralized Aichi (H3), WSN (H1), Adachi (H2), and Maryland (H13) in order of increasing activity). These results indicated that MAb S139/1 had a novel potential to neutralize the infectivity of multiple subtypes of influenza A viruses by inhibiting HA binding to the sialic acid receptors.

Protective Potential of MAb S139/1 in vivo

We then investigated the potential of MAb S139/1 to protect mice against influenza virus infection. Mice were passively immunized by intraperitoneal injection of purified MAb S139/1 one day before or after intranasal challenge with Aichi (H3) or WSN (H1). Control groups were given an irrelevant MAb (ZGP12/1.1) [18] or PBS alone. Protective efficacy was evaluated by titrating infectious virus in the lung tissues three days after challenge (Fig. 3). Mice pre-immunized with MAb S139/1 were almost completely protected from both Aichi (H3) and WSN (H1) infection, while these viruses were recovered from all the control mice at high titers (Fig. 3A). There was no significant difference between two control groups. Post-immunization with MAb S139/1 also significantly reduced both Aichi and WSN titers, as compared with control group ($p<0.05$ and $p<0.01$, respectively) (Fig. 3B). Importantly, virus was not detected from two of the five MAb S139/1-immunized mice infected with Aichi. These results indicate that passive immunization of mice with MAb S139/1 provided heterosubtypic protection against H1 and H3 viruses.

Amino Acid Substitutions of the Escape Mutants

To determine the epitope for MAb S139/1, escape mutants of Aichi (H3), WSN (H1), and Adachi (H2) were selected in the

Table 1. HI activity of MAb S139/1 with various influenza virus strains.

Virus	HI titer μg/ml [a]
A/PR/8/34 (H1N1)	>50 [b]
A/WSN RG/33 (H1N1)	1.56
A/Adachi/2/57 (H2N2)	12.5
A/Singapore/1/57 (H2N2)	6.25
A/duck/Hong Kong/836/80 (H3N1)	<0.39
A/Aichi/2/68 (H3N2)	0.78
A/Memphis/1/96 (H3N2)	>50
A/duck/Hokkaido/5/77 (H3N2)	12.5
A/chicken/Hong Kong/37/78 (H3N2)	<0.39
A/duck/Hokkaido/8/80 (H3N8)	<0.39
A/Hong Kong/483/97 (H5N1)	>50
A/rgViet Nam/1194ΔHA/2004 (H5N1)	>50
A/swan/Hokkaido/67/96 (H5N3)	>50
A/swine/Hong Kong/10/98 (H9N2)	>50
A/duck/Hong Kong/W213/97 (H9N2)	>50
A/duck/Hokkaido/49/98 (H9N2)	>50
A/gull/Maryland/704/77 (H13N6)	12.5
B/Lee/40	>50

[a]HI titers are expressed as the lowest concentrations of purified MAb S139/1 that completely inhibited hemagglutination.
[b]No detectable hemagglutination inhibition at 50 μg/ml by MAb S139/1.
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presence of this antibody as described in Materials and Methods. We confirmed that hemagglutination activities of these escape mutants were not inhibited by MAb S139/1 even at the concentration of 50 μg/ml. The nucleotide sequences of the HA genes of the parent strains and the escape mutants were determined and deduced amino acid sequences were compared among these viruses. We found amino acid substitutions at position 156, 158, or 193 (H3 numbering here and throughout the text) in these mutants (Table 2). Eleven and four escape mutants of WSN (H1) and Adachi (H2), respectively, all acquired the substitution at the same position, 193 (S193N and T193K, respectively). The amino acid residue at position 193 is located on the antigenic sites known as Sb and I-B of H1 [7] and H2 HAs [8], respectively, which correspond to HA antigenic site B of H3 HA [3]. On the other hand, the amino acid substitutions at position 156 (K156Q), 158 (G158E or G158R), or 193 (S193I or S193R) were found in the sixteen escape mutants of Aichi (H3). All these amino acid positions were involved in the conformation of HA antigenic site B [3].

Comparison of HA Amino Acid Sequences among Different HA Subtypes

We compared deduced amino acid sequences of the region including the MAb S139/1 epitope among H1, H2, H3, H5, H9, and H13 subtypes of HA (Fig. 4). Among these, WSN (H1), Adachi (H2), Aichi (H3), and Maryland (H13), which were neutralized by MAb S139/1, shared the amino acid sequence at positions 156, 158, and 193 (K, G and S/T, respectively), with one exception (N at position 158 in Maryland HA). By contrast, viruses that were not neutralized by MAb S139/1 possessed different sets of amino acids at positions 156, 158, and 193; A/PR/8/34 (PR8) (H1) (E, E and N), A/Memphis/1/96 (Memphis) (H3) (K, D, and T), A/Viet Nam/1194/2004 (VN1194) (H5) (K, N, and K), A/swan/Hokkaido/67/96 (Hok67) (H5) (K, N, and K) and, A/duck/Hong Kong/W213/97 (W213) (H9) (Q, N, and N), respectively.

Comparison of the Epitope Structure between Aichi and Other Viruses

In three-dimensional structural analysis of the Aichi (H3) HA molecule, positions of the amino acid substitutions found in the escape mutants were mapped in the globular head of the HA (Fig. 5A). We found that these three amino acids formed a conformational epitope that was adjacent to the receptor-binding site of the HA. We then compared the structure of this epitope among Aichi (H3), WSN (H1), Adachi (H2), PR8 (H1), and VN1194 (H5) HAs (Fig. 5). The epitopes formed by amino acid residues at positions 156, 158, and 193 were similar among Aichi (H3), WSN (H1), and Adachi (H2), whereas the structures of PR8

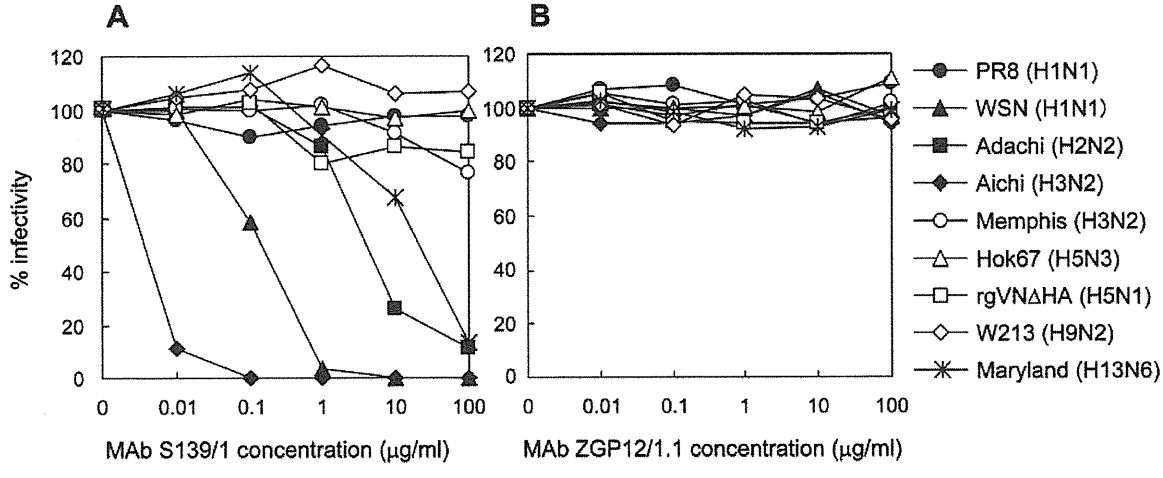

Figure 2. Neutralization activity of MAb S139/1 to various HA subtypes of influenza A virus strains. Viruses were mixed with indicated concentrations of the purified MAb S139/1 (A) or control IgG2a (ZGP12/1.1) [18] (B). Neutralization activities were evaluated by plaque reduction assays using MDCK cells.
doi:10.1371/journal.ppat.1000350.g002

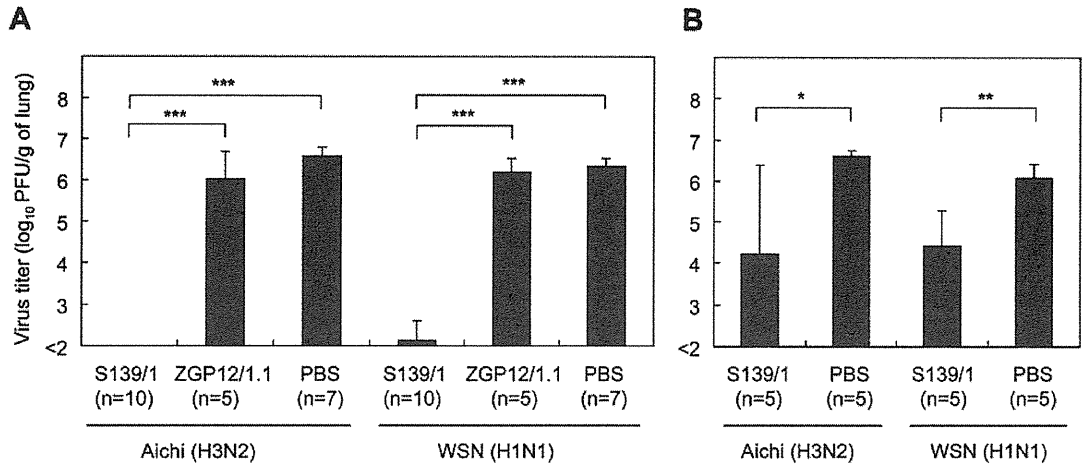

Figure 3. Protective efficacy of passive immunization with MAb S139/1 in mice. Mice were passively immunized before (A) or after (B) virus challenge with Aichi (H3) or WSN (H1). Control mice were given MAb ZGP12/1.1 or PBS. Virus titers of the lung were determined by a plaque assay. The means and standard deviations are shown. For statistical purposes, samples with undetectable virus titers were given the value 2.0 log_{10}PFU/g. The data of pre-immunized mice were analyzed using the nonparametric Kruskal-Wallis ANOVA on ranks, followed by the Mann-Whitney U-test with the Bonferroni correction for multiple comparisons. The data of post-immunized mice were analyzed using the Mann-Whitney U-test. Two-sided p values less than 0.05 were considered statistically significant. Significant differences were indicated by asterisks (*** $p<0.001$, ** $p<0.01$, * $p<0.05$). All statistical analyses were performed with the computer program R (version 2.8.1).
doi:10.1371/journal.ppat.1000350.g003

(H1) and VN1194 (H5) epitopes were distinct in the amino acid properties or side-chain orientations at positions 156, 158, and 193. A significant difference of amino acid properties at positions 158 and 193 between PR8 (H1) and Aichi (H3) was that the molecular sizes of amino acid residues E and N (PR8) were larger than G and S (Aichi). In VN1194 (H5) HA, a significant difference was found in side chain orientation, which was presumably because of electrostatic repulsion between the positively charged K156 and K193 side chains.

Neutralization of PR8 Mutants Possessing the Modified Epitope for MAb S139/1

To confirm the importance of the amino acid residues at positions 156, 158, and 193 for binding of MAb S139/1, we generated recombinant PR8 (H1) mutant viruses with modified epitopes whose amino acid sequences at these positions were replaced with those of Aichi (H3), and tested neutralizing activities of MAb S139/1 to the mutant viruses (Fig. 6). Of the seven mutants generated, three had a single substitution (E156K, E158G, or N193S), three had double substitutions (E156K/E158G, E156K/N193S, or E158G/N193S), and one had triple substitutions (E156K/E158G/N193S). We found that only the triple mutant was neutralized by MAb S139/1 (Fig. 6). Accordingly, MAb S139/1 bound to the triple mutant more efficiently than to the other mutants and parent PR8 in ELISA (data not shown). These results suggest that all three amino acids, K, G, and S at positions 156, 158, and 193, respectively, are equally important components to form this epitope (i.e., MAb S139/1 recognizes this conformational epitope through interaction with all three of these amino acid residues).

Table 2. Amino acid substitutions found in HA of WSN, Adachi, and Aichi escape mutants.

Virus	Amino acid substitution
WSN (H1)	S193N (11/11) [a]
Adachi (H2)	T193K (4/4)
Aichi (H3)	K156Q (1/16)
	G158E (10/16)
	G158R (1/16)
	S193I (2/16)
	S193R (2/16)

[a]Total numbers of escape mutants obtained in 2 independent experiments are shown (No. of variants/total escape mutants cloned.)
doi:10.1371/journal.ppat.1000350.t002

Discussion

It has been generally known that hetrosubtypic immunity can be provided by subtype cross-reactive cytotoxic T lymphocytes that recognize conserved epitopes of viral internal proteins of influenza A viruses such as nucleoprotein and matrix protein [19]. However, recent studies in mouse models suggest that humoral immunity, B cells and antibodies, also contribute to heterosubtypic protection [15,16,17,20]. In the present study, we obtained a novel monoclonal antibody, MAb S139/1, which was broadly cross-reactive to a variety of HA subtypes of influenza A viruses. MAb S139/1 most likely neutralized the viral infectivity by blocking receptor binding of the virus, since hemagglutination of the viruses was also inhibited by this antibody. Influenza virus HA subtypes are determined as serotypes based on their distinct antigenicities, and thus there are a limited number of studies reporting such heterosubtypic and cross-reactive MAb to HA [12,13,14,21,22].

For example, MAb IVA1B10 [12,21] and MAb HA1-66 [13,22], both of which recognize the HA1 region, were shown to react with H3, H4, H11, and H13 strains, but did not have HI and neutralization activities. Some MAbs recognizing the HA2 region were also shown to be cross-reactive among influenza virus strains of the same subtypes, and even among various subtypes [12,21,22,23]. However, these antibodies neither prevented hemagglutination nor neutralized infectivity of the viruses

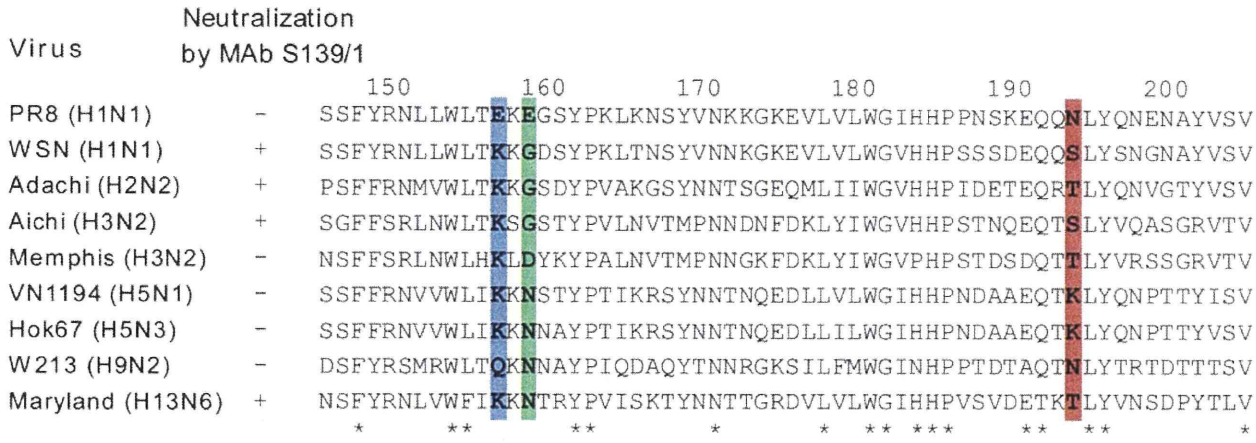

Figure 4. Comparison of the amino acid sequences of different subtypes of influenza A virus HAs. Amino acids at positions from 145 to 204 are shown. Boxed residues indicate the positions 156 (blue), 158 (green), and 193 (red). Asterisks indicate conserved amino acid residues among HA subtypes examined. Amino acid sequences for HAs, except Adachi (H2) and W213 (H9), were downloaded from the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) [48]. The NCBI accession numbers are CAA24272 (PR8), AAA43209 (WSN), AB432938 (Adachi), CAA24269 (Aichi), AAB63708 (Memphis), AAT7327 (VN1194), BAE48688 (Hok67), AB432937 (W213), and BAF46906 (Maryland).
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[12,21,22,23]. On the other hand, it was shown that cross-reactive MAb C179, specific to both H1 and H2 subtypes, neutralized viral infectivity [14], though this MAb did not show HI activity. It recognizes a conformational epitope consisting of HA1 and HA2 in the middle of the stem region, a conserved antigenic site among the subtypes, suggesting that inhibition of the low pH-induced conformational change followed by membrane fusion is the mechanism underlying the action of this antibody [14,24]. By contrast, this study for the first time demonstrates that there is a common epitope shared among multiple HA subtypes, which is recognized by a neutralizing antibody that prevents receptor binding of the virus.

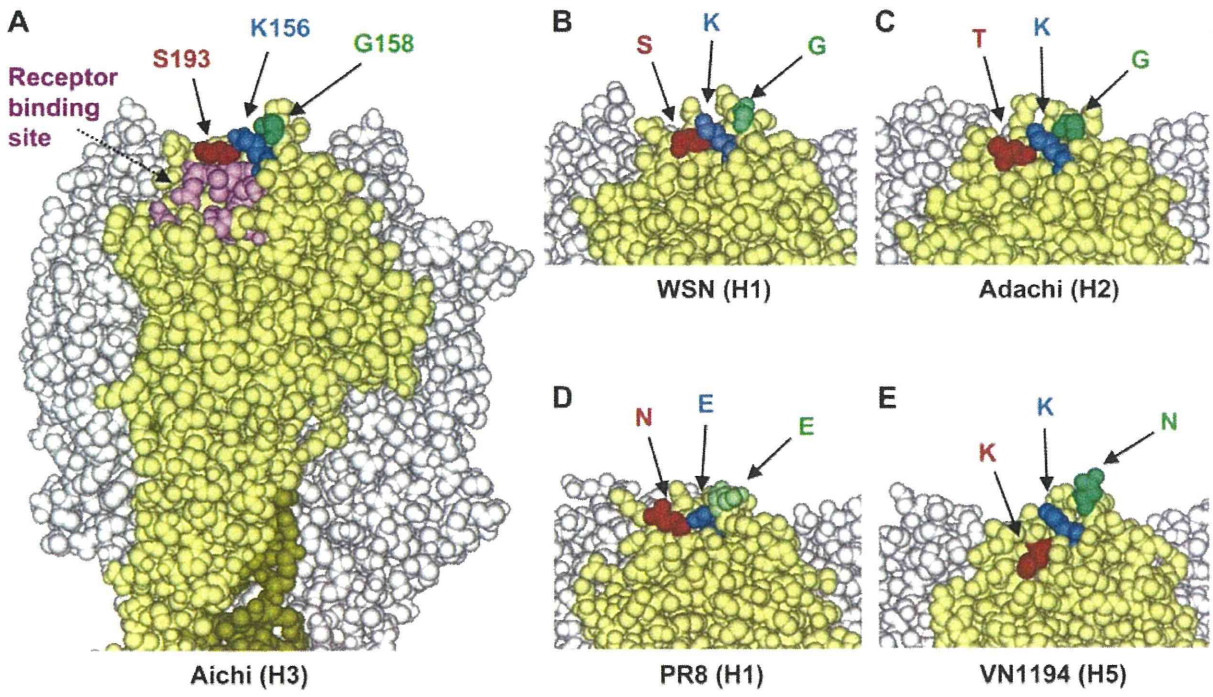

Figure 5. Structure of the MAb S139/1 epitope on the globular head of HA trimer models. Three-dimensional models of Aichi (H3) (A), PR8 (H1) (D), and VN1194 (H5) (E) HAs were constructed from the coordinates obtained from the Protein Data Bank (PDB codes: 1HGF, 1RVX, and 2IBX, respectively). The structures of WSN (H1) (B) and Adachi (H2) (C) were constructed by homology modeling as described in Materials and Methods. Images were prepared by using DS Visualizer (version 1.7, Accelrys, Inc.). Residue numbering is thoroughly on the basis of the H3 HA sequence.
doi:10.1371/journal.ppat.1000350.g005

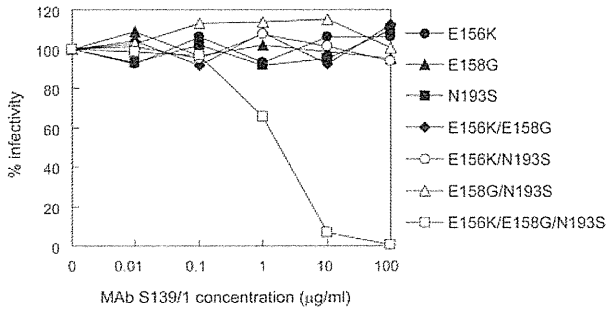

Figure 6. Neutralizing activity of MAb S139/1 to PR8 mutants with altered epitopes. Amino acids at positions 156, 158, and/or 193 of PR8 HA were substituted for by those at corresponding positions of the Aichi HA sequence. Other experimental conditions were described in Materials and Methods.
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In the present study, we found that MAb S139/1 bound to all the strains of H1, H2, H3, H5, H9, and H13 subtypes tested by ELISA, whereas it showed neutralization and HI activities to some particular strains tested. Since we assume that MAb S139/1 recognizes a single epitope on the HA molecules of all the subtypes, it is likely that the different binding affinity of MAb S139/1 to each HA subtype influences the neutralization and HI activities. Indeed, our data demonstrated that there was an appreciable correlation between its binding affinities tested by ELISA and by HI or the neutralization test. Furthermore, our sequence analyses and reverse genetics approaches revealed the major contribution of the HA amino acid residues at positions 156, 158, and 193 to the binding capacity of MAb139/1.

We found three independent substitutions at positions 156, 158, or 193 in the Aichi mutant viruses that escaped from neutralization by MAb S139/1. Three-dimensional structural analyses revealed a conformational epitope consisting of these amino acid residues. Of these, the amino acid residue at position 193 of H3 HA was shown to interact with the host receptor molecule (i.e., sialic acid-linked oligosaccharide) [5,25,26,27,28], suggesting the contribution of this residue to receptor binding of the HA. Accordingly, Aichi escape mutants with a substitution at position 193 (S193I and S193R) formed significantly smaller plaques than the Aichi parent virus (data not shown), which might have resulted from the reduced HA function of these mutants.

Three-dimensional structural analyses based on HA molecules cocrystallized with a sialylated glycan receptor analogue (pentasaccharide) suggest that molecular contacts between HA and the sialylated glycan receptor are divided into base and extension regions which include contacts with the terminal sialylgalactose moiety and the subsequent sugar rings, respectively [29,30], and that the sialylated glycan molecules bind to H1 and H3 HAs in different conformations (i.e., the H1 HA glycan binding site in the extension region form different conformation from that of H3 HA) [27,29,30,31,32,33]. Since the amino acid residue at position 193 of H3 HA seems to directly interact with a specific sugar ring in the extension region [27,31,33], mutation at this position of the H3 HA likely influence its receptor binding properties. Consistent with this hypothesis, an Aichi (H3) escape mutant (S193I) had reduced ability to grow in cell culture and in mice (data not shown). On the other hand, it has been suggested that the amino acid residues not only at position 193 but also at position 190 of H1 HA play a key role in interaction with the glycan in the extension region [27,29,31,32]. Since the amino acid substitution at position 190 of H1 HA is believed to be responsible for the alteration of receptor binding properties [29,31], a single mutation at position 193 found in WSN (H1) escape mutants might have a limited effect on the overall receptor binding capacity of the HA.

It is well-known that HAs of avian and human influenza viruses bind preferentially to α2-3 and α2-6 sialylated (SA) glycan receptors, respectively. Although it has been generally believed that amino acid substitutions at positions 226 and 228 are primarily responsible for the differences in the receptor specificity between avian and human H3 viruses, several studies have reported that the mutation at position 193 of H3 HA might also alter the receptor binding properties [5,31,33,34]. For example, S193R substitution on prototype H3 human virus HA altered binding specificity by acquiring the ability to agglutinate erythrocytes containing SAα2-3Gal linkage [5], while a single S193R substitution of H3 HA in a recent human virus enhanced SAα2-6Gal but not SAα2-3Gal recognition [34]. Thus, it is conceivable that H3 escape mutants that are naturally selected by an antibody recognizing the S139 epitope may have reduced receptor binding capacity and/or altered receptor specificity. Moreover, amino acid positions 155, 159, 190, and 225 (H3 numbering) of H1 HA, most of which cluster around this epitope, have also been demonstrated to influence receptor specificity [31]. Thus, it might be of interest to clarify whether the reactivity of MAb S139/1 to multiple HA suptypes is affected by changes of HA receptor recognition associated with substitutions around this epitope.

Recently, passive transfer of MAbs specific to viral proteins has been tested in clinical studies, providing models for the use of MAbs for prophylaxis or treatment of infectious diseases. In fact, a humanized MAb specific to RSV F protein is already approved by the US Food and Drug Administration and used in clinical cases. It has been experimentally shown that this approach is effective for influenza virus infection in mice [24,35,36,37]. The present study further indicated that the MAb S139/1 provided heterosubtypic protection of mice from H1 and H3 influenza A virus infection. It may be one of the options in the event of pandemic influenza [36]. Although MAb S139/1 neutralizes only particular strains of H1, H2, H3, and H13 subtypes, this antibody has binding capacity to other virus strains of different subtypes (Fig. 1). Since in vitro neutralization activity was not necessarily linked to the protective potential in vivo (e.g., non-neutralizing MAbs such as anti-HA2 MAbs [22,37] and anti-M2 MAbs [38,39,40] protected mice from lethal influenza A virus infection), it may be interesting to estimate the broader heterosubtypic protective efficacy of passive transfer of MAb S139/1 in animal models.

Together with previous studies by others [22,24,36,37, 38,39,40], the present study supports the notion that cross-reactive antibodies, as well as cytotoxic T lymphocytes, play some roles in heterosubtypic immunity against influenza A virus infection, and underscores the potential therapeutic utility of cross-reactive MAbs for multivalent prophylaxis and treatment against infection with influenza A viruses, including the hypothetical new pandemic influenza viruses.

Materials and Methods

Viruses and Cells

Influenza virus strains, A/PR/8/34 (PR8) (H1N1), A/WSN RG/33 (WSN) (H1N1), A/Adachi/2/57 (Adachi) (H2N2), A/Singapore/1/57 (H2N2), A/duck/Hong Kong/836/80 (H3N1), A/Aichi/2/68 (Aichi) (H3N2), A/Memphis/1/96 (Memphis) (H3N2), A/duck/Hokkaido/5/77 (H3N2), A/chicken/Hong Kong/37/78 (H3N2), A/duck/Hokkaido/8/80 (H3N8), A/Hong Kong/483/97 (H5N1), A/rgVietNam/1194ΔHA/2004 (rgVNΔHA)

(H5N1), A/swan/Hokkaido/67/96 (Hok67) (H5N3), A/swine/Hong Kong/10/98 (HK10) (H9N2), A/duck/Hong Kong/W213/97 (W213) (H9N2), A/duck/Hokkaido/49/98 (H9N2), A/gull/Maryland/704/77 (Maryland) (H13N6), and B/Lee/40 were used (Table 1). rgVNΔHA (H5N1) is a reassortant virus that has the modified H5 HA gene derived from A/Viet Nam/1194/2004 (VN1194) (H5N1) and all other genes from PR8. In this modified H5 HA, the original amino acid sequences at the cleavage site (PQRERRRKKRG) were replaced with those of A/teal/Hong Kong/W312/97 (H6N1) (PQIETRG). All infectious materials were handled in a biosafety level 2 or 3 facility under approved protocols in accord with guidelines of Hokkaido University. These viruses, except a highly pathogenic virus, A/Hong Kong/483/97, were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs at 35°C for 48 h (A/Hong Kong/483/97 was incubated in eggs for 36 hours). Some of these viruses were concentrated and purified by high-speed centrifugation of infected allantoic fluid passed through a 10 to 50% sucrose density gradient [16]. The purified viruses were resuspended in phosphate-buffered saline (PBS) and stored at −80°C until use. Madin-Darby canine kidney (MDCK) cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Monoclonal Antibodies (MAbs)

Six-week-old female BALB/c mice, were intranasally immunized twice at 2-week intervals with 100 μg of formalin (0.2%)-inactivated purified Aichi together with cholera toxin B (Sigma). Two weeks after the second immunization, the mice were intranasally boosted with inactivated virus alone. Three days later, the spleen cells from the mice and mouse myeloma Sp2/0 cells were fused and maintained according to a standard procedure [41]. Hybridomas were screened for secretion of anti-influenza-virus specific MAbs by enzyme-linked immunosorbent assay (ELISA), and then HA-specific MAbs were identified by Western blotting and immunostaining of 293T cells transfected with plasmids expressing Aichi HA. We further screened for cross-reactivity of the antibodies to other HA subtypes by ELISA, and obtained cross-reactive MAb S139/1 (IgG2a). The hybridoma producing MAb S139/1 (IgG2a) was cloned twice by limiting dilution of the cells. MAb S139/1 was purified from mouse ascites using protein A agarose columns (Bio-Rad).

Antibody Assays

ELISA was performed essentially as previously described [41]. Briefly, purified viruses were disrupted with 50 mM Tris–HCl (pH 7.8) containing 0.5% Triton X-100 and 0.6 M KCl, diluted by PBS, and used for antigen coating (20 μg protein/ml in PBS, 50 μl/well), followed by blocking with BSA. Binding of MAb S139/1 was detected by using peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson) and *o*-phenylendiamine dihydrochloride (Sigma). HI activity of the purified MAb was tested by the standard method using 0.5% chicken erythrocytes. Neutralizing activity of the MAb was measured by a plaque reduction assay using MDCK cells. Ten-fold dilutions of MAb were mixed with 100–200 plaque forming unit (PFU) of viruses and incubated for 1 h at room temperature. The confluent monolayers of MDCK cells on 12-well plates were inoculated with the mixture. After 1 h adsorption, the virus inoculums were removed and the cells were overlaid with MEM containing 1% Bact-agar and trypsin (5 μg/ml). The plaques were enumerated after incubation at 35°C, 5% CO_2 for 2 days. Western blotting was performed as follows. Virus proteins were separated by 10% SDS-polyacrylamide gel electrophoresis under reducing or non-reducing conditions, and transferred to PVDF membranes (Millipore). The membranes were blocked with 3% skim-milk (SM) in PBS containing 0.05% Tween-20 (PBST) and exposed to MAb S139/1 (1 μg/ml) in 1% SM-PBST, and then probed with horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson), and the reacted bands were visualized by Immunostaining HRP-1000 (Konica Minolta).

Passive Immunization and Protection Tests

The experimental protocols were reviewed and approved by the Hokkaido University Animal Care and Use Committee (08-0234). Six-week-old female BALB/c mice were passively immunized by intraperitoneal injection with 200 μg of purified MAb S139/1 or ZGP12/1.1 (IgG2a) [18] in 0.5 ml of PBS. Twenty four hours before or after immunization, mice were challenged intranasally with 50 μl of 10×50% mouse infectious dose of Aichi (H3) or WSN (H1) under anesthesia with isoflurane. Three days after the challenge, mice were euthanized to obtain the lung tissue samples. The lung homogenates (10% w/v) prepared in MEM were disrupted and centrifuged at 3,000×g for 10 min, and then the supernatants were examined for virus infectivity. Virus titers were measured by a plaque assay using MDCK cells.

Sequence Analysis of the HA Genes

Viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen). After reverse transcription with M-MLV reverse transcriptase (Invitrogen) using Uni12 primer (5′-AGCAAAAG-CAGG), HA genes were amplified by PCR using gene-specific primer sets [42]. PCR products were purified with a QIAQuick PCR purification kit (Qiagen) and nucleotide sequences were analyzed using a dye-terminator cycle sequencing system with an ABI sequencer (Perkin-Elmer, Applied Biosystems).

Selection of Escape Mutants

Escape mutants were selected by culturing WSN (H1), Adachi (H2), and Aichi (H3) strains in MDCK cells in the presence of MAb S139/1. Viruses were incubated with purified MAb S139/1 (final concentration of 10 μg/ml) for 1 h, and then the mixtures were inoculated into confluent MDCK cells in 6-well tissue culture plates. After 1 h adsorption, the cells were overlaid with MEM containing 1% Bacto-Agar (Difco) and MAb S139/1 ascites (final dilution of 1:200) and trypsin (5 μg/ml), and then incubated for 2 days at 35°C. Escape mutants were purified from single isolated plaques, and propagated in MDCK cells with serum-free MEM containing trypsin. The nucleotide sequences of the HA genes of the parent strains and the escape mutants were determined and deduced amino acid sequences were compared among these viruses.

Generation of Recombinant Mutant Viruses by Reverse Genetics

The plasmid pWH194-HA [37] expressing PR8 HA was modified using a QuickChange II Site-Directed Mutagenesis kit (Stratagene). HA mutant viruses were generated by the reverse genetics method as described previously [43]. Briefly, 293T and MDCK cells were cocultured on 6-well plate and transfected with a set of eight influenza virus plasmids allowing the rescue of the recombinant PR8 (H1N1) for generating all HA mutants. The recombinant viruses produced from transfection were amplified in MDCK cells, and stored at −80°C. The HA genes of the recombinant viruses were sequenced to verify the presence of the desired mutations and the absence of other changes.

Molecular Modeling

The HA structures of WSN (H1) and Adachi (H2) were constructed using Modeller 8v2 [44] based on the crystal structures of H1 (PDB code: 1RU7) and H5 (PDB code: 2FK0) HA molecules, respectively. After one hundred models of the HA trimer were generated, we selected the model with the best probability distribution function (PDF) score. The HA model was evaluated by using PROCHECK [45], WHATCHECK [46], and VERIFY-3D [47].

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Author Contributions

Conceived and designed the experiments: RY AT. Performed the experiments: RY HO NK DT AT. Analyzed the data: RY MI HK KI AT. Wrote the paper: RY MI HO AT. Performed the molecular modeling: MI.

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Novel trivalent anti-influenza reagent

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ABSTRACT

We designed and synthesized novel trivalent anti-influenza reagents. Sialyllactose was located at the terminal of each valence which aimed to block each receptor-binding site of the hemagglutinin (HA) trimer on the surface of the virus. Structural analyses were carried out with a model which was constructed with a computer simulation. A previously reported cyclic glycopeptide blocker [Ohta, T.; Miura, N.; Fujitani, N.; Nakajima, F.; Niikura, K.; Sadamoto, R.; Guo, C.-T.; Suzuki, T.; Suzuki, Y.; Monde, K.; Nishimura, S.-I. *Angew. Chem. Int. Ed.*, **2003**, *42*, 5186] bound to the HA in the model. The analyses suggest that the glutamine residue in the cyclic peptide bearing Neu5Acα2,3Galβ1,4Glc trisaccharide via a linker interacts with the Gln189 in HA through hydrogen bonding. The present anti-influenza reagents likely interact with a glutamine residue included in the vicinity of Gln189. A plague reduction assay of the influenza virus, A/PR/8/1934 (H1N1), was performed in MDCK cells to evaluate for the synthesized compounds to inhibit viral replication. One of the compounds showed approximately 85% inhibition at the concentration of 400 μM at 4 °C.

An influenza outbreak poses a significant threat to public the health worldwide as highlighted by the novel swine origin influenza A (H1N1). Since the first detection in April 2009 until November 2009, the human case of infection with the virus has been confirmed over 6770 deaths in 206 countries by the WHO.[1] The pandemic of novel H1N1 virus hasten the development of new anti-virus reagents that are effective for various subtypes of influenza A virus. Oseltamivir and zanamivir as neuraminidase (NA) inhibitors are now widely used as effective anti-influenza drugs. However, strains resistant to these inhibitors have been selected mutations of NA result in inhibitor resistance.[2,3] Therefore, novel inhibitors of influenza virus are strongly required.

Influenza virus expresses the other glycoprotein, hemagglutinin (HA), which plays a fundamental role in the initial step of the infection process.[4] HA binds to the receptor carbohydrate chain terminated with neuraminic acid on the host cell-surface. Thus, HA is a potential target of anti-influenza virus reagents. Inhibition of the binding between HA and the receptor on the cell-surface should prevent humans from being infected by virus. HA blocker is expected to act as a prophylactic reagent. The structure of the HA has been analyzed in detail by extensive biochemical and crystallographic studies.[5] These studies indicated that the HA has three identical receptor-binding sites on the top of the trimer that protruded from the viral envelope.[6] In addition, sialyllactose binds to the HA of influenza virus more strongly than one neuraminic acid unit.[7] It is important to efficiently occupy the binding sites of the HA. We have reported that a trivalent glycopeptide inhibits efficiently hemagglutination mediated by viral hemagglutinin.[8] Several groups also demonstrated that synthetic polymers bearing multiple neuraminic acids showed greatly enhanced affinity to influenza HA.[9] In recent years, multivalent blockers designed with polyamino acid have also been developed.[10] Since multivalent glycoconjugate blockers have densely displayed neuraminic acids at the terminal of the sugar chain, it is thought to amplify the affinity between the HA and the blockers. Since syntheses of glycoproteins and glycopeptides are quite expensive, complicated and difficult to complete in short period of time with the large quantity, it is defective for HA blockers as preventive medicines. In this Letter, we report the synthesis and evaluation of the inhibitory effect of novel trivalent blockers against influenza virus A/PR/8/1934 (H1N1).

We have developed much more simple and practical trivalent blockers with siallyllactose at each terminal of valence (Fig. 1). In our previous Letter, we reported that the monovalent glycopeptide showed no inhibition of hemagglutination. Therefore, in this study, we only designed trivalent blockers. These blockers were

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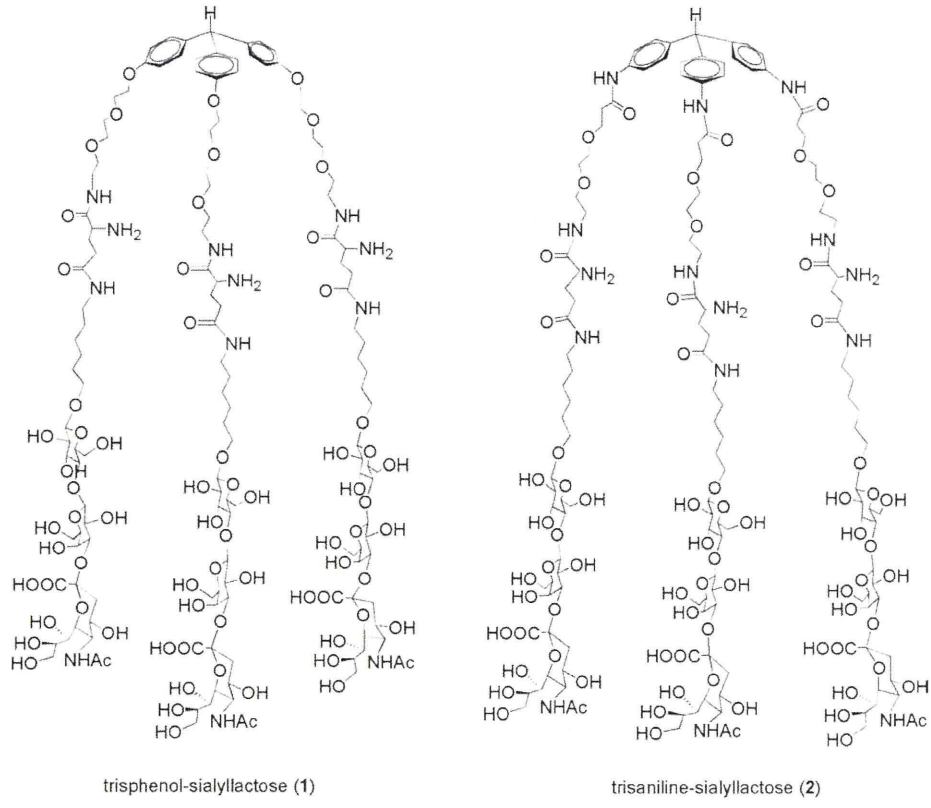


Figure 1. Chemical structures of novel trivalent anti-influenza reagetns.

designed with structure-based methods shown in Figure 2a and b. Geometrical observation was performed with the model structures which were constructed with the HA (PDBID is 1HGG) and our previously developed glycopeptides. The major character of the binding between HA and the blocker were kept in the model structure. Then novel alternative core component were

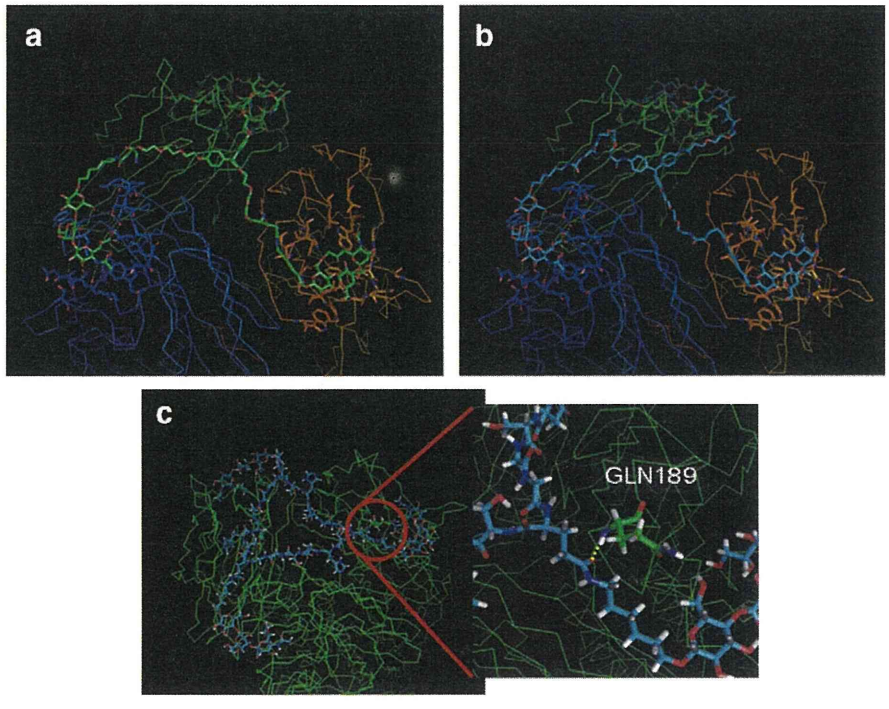


Figure 2. Model structures of binding between trivalent trivalent anti-influenza reagents and HA. (a) trisphenol type, (b) trisaniline type, and (c) previously synthesized HA blocker (Ref. 8).

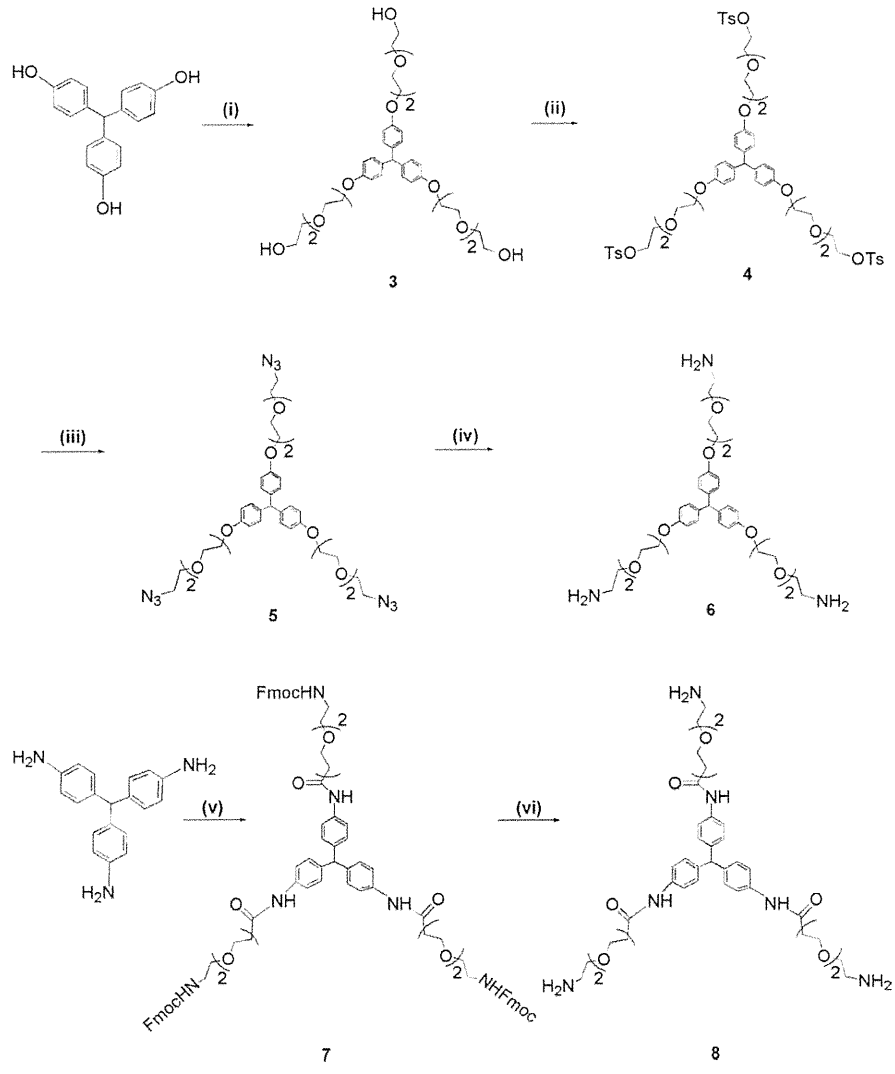
examined. The length of the linker was determined and the trivalent blockers were designed and synthesized. In order to induce an interaction with the Q189 residue as shown in Figure 2c, glutamine residue was introduced in the linker part as connectors between aglycon core and trisaccharides in addition to the interaction between sialyllactose and the HA receptor site.

Our designed compounds bound to the HA (PDB ID:1HGG) on model structures with computer simulations. We carried out short molecular dynamics simulations (200 ps) at 1000 K with the time step of 1 fs by using MMFF94S force fields. During the simulation, the geometrical position of sialylllactoses and the HA were fixed. The conformation with the lowest potential energy as shown in Figure 2 was selected and observed. This observation suggests that the lengths of the linkers and the position of the glutamine residue were appropriate and the designed compounds could bind efficiently with HA.

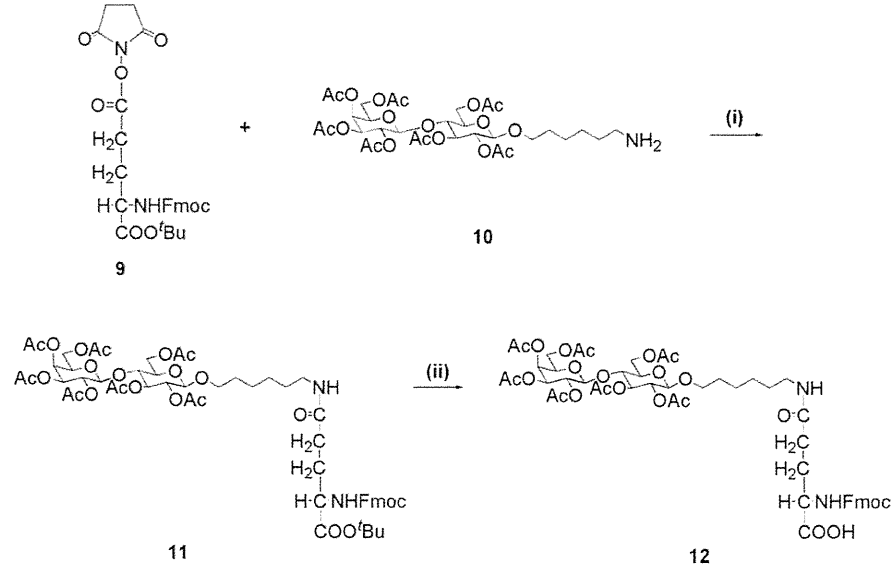
Chemical synthesis of the core parts of the HA blockers is outlined in Scheme 1. Trisphenol and trisaniline skeleton were adopted as the starting materials because both have rigid conformations because of the existence of a center sp^3 carbon, so three valences can spread out evenly. The flexible hydrophilic linker between trisaccharide and the core was built from oligoethyleneglycol structure derived from 2-[2-(2-chloroethoxy)ethoxy]ethanol and 3-{2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-ethoxy]-ethoxy}-propionic acid. First, **3** and **7** were synthesized according to the above design. Then the tosyl group was introduced into **3** as a leaving group in order to convert it to the azide compound **5** which was transformed to the trisamine **6** by a single reduction reaction. Another core aglycon **8** was built briefly in two steps by normal deprotection of Fmoc in **7**.

N-α-Fluorenylmethoxycarbonyl-*O*-*t*-butyl L-glutamic acid (Fmoc-Glu-O^{t}Bu) was activated into its *N*-succinimidyl ester intermediate **9** and coupled with the full-protected lactose derivative **10**[11] to give the key intermediate **11** which was converted effectively into the corresponding acid form **12** by TFA (Scheme 2).

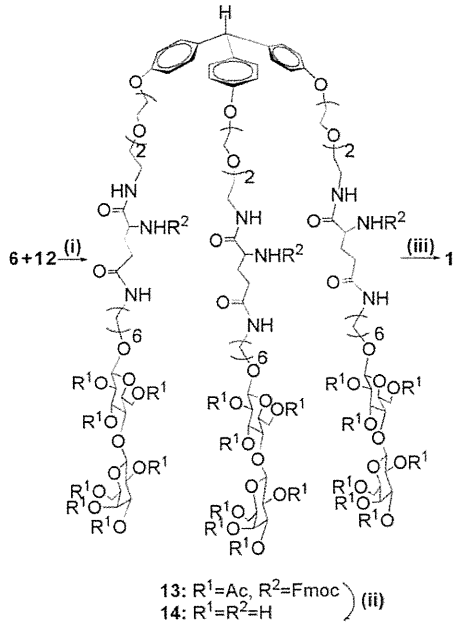
As depicted in Schemes 3 and 4, the key condensation reactions between trisamine (**6** or **8**) and acid **12** were carried out by the use of condensing agents EDC and DPPA, respectively. After the Fmoc protecting group at amino group and the acetyl group on sugar were removed smoothly, sialic acid was introduced by



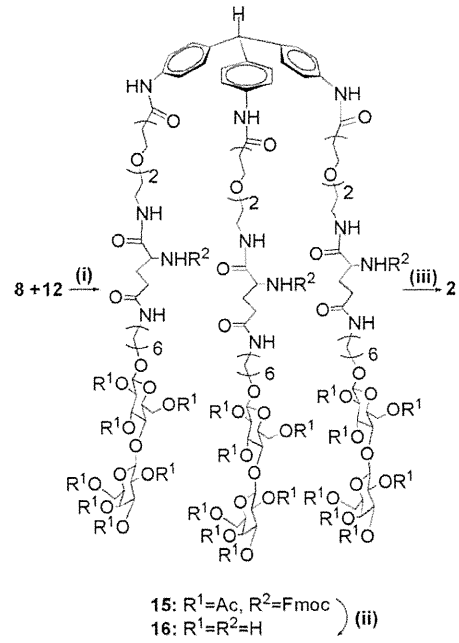
Scheme 1. Reagents and conditions: (i) 2-[2-(2-chloroethoxy)ethoxy]ethanol, K_2CO_3, KI, DMF, 100 °C, 46 h, 65%; (ii) TsCl, TEA, CH_2Cl_2, rt, 3 h, 67%; (iii) NaN_3, DMF, 60 °C, 9 h, 94%; (iv) H_2 gas, 10% Pd–C, MeOH/EtOAc = 2:1, rt, 2 h, 91%; (v) 3-{2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-ethoxy]- ethoxy}-propionic acid, DCC, DMAP, CH_2Cl_2, rt, 6 h, 83%; (vi) piperidine, CH_3CN–$CHCl_3$, rt, 1.5 h, 60%.



Scheme 2. Reagents and conditions: (i) TEA, $CHCl_3$, rt., 24 h, 56%); (ii) TFA, CH_2Cl_2, rt, 3 h, 94%.



Scheme 3. Reagents and conditions: (i) EDC, DMAP, CH_2Cl_2, rt, 22 h, 59%; (ii) (a) piperidine, CH_3CN, rt, 1.5 h; (b) NaOMe–MeOH, rt, 1 h, 87% (two steps); (iii) α2,3-(*N*)-sialyltransferase, CMP-NANA, cacolylate buffer, $MnCl_2$, 37 °C, 28 h, 45%.



Scheme 4. Reagents and conditions: (i) DPPA, TEA, DMF, rt, 18 h, 32%; (ii) (a) piperidine, CH_3CN, rt, 1.5 h; (b) NaOMe–MeOH, rt, 1 h, 80% (two steps); (iii) α2,3-(*N*)-sialyltransferase, CMP-NANA, cacolylate buffer, $MnCl_2$, 37 °C, 64 h, 46%.

an enzymatic method with α2,3-sialyltransferase under a mild condition to give the target trisphenol–sialyllactose **1** and trisaniline–sialyllactose **2**. Since α2,3-(*N*)-sialyltransferase was easy to purchase, we used the enzyme and synthesized the compounds with the Neu5Ac-α2,3Gal terminal. The final target compounds were purified by reverse phase high-performance liquid chromatography, followed by lyophilization. All of the structures of the synthesized compounds were confirmed by ^1H, ^{13}C NMR and MS measurements.[12]

Four synthesized substances, **1**, **2**, **14** and **16** showed higher water solubility, thus the test was facilitated in an aqueous system. The inhibition effects against human influenza A virus infection were investigated by means of inhibition of the cytopathic effect on MDCK cells.[13] The employed virus strain, A/PR/8/1934 (H1N1), recognized the receptor with saccharide terminated in Neu5Ac-α2,3Gal and Neu5Ac-α2,6Gal.[14] Since the amount of the synthesized compounds **1** and **2** is quite small, the inhibition activity against the concentration of the compounds could not be observed. Thus, we could not evaluate the IC_{50} of the inhibition. We evaluated the inhibition effect with MDCK cells at a certain concentration of 400 μM. As the results are shown in Table 1, **1** showed a significant inhibitory effect, particularly at lower temperature 4 °C, as the number of plaques was reduced and approximately 85% inhibition of plaque formation while **2** gave a weaker effect than **1**. Although **14** and **16** possess the same trivalent skeleton

Table 1
Blocking effects of novel trivalent blockers on virus replication

	Numbers of plagues at different adsorption temperature (percentage of plaque [%])	
	4 °C	35 °C
Trisphenol–sialyllactose (**1**)	9 × 10 (15.5)	38 × 10 (45.6)
Trisaniline–sialyllactose (**2**)	39 × 10 (67.2)	47 × 10 (56.6)
Trisphenol–lactose (**14**)	42 × 10 (72.4)	64 × 10 (77.1)
Trisaniline–lactose (**16**)	45 × 10 (77.6)	68 × 10 (81.9)
Virus only	58 × 10 (100.0)	83 × 10 (100.0)
No virus (inoculation)	0 (0.0)	0 (0.0)

as **1** and **2** with no neuraminic acid they indicated the small blocking effect. It suggests that there are no-specific interaction between **14** or **16** and virus. **1** showed a higher activity at a low temperature (4 °C) than it was at 35 °C. This suggests that neuraminidase activity was suppressed at this temperature. Terminal neuraminic acid might be partly cleaved by neuraminidase at 35 °C.

Unexpectedly, **1** and **2** exhibited no hemagglutination-inhibition assay (HI) activity (data not shown). In the molecular simulations, we investigated a geometrical aspect of the complex between the compounds and hemagglutinin. In this study, we challenged to simplify the complex HA blocker which previously designed. The compounds **1** and **2** were quite simple compared with the previously designed glycopeptides type HA blocker. Our compounds can suppress the replication of the influenza virus. It is necessary to elucidate the interaction between virus and the anti-influenza reagents. This study was the first step of such a computer assisted drug design. We could not perform the experiment to evaluate the interaction between virus and our synthesized compounds, because the amount of obtained compounds was quite small. We also could not determine the reason why **2** had less virus replication activity than **1**. The two compounds carried three equivalent neuraminic acid sugar units. They only differed in core structure. Exceedingly, the freedom of the sugar moieties in **2** was different from that of **1** because of the stiff amido bond, hence the trisaccharide in **2** was unable to approach the viral HA binding sites efficiently. Perhaps some other steric factors were limited the conformational flexibility of **2**.

In conclusion, novel trivalent anti-influenza reagents were designed and constructed effectively. The inhibition effects were examined in the virus replication of the A/PR/8/34 (H1N1) virus strain in MDCK cells. Based on the significant activity of trisphenol–sialyllactose **1** at 400 μM, this may be a potential candidate for anti-influenza drug. It is impressed that our compounds has inhibition of virus replication without HAI activity. It suggests that the further investigation to understand the detail of interaction between the anti-influenza blocker and virus were indispensable to better design of novel anti-influenza reagents. The temperature dependence of the MDCK assay indicated that the NA activity of the influenza virus removes the terminal neuraminic acid in each valence. Therefore, unnatural glycosidic bonds have to be examined to prevent the neuraminic acid being removed by NA. On the basis of the results from this study, we are examining the more effective anti-influenza reagents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.04.060.

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Influenza A Virus Polymerase Inhibits Type I Interferon Induction by Binding to Interferon β Promoter Stimulator 1*

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Type I interferons (IFNs) are known to be critical factors in the activation of host antiviral responses and are also important in protection from influenza A virus infection. Especially, the RIG-I- and IPS-1-mediated intracellular type I IFN-inducing pathway is essential in the activation of antiviral responses in cells infected by influenza A virus. Previously, it has been reported that influenza A virus NS1 is involved in the inhibition of this pathway. We show in this report that the influenza A virus utilizes another critical inhibitory mechanism in this pathway. In fact, the viral polymerase complex exhibited an inhibitory activity on *IFNβ* promoter activation mediated by RIG-I and IPS-1, and this activity was not competitive with the function of NS1. Co-immunoprecipitation analysis revealed that each polymerase subunit bound to IPS-1 in mammalian cells, and each subunit inhibited the activation of *IFNβ* promoter by IPS-1 independently. In addition, by a combinational expression of each polymerase subunit, IPS-1-induced activation of *IFNβ* promoter was more efficiently inhibited by the expression of PB2 or PB2-containing complex. Moreover, the expression of PB2 inhibited the transcription of the endogenous *IFNβ* gene induced after influenza A virus infection. These findings demonstrate that the viral polymerase plays an important role for regulating host anti-viral response through the binding to IPS-1 and inhibition of IFNβ production.

Influenza A virus, which belongs to the *Orthomyxoviridae*, is a negative sense, single-stranded RNA virus, which causes respiratory disease in humans. Influenza A virus carries eight segmented RNA as its genome, and viral proteins are translated from viral mRNA transcripted by the RNA-dependent RNA polymerase of influenza A virus. The influenza A virus polymerase complex is a heterotrimer consisting of PA (polymerase acidic protein), PB1 (polymerase basic protein 1), and PB2, and each component is crucial in order for the virus to replicate (1).

Type I interferons (IFNs) represented by IFNα and IFNβ are known to be important factors in the activation of host defensive mechanisms against invasion of exogenous pathogens. Type I IFNs are produced by various cells and activate transcription of IFN-stimulated genes through the IFNα receptor, JAK (Janus kinase), and STAT (signal transducers and activator of transcription) (2, 3). Production of proteins from IFN-stimulated genes is also important in the acquisition of resistance to influenza A virus infection. For instance, mouse *Mx1* (myxovirus resistance 1) or its human homologue *MxA*, which is known to be a member of the IFN-stimulated genes, displays a strong antiviral activity against influenza A virus infection by overexpression (4–6).

The induction pathway for Type I IFN is activated by cellular sensors, which recognize exogenous pathogens, such as Toll-like receptor family receptors (7, 8). Recently, the gene products of *RIG-I* (retinoic acid-inducible gene I) and *MDA5* (melanoma differentiation-associated gene-5) were identified as intracellular RNA sensors, which are responsible for the activation of IFN induction in cells infected with viruses (9, 10). These molecules recognize distinct viruses in each other (*e.g.* RIG-I recognizes *Flaviviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, and *Reoviridae*, whereas MDA5 recognizes *Picornaviridae*) (11–13) and bind to IPS-1 (IFNβ promoter stimulator 1; also called MAVS, Cardif, or VISA). IPS-1 is known to be a downstream mitochondrial adapter protein that transmits the induction signal of type I IFN through the activation of transcription factors IRF3 (IFN regulatory factor 3), IRF7, and NF-κB (nuclear factor κB) (14–17). These molecules are assumed to be physiologically important in the inhibition of viral replication; in fact, the functions of these molecules are inhibited by a variety of viruses (18–22). In addition, mouse embryonic fibroblasts derived from IPS-1-deficient mice do not completely produce type I IFN against influ-

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