Monoclonal antibody, but not synthetic peptide, targeting the ectodomain of influenza B virus M2 proton channel has antiviral activity

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SUMMARY

The proton channels of influenza A virus (A/M2) and influenza B virus (BM2) are essential for viral replication. Previously we have shown that monoclonal antibodies targeting the ectodomain of the A/M2 proton channel have antiviral activity *in vitro*. In this study, we generated both monoclonal antibody and phage displayed peptide against the eight amino acids comprising the ectodomain of the BM2 proton channel and investigated their antiviral activities *in vitro*. A cytopathic assay showed that the monoclonal antibody potently protected MDCK cells from homologous, but not heterologous, virus infections. A plaque forming assay showed that viral replication was not completely neutralized, but greatly inhibited, by the monoclonal antibody. In contrast, no antiviral activity was observed for the synthetic native or engineered peptides. These results indicate that antibody targeting the M2 proton channel is a promising therapeutic candidate for treating influenza virus infections, and that antibody structure is important for antiviral activity.

KEY WORDS: Influenza B, M2, Monoclonal antibody, Peptide, Antiviral

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INTRODUCTION

The proton channels of influenza A virus (A/M2) and influenza B virus (BM2) are essential for viral replication and are among the smallest *bona fide* ion channel proteins (Pinto and Lamb, 2006a). Both proteins are homotetrameric, type III integral membrane proteins possessing the properties of ion selectivity and activation, which are essential for dissociation of virus-specific ribonucleoprotein (vRNP) from the M1 protein during the uncoating process and for the preservation of the native conformation of hemagglutinin (HA) during transport to the cell surface (Mould *et al.,* 2003; Pinto and Lamb, 2006b). The predicted A/M2 and BM2 proteins comprise N-terminal ectodomains of 24 (A/M2) or 8 (BM2) residues, membrane-spanning domains of 20 residues for both proteins, and long cytoplasmic tails of 53 (A/M2) or 82 (BM2) residues (Pinto and Lamb, 2006b).

We and others have demonstrated that antibodies targeting the N terminal of the A/M2 protein ectodomain potently inhibited the replication of influenza A viruses *in vitro* and *in vivo* (Treanor *et al.,* 1990; Liu *et al.,* 2003; Liu *et al.,* 2004; Wang *et al.,* 2008; Wang *et al.*, 2009). The inhibition mechanism has not yet been elucidated, but is likely to occur via blocking of the ion channel function. Although the A/M2 and BM2 amino acid sequences share little homology, the two proteins possess similar structural and functional properties (Balannik *et al.,* 2008).

During the past two decades, there have been efforts to develop universal vaccines and therapeutic agents against influenza A virus infection

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which have targeted the ectodomain of A/M2 protein (M2e), however, none of these studies identified the BM2 proton channel as an antiviral target. Moreover, the antiviral drug amantadine only inhibits the ion channel activity of A/M2, not BM2 (Pinto *et al.,* 1992; Paterson *et al.,* 2003).

In this study, we produced a monoclonal antibody (mAb) and a phage displayed peptide against the eight amino acids that comprise the ectodomain of BM2 protein. We demonstrated that the mAb, but not the synthetic native or engineered peptides, had antiviral activity against influenza B viruses *in vitro*.

MATERIALS AND METHODS

Cells, viruses and peptides

Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (ExCell Bio, New Zealand). Influenza viruses B/Lee/40, B/Shanghai/6001/06 and A/WSN/33(H1N1) were used in this study. A/WSN/33 viruses were generated by reverse genetics as described previously (Neumann *et al.*, 1999).

Peptides BM2e-BSA (MLEPFQIL conjugated with bovine serum albumin (BSA) at the C terminal), BM2e-Ligand (VSFTPSF) and BM2e-Ligand-MAP (multiple antigenic peptide-conjugated BM2e-Ligand) were synthesized with a purity >95% by ShineGene Bio-Technologies, Inc. (Shanghai, China).

Production of monoclonal antibodies

The specific anti-BM2e mAbs were produced as previously described (Wang *et al.,* 2009). In brief, Balb/c mice were immunized three times with BM2e–BSA protein at two-weekly intervals. Mouse myeloma cells (SP2/0) were fused with spleen cells of the immunized mice. BM2e–BSA positive and BSA negative mAb-secreting hybridomas were screened by ELISA and then amplified.

The isotype of the screened mAbs was determined using mouse mAb isotype reagents (Sigma). The crude ascitic fluids were precipitated by $(NH_4)_2SO_4$, followed by purification using a HiTrap IgM purification column according to manufacturer's instructions (Amershan Bio Sciences, Sweden). The antibody concentration was titrated using a BCA Protein Assay Kit (Bio-Rad).

Phage display assay

Screening of the seven-mer (Ph.D.-7™) random phage display peptide library (New England Biolabs) was performed as described by Zou *et al*. (Zou *et al.,* 2008). Briefly, a microtiter plate (Costar) was coated with 100 mg/ml of BM2e–BSA peptide overnight at 4ºC. After blocking with 5 mg/ml BSA, the Ph.D.-7™ phage display peptide library was added and three biopannings were performed according to the standard procedure provided by the manufacture. For the third-round panning, the titer of the eluate was directly determined without amplification. The separate blue plaques were amplified and sequenced. The target of the selected phage was confirmed by ELISA according to the manufacturer's instructions (New England Biolabs). BM2e-BSA and BSA proteins were coated parallely in microtiter plate. After blocking with BSA, amplified phage were 10-fold serial diluted from 1×10^{13} colony forming units (cfu) to 1×10^{7} cfu and were used to inoculate each well of the microtiter plate. Mouse anti-M13 phage mAb (New England Biolabs) was used to detect bound phage and peroxidase-conjugated goat anti-mouse immunoglobulins (Invitrogen) were used as the second antibody. The plate was visualized by addition of o-phenylenediaminde dihydrochloride peroxide solution (Sigma) and stopped by addition of 3% H_2SO_4.

Inhibition assay

An inhibition assay based on determining a cytopathic effect (CPE) was performed as described by Schmidtke *et al*. with modifications (Schmidtke *et al.,* 2001). Briefly, purified mAb BM2e-2 was two-fold diluted from 10 µg/ml to 0.313 µg/ml, and peptides BM2e-Ligand and BM2e-Ligand-MAP were 10-fold diluted from 10 µM to 1 nM in DMEM. An unrelated peptide, the heptide repeat 2 region (HR2) of Spike protein derived from coronavirus strain 229E, and mAb against the HR2 peptide were used as controls. The solutions were incubated with MDCK cells for four days to observe their cytotoxicity. The solutions were then mixed with 50 of 50% tissue culture infective doses ($TCID_{50}$) of B/Shanghai/

6001/06, B/Lee/40 and A/WSN/33 viruses and incubated for 30 min at 37°C. The mixture was then used to inoculate MDCK cells and was incubated for 1 h before being replaced by fresh medium containing 0.5% BSA and 1.5 µg/ml of TPCK treated trypsin (Thermo Scientific). Seventy-two hours later, the cells was washed and stained with crystal violet staining buffer.

The optical density (OD) value at 630 nm was quantified using a spectrophotometer (Thermo Scientific). For each treatment, two parallel wells were prepared. All experiments were repeated three times independently. The protective rate was calculated as described previously (Wang *et al.*, 2009). For the plaque form unit (PFU) assay, MDCK cells were infected with 100 pfu of antibody or peptide treated B/Lee/40 virus as described above and an overlay containing 0.8% agarose was added. Six days later, the agarose overlay was removed and the cells were stained with crystal violet.

RESULTS

Generation of mAbs against the ectodomain of BM2 protein

We previously showed that BSA-conjugated short peptides were immunogenic and effective for screening mAbs (Wang *et al.*, 2009). In this study, we conjugated the eight amino acids that comprise the ectodomain of BM2 protein to the carrier protein BSA for generation of anti-BM2e mAbs. Three BM2e–BSA positive, but BSA negative, mAb secreting hybridomas were obtained, one of which, designated BM2e-2, which secreted IgM mAbs, was subcloned and used to produce ascitic fluids. The crude ascitic fluids were further purified and examined by SDS-PAGE (Figure 1).

Generation of peptide ligand to BM2e peptide

The seven-mer random phage display peptide library was used for BM2e–BSA ligand mapping. After three rounds of panning, three specific peptide sequences, HAIYPRH, VSFTPSF and AMSSRSL, were obtained by sequencing 30 phage colonies. ELISA showed that only the VSFTPSF peptide, designated BM2e-Ligand, was a ligand to BM2e peptide. The other two peptides

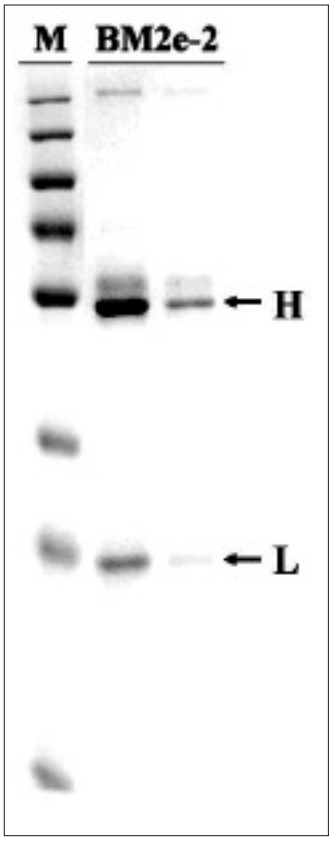

FIGURE 1 - *Purification of mAb BM2e-2 by ion-exchange chromatography. Molecular marker, heavy chain (H) and light chain (L) are indicated.*

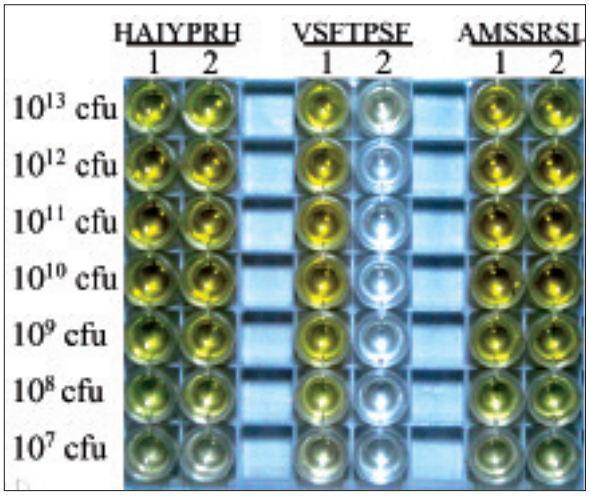

FIGURE 2 - *Verification of the phage bound target by ELISA. BM2e-BSA (1) or BSA (2) proteins were used to coat a microtiter plate. Three recombinant phages with the insertion sequences HAIYPRH, VSFTPSF and AMSSRSL, at concentrations ranging from 10^{13} cfu to 10^{7} cfu, were added. Bound phages were detected using the anti-M13 phage mAb.*

were ligands to BSA protein and were therefore excluded from subsequent studies (Figure 2).

Inhibition of virus replication by the mAb, as determined by the CPE assay

The antiviral activity of the mAb BM2e-2, the synthetic peptide BM2e-Ligand and BM2e-Ligand-MAP, were investigated by a standard CPE assay. The cytotoxicity was examined on MDCK cells prior to viral challenge. No obvious cell lysis or inhibition of cell growth was observed for the mAb or peptide-treated cells, even at a mAb concentration of 10 μg/ml or a peptide concentration of 10 μM (data not shown). B/Lee/40, B/Shanghai/6001/06 and A/WSN/33(H1N1) viruses were then pretreated with the mAb or peptide solutions and used to challenge MDCK cells. As shown in Figure 3, mAb BM2e-2 efficiently inhibited influenza B virus replication by preventing MDCK cell lysis caused by viral challenge. The mAb protected 80-95% of MDCK cells from infection of influenza B viruses B/Lee/40 and B/Shanghai/

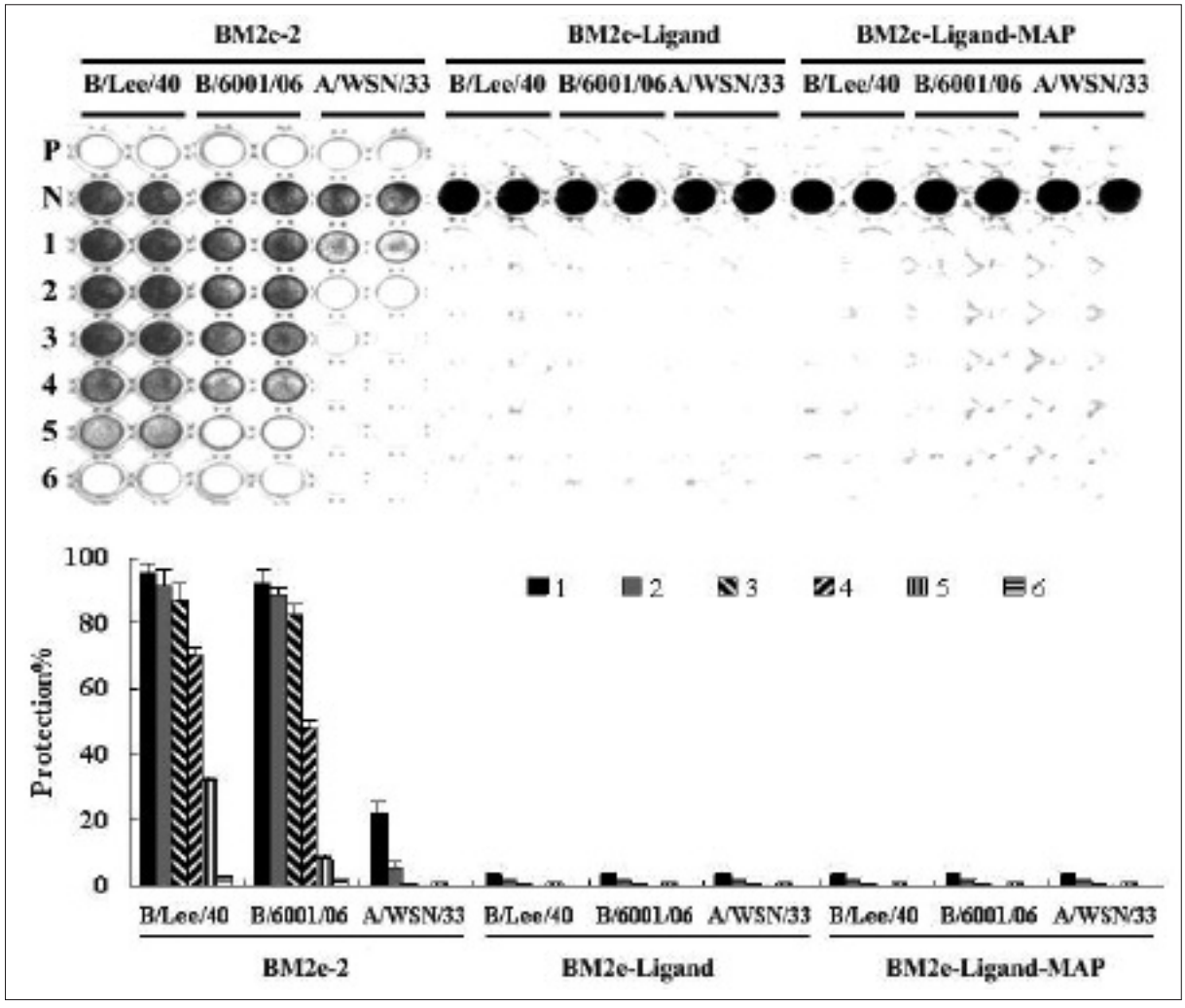

FIGURE 3 - *Inhibition of influenza virus replication by mAb BM2e-2, peptide BM2e-Ligand and BM2e-Ligand-MAP. The MDCK CPE assay (upper panel) and the % protection (lower panel) are shown. P: positive control, 50 $TCID_{50}$ viruses treated with anti-HR2 mAb (for BM2e-2, 10 μg/ml) or HR2 peptide (for BM2e-Ligand and BM2e-Ligand-MAP); N: negative control, cells treated with anti-HR2 mAb or HR2 peptide; 1-6: viruses pretreated with mAb BM2e-2 at a concentration of 10, 5, 2.5, 1.25, 0.625 and 0.313 μg/ml, or with peptide at a dilution of 100 μM, 10 μM, 1 μM, 100 nM, 10 nM and 1 nM (BM2e-Ligand and BM2e-Ligand-MAP), respectively. Error bars indicate the standard errors for the double treatments in three individual experiments.*

6001/06 at concentrations of 10 µg/ml to 2.5 µg/ml. Over 70% and 30% protection were observed against B/Lee/40 infection at mAb concentrations of 1.25 µg/ml and 0.625 µg/ml, respectively. However, the mAb provided only about 50% and 10% protection to B/Shanghai/6001/06 virus infection at concentrations of 1.25 µg/ml and 0.625 µg/ml. Little antiviral activity was observed at mAb concentration of 0.313 µg/ml. Little inhibitory effect was observed against A/WSN/33 virus infection for the mAb. In contrast to the antiviral activity detected with mAb BM2e-2, no obvious antiviral activity was observed for the synthetic peptide BM2e-Ligand. Similarly, no antiviral activity was observed with the eight BM2e-Ligand peptides conjugated to a lysine chain (BM2e-Ligand-MAP), even at the highest concentration of 100 µM (Figure 3).

Inhibition of virus replication by the mAb, as determined by the PFU assay

Antibodies against the influenza A virus M2 proton channel can inhibit virus replication rather than neutralize the virus (Zebedee and Lamb, 1988). To investigate the neutralizing properties of mAb BM2e-2 and synthetic peptide BM2e-Ligand, we performed a PFU assay against B/Lee/40 virus which steadily forms plaques in MDCK cells. As shown in Figure 4, no significant difference was found in the total number of plaques formed by B/Lee/40 virus treated with or without mAb BM2e-2. However, the size of the plaques was significantly inhibited by mAb BM2e-2 treatment. B/Lee/40 virus generally formed plaques of around 5 mm in diameter (Figure 4.2). However, mAb BM2e-2 treated viruses formed plaques less than 1 mm in diameter (Fig. 4.3). In contrast, no significant inhibitory effect was observed for the BM2e-Ligand peptide treated B/Lee/40 virus both in the total number of plaques and the size of the plaques (Figure 4.4).

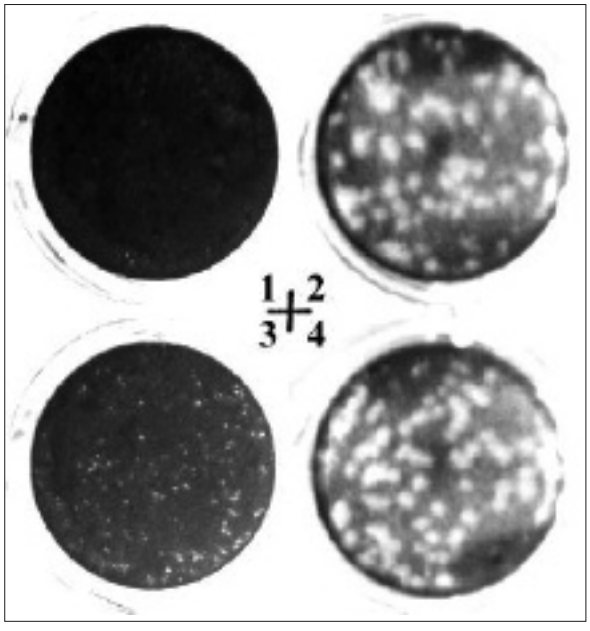

FIGURE 4 - *Inhibition of plaque size by mAb BM2e-2. 1: MDCK cell control; 2: MDCK cells infected with 100 pfu of B/Lee/40 virus; 3 and 4: B/Lee/40 viruses pretreated with mAb BM2e-2 at a five-fold dilution (3) or peptide BM2e-Ligand at 100 M (4).*

DISCUSSION

The highly pathogenic avian influenza viruses pose a substantial threat to human health, especially those from wild birds (Niqueux *et al.,* 2010). In contrast, influenza B viruses are known to predominantly infect humans and generally cause mild disease. In some cases, however, influenza B virus infections can lead to severe disease requiring hospitalization (Nicholson *et al.,* 2003). The frequent antigenic drift and reassortment that occurs among different strains of influenza B virus make it a significant potential threat to human health (McCullers *et al.,* 2004). Vaccination is the only means of protecting individuals against influenza B virus infection. Moreover, the presence of influenza antibodies in vaccinated people significantly reduced the severeness in patients with chronic obstructive pulmonary disease (Anar *et al.,* 2010). Antiviral drugs against influenza A viruses do not inhibit influenza B virus infections (Palese, 2006). Previously, our group and others reported that mAbs targeting the ectodomain of the influenza A virus M2 ion channel displayed antiviral activities (Treanor *et al.,* 1990; Liu *et al.,* 2003; Liu *et al.,* 2004; Wang *et al.,* 2008; Wang *et al.,* 2009). Here we report that a mAb against the eight amino acids that comprise the ectodomain of influenza B virus effectively inhibits virus replication *in vitro*. Although mAb BM2e-2 showed no neutralizing effect in a PFU assay, a common feature of antibodies against the influenza A virus

M2 proton channel (Zebedee and Lamb, 1988), it greatly inhibited the enlargement of plaques. Furthermore, protection of cells from virus infection in a CPE assay demonstrated the potent antiviral activity of the mAb BM2e-2. The ectodomain of influenza B virus M2 protein is remarkably conserved among strains of all lineages, and it may therefore be a potential candidate for antibody therapy against seasonal and emerging reassorted influenza B virus infections.

In contrast to the antiviral activity of mAb BM2e-2, the synthetic peptide ligand of the M2 protein ectodomain failed to inhibit influenza B virus replication in MDCK cells in both the CPE and PFU assays. Although the structure of the A/M2 proton channel has been elucidated (Sakaguchi *et al.,* 1997; Stouffer *et al.,* 2008; Schnell and Chou, 2008), little is known about the structural and functional properties of BM2. It is likely that BM2 protein can be chemically cross-linked by oligomerization to form a tetrameric structure (Paterson *et al.,* 2003; Balannik *et al.,* 2008). However, unlike A/M2, which has disulfide bonds in the ectodomain to stabilize the homotetramer, BM2 does not contain cysteine residues in its ectodomain and the structure of BM2 is likely to differ greatly due to the absence of disulfide bonds (Balannik *et al.,* 2008). Antibodies are large molecules with stable structures that have potential advantages for the blocking of ion channel function. In this study, we also attempted to develop a peptide inhibitor against the BM2 proton channel. We generated peptide ligand to the ectodomain of BM2 protein using phage display techniques, and engineered this peptide into a more complex structure by conjugation with MAP. However, no antiviral activity was detected for the BM2e peptide ligand and the engineered peptide. Further studies to analyze the structural basis of the interaction between the peptide ligand and the BM2 ion channel are required.

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