

Hemagglutinin Immunoglobulin M (IgM) Monoclonal Antibody that Neutralizes Multiple Clades of Avian H5N1 Influenza A Virus

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Abstract

The hemagglutinin (HA) of influenza A virus plays an essential role in mediating the entry of the virus into host cells. In this study, 4 HA monoclonal antibodies (MAbs) of immunoglobulin M (IgM) isotype were generated by using recombinant full-length HA protein, which was expressed and purified from the baculovirus-insect cell system, from a H5N1 isolate (A/chicken/hatay/2004(H5N1)). Western blot analysis showed that these IgM MAbs bind the HA1 subunit and prevent HA-induced agglutination of erythrocytes. Consistently, the IgM MAbs inhibits the entry of HA pseudotyped lentiviral particles into Madin-Darby Canine Kidney (MDCK) cells. The most potent MAb, MAb 4F3, was further shown to efficiently neutralize multiple clades of H5N1 influenza A virus. To our knowledge, there are few studies documenting the properties of H5N1 neutralizing antibodies of IgM isotype. Thus, this panel of MAb adds diversity to the repertoire of broadly neutralizing monoclonal antibodies that are useful for developing novel therapeutics for combating future outbreaks of H5N1.

Keywords: Baculovirus; Hemagglutination inhibition; Neutralization activity; Immunoglobulin M (IgM) isotype; Monoclonal antibody; HA pseudotyped lentiviral particles

Introduction

The vast spread of avian H5N1 virus and 2009 pandemic influenza A (H1N1) virus across much of the globe highlights the vulnerability of humans to the emergence of the novel subtypes of influenza A virus, which belongs to the *Orthomyxoviridae* family. Studies confirm that there is an unseen network of influenza viruses among the migratory birds that span the world (Olsen et al., 2006). Since 2003, numerous countries in Asia, Europe and Africa have reported outbreaks of highly pathogenic avian H5N1 influenza virus among poultry flocks. H5N1 has engendered alarm not only because it is unusually virulent and causing severe economic losses but also because of reported infections in humans and other mammals. In order to prevent spread of influenza viruses, emphasis must be placed on biosecurity and flock management practices, the development of rapid diagnostics, novel antiviral therapy and vaccination strategies (Beigel et al., 2005; Lipatov et al., 2004; Peiris et al., 2007).

The hemagglutinin (HA) is the major surface glycoprotein of the influenza virus and is responsible for viral attachment to host cells and viral entry (Kilbourne, 1987). Hence, HA is the primary target

for novel antiviral therapy and vaccination strategies. The H5N1 viruses have now appeared in at least 53 countries on three continents and continue to evolve and diversify. Based upon the evolution of the HA gene, the H5N1 viruses could be grouped into numerous clades, representing emerging lineages and multiple genotypes (see recent review by (Guan et al., 2009)). This continuous evolution of the H5N1 virus, which is endemic in the poultry population of some countries, poses a challenge to the development of vaccine or antiviral therapy that has to remain efficient against newly emerged antigenic variants.

In this study, a panel of immunoglobulin M (IgM) monoclonal antibodies (MAbs) was generated using recombinant full-length H5N1 HA protein expressed using the insect cell-baculovirus system. The abilities of the MAbs to bind the HA protein and inhibit HA-induced agglutination of erythrocytes were evaluated. The neutralization activities of these MAbs were also determined using pseudotyped lentiviral particles expressing HA from the homologous H5N1 virus, which is A/chicken/hatay/2004(H5N1) (GenBank accession number AJ867074). The most potent IgM MAb was further tested for its specificity in preventing viral entry as well as its ability to neutralize multiple clades of H5N1 influenza A virus.

Materials and Methods

Immunization of mice and generation of hybridomas

Recombinant baculovirus carrying the HA gene from a H5N1 isolate (A/chicken/hatay/2004(H5N1), GenBank accession number AJ867074) was generated and used to express the HA protein as previously described (Shen et al., 2008). The purified HA protein was then used to immunize Balb/c mice and generate hybridomas as previously described (Lip et al., 2006).

Western blot analysis

The N- and C-terminal fragments of HA were expressed in *E.*

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coli and purified as previously described (Shen et al., 2008). Equal amount of the purified proteins were separated on SDS–polyacrylamide gels and transferred onto nitrocellulose Hybond C membrane (GE Healthcare, Uppsala, Sweden). The membranes were blocked with 5 % non-fat dry milk for 30 min, followed by overnight incubation at 4°C with the primary antibody. Then, the membranes were washed extensively with PBST (PBS containing 0.05 % Tween-20), followed by incubation with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce, Rockford, USA) for 1 h at room temperature, washing and detection using an enhanced chemiluminescence method (Pierce, Rockford, USA). The mouse anti-GST antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Hemagglutination inhibition assay

The purified recombinant full-length HA protein was previously shown to have hemagglutination activity (Shen et al., 2008). Due to the lack of high containment biosafety facility in our laboratories, we used the purified recombinant full-length HA protein to determine if the MAbs have hemagglutination inhibition activities. Hemagglutination assay was performed using 0.5% turkey red blood cells (RBCs) (i-DNA biotechnology Pte Ltd, Singapore) in a V-bottomed 96-well microtiter plate. First, 50 µl of the purified recombinant full-length HA protein was added to the first reaction well which was serially diluted 2 folds into subsequent wells. 50 µl of 0.5% turkey RBCs were added to each well and the plate was left undisturbed at room temperature for 30 min before it was viewed under a microscope. The amount of HA required to achieve 4 HA units was then used for the hemagglutination inhibition assay where MAbs were mixed with HA for 1 h at room temperature before 0.5% turkey RBCs were added. Each MAbs was serially diluted 2 folds before mixing with HA.

Neutralization assay using pseudotyped lentiviral particles

Due to the lack of high containment biosafety facility in our laboratories, the neutralization titers of the MAbs were determined using pseudotyped lentiviral particles expressing HA (abbreviated as HA-pp). HA-pp was produced in 293T co-transfected with pXJ3'-HA and pNL4-3.Luc.R-E- plasmids (Connor et al., 1995; He et al., 1995) with one modification from the method previously described (Shen et al., 2008). The modification involved transfection of another plasmid pXJ3'-NA instead of using recombinant neuraminidase to aid viral release from the 293T cells. The pXJ3'-NA was constructed using the neuraminidase gene from A/chicken/hatay/2004(H5N1) (GenBank accession number AJ867075). The following HA genes were used to generate HA-pp: A/chicken/hatay/2004(H5N1) (GenBank accession number AJ867074); A/Vietnam/1203/2004(H5N1) (GenBank accession number AY818135); A/Indonesia/05/2005(H5N1) Los Alamos database number ISDN125873); A/India/2006(H5N1) (GenBank accession no. EF362418). Pseudotyped lentiviral particles expressing the spike protein of the severe acute respiratory syndrome coronavirus was generated as previously described (Åkerström, 2009) and used to test the specificity of the MAb's neutralizing activity. In this case, Chinese Hamster Ovarian (CHO) cells stably expressing ACE2, CHO-ACE2 cells, were used for infection as previously described (Åkerström, 2009).

All MAbs were heated at 56°C for 0.5 h before use. MAbs were diluted 2-fold serially in DMEM medium starting from dilution 1 in 20. Equal volume (0.2 ml) of pseudotyped viruses was mixed

with the diluted antibodies and incubated at room temperature for 1 h. The antibody-virus mixtures were added to Madin-Darby Canine Kidney (MDCK) cell layer in wells of 24-well plates and incubated at 37°C for 2 days. The infected cells were detached from the wells with 0.125% trypsin/versene, transferred to 1.5 ml tubes and washed 2 times with PBS by centrifugation at 6,000 rpm. Cell pellets from each well were treated with 120 µl of Cell Culture Lysis Reagent (Promega) and incubated on ice for 10 min. The cell lysates in 1.5 ml tubes were centrifuged at 13,000 rpm at 4°C for 20 min. Supernatant of cell lysate was added to duplicate wells of 96-well white opaque plates (Costar, USA). Equal volume (50 µl) of Luciferase Assay Substrate (Promega) was added to each well and density was read immediately using Microplate Lumimometer (Turner Biosystems, Research Instruments). Samples derived from cells infected with pseudotyped virus without viral envelope were used as negative control. Infectivity was measured by luciferase activity and expressed as percentage of the luciferase activity in the absence of antibody.

Quantitative determination of IgM antibody in ascites fluid

Quantitative determination of IgM antibody in ascites fluid was performed using the mouse IgM ELISA kit (Alpha Diagnostic International, San Antonio, TX, USA) according to the manufacturer's instructions.

Purification of IgM antibody from ascites fluid

Purification of IgM antibody from the ascites fluid was performed using the IgM HiTrap columns (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The concentration of the purified antibody was determined using Coomassie Plus reagent from Pierce (Rockford, IL, USA).

Results

A panel of IgM MAbs with the ability to bind to the HA1 subunit

The HA protein is synthesized as a precursor form (HA0) that is then cleaved by host proteases into the disulfide-linked HA1 and HA2 subunits, resulting in membrane fusion potential and virus infectivity (Klenk et al., 1975; Lazarowitz and Choppin, 1975). In this study, we generated a panel of IgM MAbs using recombinant full-length HA, which was expressed using the insect cell-baculovirus system, of a clade 1 influenza A (H5N1) virus. The HA1 and HA2 fragments were expressed separately as GST-fusion proteins and used to test the reactivities of these MAbs. Western blot analysis showed that all 4 IgM MAbs, namely 4F3, 4F8, 5D9 and 6C1, bound to the HA1 subunit but not the HA2 subunit (Figure 1). The anti-GST antibody could detect both the HA1 and HA2 fragments as they were expressed as GST-fusion proteins. Coomassie staining showed that the HA1 and HA2 proteins are of high purity.

HA MAbs inhibit the hemagglutination activity of the HA Protein

As insect cells have similar protein processing capabilities to that of higher eukaryotes (Kost et al., 2005), the baculovirus-expressed recombinant HA proteins have been shown to be biologically active. For example, the immunogenicity of the antigen produced by this system was demonstrated in clinical trials for a vaccine formula carrying baculovirus-expressed recombinant HA of a H5N1 isolate (Treanor et al., 2001). We have also previously shown that

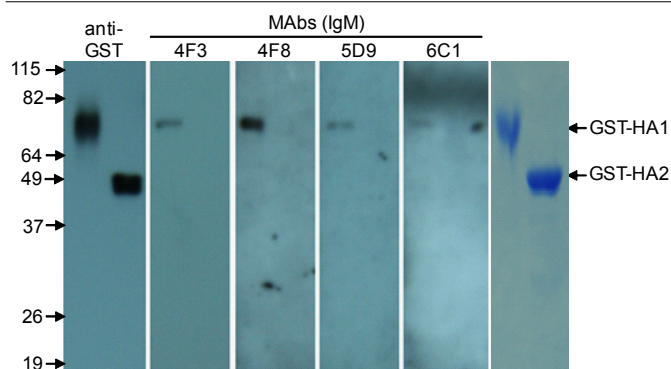


Figure 1: Reactivities of MAbs to HA1 domain.

Purified fragments of HA corresponding to the HA1 and HA2 domains were separated on SDS-polyacrylamide gels and analyzed in Western blots using ascites fluid for each MAb. For the last gel, Coomassie blue staining was performed to ascertain the purity of the HA1 and HA2 proteins used.

our purified recombinant HA is immunogenic and has the ability to cause agglutination of erythrocytes (Shen et al., 2008).

As the hemagglutination activity of the HA protein is dependent on the interaction between receptor binding domain in HA1 and sialic acid receptors on the surface of the erythrocyte, the HA MAbs were tested for hemagglutination inhibition activity. As shown in Figure 2, ascites fluid of 4F3, 5D9 and 4F8 exhibited hemagglutination inhibition activity at dilutions of 1:128 and below while ascites fluid of 6C1 exhibited hemagglutination inhibition activity at dilutions of 1:64 and below. On the other hand, an irrelevant IgM MAb did not have hemagglutination inhibition activity.

HA MAbs inhibit the entry of HA-pseudotyped virus into MDCK cells

Next, the neutralization titers of the MAbs were determined using pseudotyped lentiviral particles carrying HA from the homologous A/chicken/hatay/2004(H5N1) virus (abbreviated as Hatay-HA-pp) as such viral particles has been shown to have similar cell entry characteristics as live H5N1 influenza virus (Alberini et al., 2009; Nefkens et al., 2007; Temperton et al., 2007). As shown in Figure 3, the ascites fluid of the HA MAbs prevented the entry of

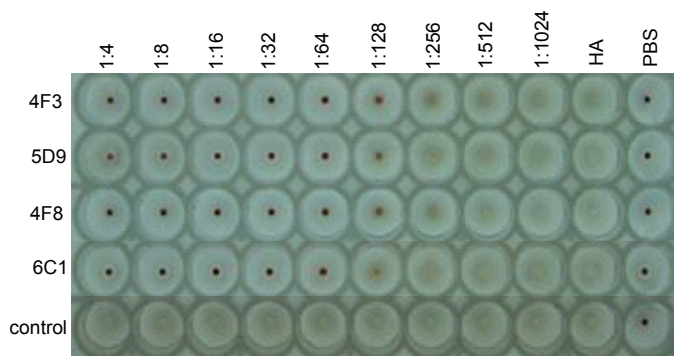


Figure 2: Hemagglutination inhibition activities of MAbs.

Equal amounts of purified recombinant full-length HA protein were incubated with different dilutions of ascites fluid for each MAb for 1h. Then, they were incubated with turkey red blood cells for 30 min at room temperature and observed under a microscope for hemagglutination. "Control" refers to an irrelevant MAb of IgM isotype while "PBS" refers to turkey red blood cells treated with PBS only.

Hatay-HA-pp by more than 50% at dilutions of 1:20 to 1:80.

MAb 4F3 inhibits the entry of pseudotyped viruses carrying HA from multiple clades of avian H5N1 influenza a virus

From the experiments performed using ascites fluid (Figures 2 and 3), it appears that the MAbs 4F3, 5D9, 4F8 and 6C1 have similar neutralization activities. An ELISA assay was used to quantify the amount of antibody in the ascites fluid. The results showed that MAb 4F3 has the lowest antibody concentration (data not shown), suggesting that it is most efficient in inhibiting viral entry. Thus, MAb 4F3 was purified from ascites fluid and re-tested in the neutralization assay. As shown in Figure 4A, MAb 4F3 inhibited the viral entry of Hatay-HA-pp by more than 50% for antibody concentrations greater than 4 µg/ml. To determine if the inhibition by MAb 4F3 is specific, neutralization assay was performed using pseudotyped virus carrying the spike protein of the severe acute respiratory syndrome coronavirus (SARS-S-pp). Although MAb 4F3 has some effect on the viral entry of SARS-S-pp, it did not reduce the viral entry by more than 50% at any of the concentrations tested (Figure 4A).

Finally, pseudotyped viruses carrying HA from other isolates of H5N1 were generated. These are A/Vietnam/1203/2004(H5N1) (clade 1; abbreviated as VN1203-HA-pp), A/Indonesia/05/2005(H5N1) (clade 2.1; abbreviated as IN5/05-HA-pp) and A/India/2006(H5N1) (clade 2.2; abbreviated as India-HA-pp). Interestingly, 4 µg/ml of MAb 4F3 inhibited the viral entry of all these isolates by more than 50% (Figure 4B). On the other hand, an irrelevant IgM MAb did not reduced viral entry of any of the isolates significantly (Figure 4B).

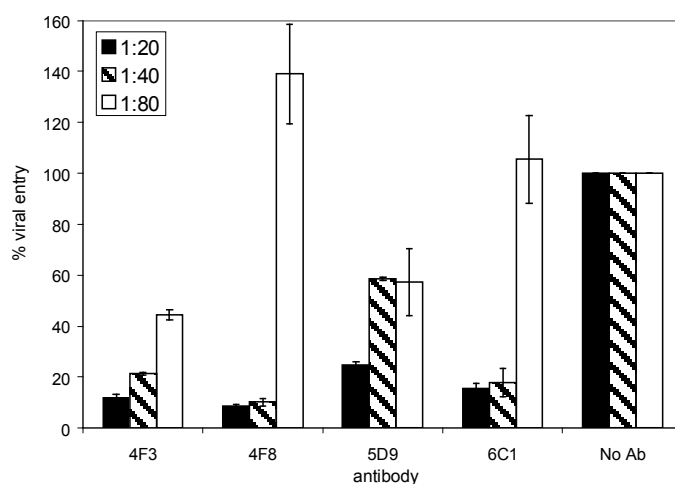


Figure 3: Neutralization activities against Hatay-HA-pseudotyped virus.

Equal amounts of pseudotyped virus carrying the HA protein of A/chicken/hatay/2004(H5N1) were incubated with ascites fluid for each MAb (at 1:20, 1:40 or 1:80 dilution) at room temperature for 2 h. The mixtures were inoculated into monolayer of MDCK cells and luciferase activity of cell lysates was determined 2 days later. Luciferase activity was expressed as percentage of those of inoculations in the absence of antibody, which is considered 100%. The experiments were repeated more than three times. Data are means of duplicate wells of one representing experiment. The error bars represent the standard errors of the mean.

Discussion

Currently, the control of influenza viruses relies on two options, vaccination and treatment with small molecule antiviral drugs like the M2 ion channel inhibitors and neuraminidase inhibitors. However, there is evidence that polyclonal immunoglobulins isolated from convalescent donors conferred protection against highly pathogenic influenza A viruses like the 1918 pandemic strain or newly-emerged H5N1 strain (Luke et al., 2006; Zhou et al., 2007). As the

use of polyclonal immunoglobulins for treatment has numerous problems, monoclonal antibodies that can neutralize viruses are emerging as promising new antiviral therapeutics (see recent reviews by (Lanzavecchia et al., 2007; Zhu et al., 2006)).

In this study, a panel of IgM MAbs has been generated from mice immunized with recombinant full-length HA of H5N1 influenza A virus expressed in insect cells. They were found to bind the HA1 domain, prevent HA-induced agglutination of erythrocytes and inhibit the entry of pseudotyped lentiviral particles expressing the homologous HA protein into MDCK cells. Interestingly, IgM antibodies have been shown to play protective roles in influenza A virus mice challenge studies (Baumgarth et al., 2000; Harada et al., 2003).

Recently, Throsby and co-workers isolated MAbs from combinatorial libraries built from IgM⁺ B cells in recent seasonal influenza vaccines and showed that these MAbs can neutralize many subtypes of influenza A virus including H5, H1, H2, H6, H8 and H9 (Throsby et al., 2008). These MAbs bind to a highly conserved antigenic site in the stem domain of HA. The most potent IgM MAb, MAb 4F3, generated in this study was further evaluated and found to prevent the viral entry of pseudotyped lentiviral particles expressing heterologous HA proteins from different clades of H5N1 influenza A virus, including a clade 2.2 virus isolated in India in the year 2006 (Ray et al., 2008). Consistently, this result suggests that MAb 4F3, which was produced using HA protein from a clade 1 virus, binds to a well-conserved epitope in the HA1 domain and can probably neutralize multiple clades of H5N1 influenza A virus. Thus, it seems that IgM⁺ B subset contains a diverse repertoire of antibodies against conserved epitopes in the HA protein. As most of H5N1 neutralizing MAbs characterized so far are of IgG isotypes, the panel of HA IgM MAbs described here contributes to the diversity of MAbs that may be useful for the development of passive antibody therapy. Future studies will be performed to determine their protective efficacies in animal challenge models.

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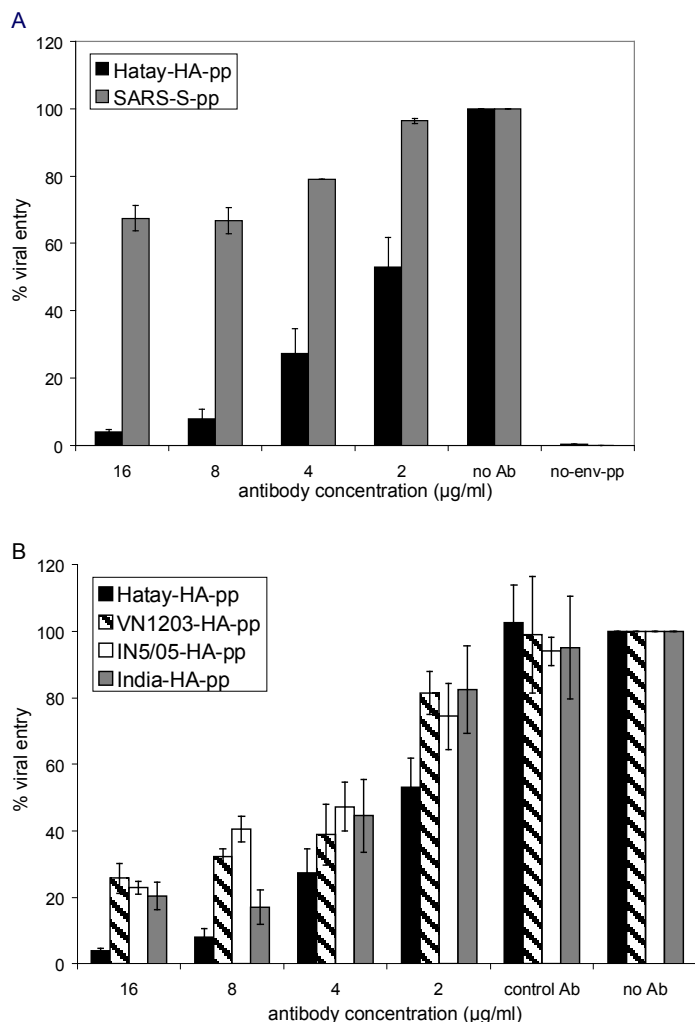


Figure 4: Specificity and cross-clade neutralization activity of MAb 4F3.

(A) Neutralization assays were performed as in figure 3 except that purified MAb 4F3 was used. Different concentrations of MAb 4F3 were used to inhibit the entry of Hatay-HA-pp into MDCK cells or SARS-S-pp into CHO-ACE2 cells. MDCK and CHO-ACE2 cells were also infected with pseudotyped virus without envelope protein (no-env-pp). (B) Different concentrations of MAb 4F3 were used to inhibit the entry of pseudotyped viruses carrying HA from other isolates of H5N1. These are A/Vietnam/1203/2004(H5N1) (clade 1; abbreviated as VN1203-HA-pp), A/Indonesia/05/2005(H5N1) (clade 2.1; abbreviated as IN5/05-HA-pp) and A/India/2006(H5N1) (clade 2.2; abbreviated as India-HA-pp). An irrelevant MAb of IgM isotype was used as negative control. For both (A) and (B), luciferase activity was expressed as percentage of those of inoculations in the absence of antibody, which is considered 100%. The experiments were repeated more than three times. Data are means of duplicate wells of one representing experiment. The error bars represent the standard errors of the mean.

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