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Effective intranasal therapeutics and prophylactics with monoclonal antibody against lethal infection of H7N7 influenza virus

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ABSTRACT

Recurrence of highly pathogenic avian influenza (HPAI) virus subtype H7 in humans and poultry continues to be a serious concern to public health. No effective prevention and treatment are currently available against H7 infection. One H7 monoclonal antibody, Mab 62 was selected and characterized. Mab 62 presented efficient neutralization activity against all six representative H7 strains tested, including the H7N9 strain from the recent outbreak in China. The epitope of 62 identified on H7 HA1 exists in all the human H7 strains, including the recent H7N9 strains from China. Mab 62 when administered passively, pre or post challenge with 5 MLD50 (50% mouse lethal dose) HPAI H7N7 influenza viruses could protect 100% of the mice from death. The efficacy of intranasal administration of the Mab was evaluated versus the intraperitoneal route. In the therapeutic study, body weight loss and virus load were reduced in intranasally inoculated mice, as compared to the intraperitoneal group. Intranasal administration results in early clearance of the virus from the lungs and completely prevents lung pathology of H7N7. The study confirmed that intranasal administration of Mab 62 is either an effective prophylactic or therapeutic means against H7 lethal infection. The results of epitope analysis suggest the potential of Mab 62 to be used for the efficacious prevention and treatment against the recent human H7N9 strains.

1. Introduction

Human cases and pandemic potential caused by highly pathogenic avian influenza (HPAI) viruses continue to pose a significant threat to public health (Horimoto and Kawaoka, 2001). Human infections with avian influenza virus have been mainly caused by viruses of the H5, H7, and H9 subtypes (Malik Peiris, 2009). The most recent outbreak of H7N9 strains in eastern China resulted in more than 130 human cases, including 37 deaths, making H7 subtype HPAI viruses the focus of public attention. Before 2002, only occasional case reports of human H7 influenza virus infections occurred as a result of direct animal-to-human transmission or laboratory accidents (Webster et al., 1981) and most of these infections resulted in conjunctivitis and/or mild influenza-like illness (Lee et al., 2006; Spackman et al., 2003). Outbreaks associated with human infections were reported in 2002 and 2003 in the United States, 2004 in Canada, in 1995 and 2006–2007 in the United Kingdom, in 2002 in Italy, and in 2003 in the Netherlands (Belser et al., 2009). The 2003 outbreak in the Netherlands was caused by an HPAI H7N7 subtype and was the source of infection for 89 people exposed to affected poultry, including one death and three cases of possible human-to-human virus transmission (Belser et al., 2007; Fouchier et al., 2004). An increase in isolation of avian influenza A H7 viruses from poultry outbreaks and humans has raised concerns that additional zoonotic transmissions of influenza viruses from poultry to humans will occur (Koopmans et al., 2004; Lee et al., 2006; Velumani et al., 2008). In previous studies, the virus isolated from the fatal case in 2003, A/Netherlands/219/03 (NL219) caused a lethal infection in a mouse model, which presented with increased replication efficiency and a broader tissue distribution than nonlethal isolates from the same outbreak.

Currently, there are no effective treatment and prevention agents available against H7 infection (Swayne, 2012). Vaccine strategies requiring endogenous synthesis of antibodies will not provide the immediate protection needed against H7 infections in the event of a pandemic. Isolation of drug-resistant viral strains in the recent past years and the risk of side effects from drugs warrant an urgent search for alternative strategies for treatment and prophylaxis. Antibody based therapy is one of the alternative approaches for the immunoprophylaxis or the treatment of influenza and other infections. Unfortunately, the polyclonal immunoglobulins are less than ideal therapies due to limited supply, cost and

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toxicity issues. The pooled sera used to manufacture immunoglobulin also carry the potential risk of contamination with blood-borne pathogens. Therefore, passive administration of monoclonal antibodies against neutralizing epitopes of H7 may be an attractive alternative for passive vaccination of humans, in particular for those individuals who are at high risk from influenza infection such as immuno-compromised patients or the elderly who do not respond well to active immunization (Casadevall et al., 2004; Keller and Stiehm, 2000).

Monoclonal antibody based prophylactics and therapeutics can be further optimized by using a mucosal administration strategy (Ye et al., 2010). Intranasal administration is useful for the treatment of infectious respiratory diseases in the upper airways where antibody is most important for protection against viral infection (Weltzin and Monath, 1999). Intranasal administration presents advantages over subcutaneous or intramuscular injection by its ease of delivery, non-pain procedure and low risk of allergy or other side effects (Vujanic et al., 2012). Murine monoclonal antibodies are accepted to be administrated to humans via the mucosal route without humanization (Weltzin and Monath, 1999), a procedure which is costly and time-consuming (Marasco and Sui, 2007; Weltzin and Monath, 1999). The aim of this study is to develop mucosal treatment and prevention methods against HPAI H7 virus using monoclonal antibody. In the presented research, a H7 neutralizing monoclonal antibody 62 was characterized and selected for passive immunization against H7N7 AIV lethal challenge in mice. The efficacy of the Mab in prophylactics and therapeutics via either intranasal or intraperitoneal route was evaluated. Based on the sequence analysis of the 62 epitope and the neutralization test, it was suggested that Mab 62 can also be used for effective treatments against the recent human H7N9.

2. Materials and methods

2.1. Ethics statement

All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of the National Institute of Infectious Diseases (NIID). Experimental protocols were reviewed and approved by Institutional Animal Care and Use Committee of the Temasek Life Sciences Laboratory, National University of Singapore, Singapore. (IACUC approval number TLL-12-012). Mice were housed in individually ventilated cages (Tecniplast Sealsafe) provisioned with water and standard food, and monitored daily for health and condition. More than 25% body weight loss was used as the criterion for early euthanasia. The animals were euthanized by CO_2 inhalation for 5 min.

2.2. Viruses and cell lines

H7N1 (A/Chicken/Malaysia/94) was obtained from the Agri-Food and Veterinary Authority of Singapore. Reassortant influenza virus H7N9 (A/Shanghai/1/13), H7N3 (A/Canada/rv504/04), H7N6 (A/quail/Aichi/3/09), H7N7 (A/duck/Hokkaido/1/10) and H7N7 (A/Netherlands/219/03) were generated by reverse genetics as described previously Ho et al. (2009). Briefly, the complementary DNA of the HA and NA genes of H7 viruses were synthesized based on the sequences from the NCBI influenza database while the six cDNAs of the internal genes were synthesized based on the PR8 (A/Puerto Rico/8/1934) virus sequence (GenScript, USA). The cDNA of each of the eight influenza virus gene segments was inserted between the pol I promoter (pIh) and the pol I terminator of pClpolsaplT vector (kindly provided by Ruben Donis, CDC, USA) and cotransfected into cocultured 293T human embryonic kidney cells and Madin–Darby canine kidney (MDCK) cells using lipofectamine 2000 (Life Technologies, USA). MDCK cells were maintained in Dulbeccos Modified Eagle Medium (DMEM; Life Technologies, USA) containing 10% Fetal Bovine Serum (FBS; Life Technologies, USA). 293T were maintained in Opti-MEMI (Life Technologies, USA) containing 5% FBS. After 48 h the transfected supernatants were collected and virus titers were determined by standard hemagglutination assays. The sequences were confirmed using a specific set of universal primers as described previously Prabakaran et al. (2009a). Viruses were propagated in 10 days old specific pathogen free embroyonated chicken eggs at 37 °C. The tissue culture infectious dose 50 (TCID50) of reassortant virus was then calculated by the Muench–Reed method (1938).

All experiments involving human H7 strains were performed in a biosafety level 3 (BSL-3) containment laboratory in compliance with CDC/NIH and WHO recommendations and were approved by the Agri Veterinary Authority (AVA) of Singapore.

2.3. Production and characterization of Mab

BALB/c mice were immunized twice subcutaneously at intervals of 2 weeks with BEI (binary ethylenimine) inactivated H7N1 (A/Chicken/Malaysia/94) and adjuvant (SEPPIC, France). Mice were boosted with the same viral antigen, 3 days before the fusion of splenocytes with SP2/0 cells (He et al., 2009). The fused cells were seeded in 96-well plates, and their supernatants were screened by immunofluorescence assays as described below. The hybridomas that produced the Mabs were cloned by limiting dilution at least three times. The positive Mabs were tested for their hemagglutination inhibition activity as described below. Immunoglobulins from selected positive Mabs were isotyped using a commercial isotyping kit (Amersham Bioscience, England) as described in the manufacturer's protocol. The hybridoma suspension was harvested 3 days postseeding and cell debris pelleted by centrifugation at 400*g* for 10 min, followed by collection of the supernatant and storage at −20 °C. Mabs were purified with Montage kit Prosep-G (Millipore) for IgG.

2.4. Immunofluorescence assay (IFA)

MDCK cells cultured in 96-well plates were infected with AIV H7 strains. At 24 h post-infection, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed thrice with phosphate buffered saline (PBS), pH 7.4. Fixed cells were incubated with hybridoma culture supernatant at 37 °C for 1 h, rinsed with PBS and then incubated with a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Immunoglobulin (Dako, Denmark). Cells were rinsed again in PBS and antibody binding was evaluated by wide-field epi-fluorescence microscopy (Olympus IX71).

2.5. Hemagglutination inhibition assay

Hemagglutination inhibition (HI) assays were performed as described previously Prabhu et al. (2009). Briefly, Mabs were serially diluted (2-fold) in V-bottom 96-well plates and mixed with 4 HA units of H7 virus. Plates were incubated for 30 min at room temperature, and 1% chicken RBCs were added to each well. The hemagglutination inhibition endpoint was the highest Mab dilution in which agglutination was not observed.

2.6. Isolation and analysis of escape mutants

The epitope recognized by Mab 62 was mapped by characterization of escape mutants as described previously He et al. (2010). Briefly, H7N1 parental viruses were incubated with an excess of Mab for 1 h and then inoculated into 11 days old

embryonated chicken eggs. The eggs were incubated at 37 °C for 48 h. Virus was harvested and used for cloning in limiting dilution in embryonated chicken eggs and the escape mutants were plaque purified. The HA gene mutations were then identified by sequencing and comparison with the sequence of the parental virus.

2.7. Microneutralization assay

Neutralization activity of Mab against H7 strains was analyzed by microneutralization assay as previously described He and Kwang (2013). Briefly, Mab was serially twofold diluted and incubated with 100 TCID50 of different clades of H7 strains for 1 h at room temperature and plated in duplicate onto MDCK cells grown in a 96-well plate. The neutralizing titer was assessed as the highest Mab dilution in which no cytopathic effect was observed by light microscopy.

2.8. Immunization and challenge

For challenge experiments H7N7 reassortant virus was mouse adapted by three sequential lung to lung passages, as described previously Brown (1990). Virus present in the lung passage was propagated in the allantoic cavities of 10 days old chicken eggs for 48 h at 37 °C to prepare a virus stock. 50% mouse lethal dose (MLD50) was calculated as described by Reed and Munch method. Groups of SPF female BALB/c mice aged 4–6 weeks were used for the challenge studies. Mice (n = 5 per group) were inoculated intranasally with 5 or 10 MLD50 (Mouse lethal dose 50%) of pathogenic H7N7 (A/Netherlands/219/03) strain.

For intranasal immunization, mice were first anesthetized intraperitoneally with 100 ul of saline with ketamine (10 mg/ml) and xylazine (1 mg/ml). Each mouse was inoculated intranasally with 100 ul of Mab or virus in PBS.

2.9. Prophylactic efficacy

To determine the prophylactic efficacy, mice were pre-treated intranasally or intraperitoneally with 2.5, 5 or 10 mg/kg of purified Mab 62, prior to the viral challenge. Control mice were treated intranasally with PBS only. After 24 h, mice were challenged with 5 MLD50 of the H7N7 strain. Mice were observed daily to monitor body weight and mortality until death or day 14 after challenge.

2.10. Therapeutic efficacy

To determine the therapeutic efficacy, each group of mice was experimentally infected with 5 MLD50 of the H7N7 strain. 1, 2 or 7 days after viral infection, the mice groups were treated either via intranasal or intraperitoneal route with 5, 10 or 15 mg/kg of Mab 62. Infected control mice were treated intranasally with PBS only. Mice were observed daily to monitor body weight and mortality until death or day 14 after challenge.

2.11. Histopathological analysis

Lung samples for histological examination were harvested from each mice group (N = 3) on day 2, day 4 and day 6 post challenge and fixed in 10% buffered formalin (pH 7.4), embedded in paraffin and sectioned at 4 um. The sections were deparaffinized using Hist-choice (Amersco) and rehydrated in sequentially graduated ethanol baths. The slides were stained with hematoxylin and eosin and pathological evaluation was performed by light microscope (Olympus, UK). The images were captured by digital imaging system (Nikon, USA).

2.12. Statistical analysis

The data are expressed as arithmetic means and standard deviations (SD). An unpaired two-tailed Student's t test was performed to determine the level of significance in the difference between the means of two groups. One-way analysis of variance (ANOVA) was also used to test for differences between groups. All statistical analyses were done with SigmaStat 2.0 (Jandel Corporation) software.

3. Results

3.1. Mab 62 is an efficient neutralizing antibody against different H7 strains

A panel of Mabs against influenza hemagglutinin was screened for efficient recognition of different strains of H7 viruses. Based on the results of the HI assay, Mab 62 was selected for further studies due to its high HI activity (Table 1) against a wide range of H7 viruses from birds and humans. The Mab belongs to the IgG1 isotype. The virus neutralizing activity of Mab 62 was further confirmed to be positive against six representative H7 strains, including one human H7N9 strain (Table 1). Based on this, the amino acids involved in forming the epitope of Mab 62 were analyzed using selection of neutralization escape mutants. A/chicken/Malaysia/94 H7N1 virus was used as parental virus for the selection. Sequences of the complete HA genes isolated from multiple escape variants were compared with the parental virus. It was found that mutants with Mab 62 have a single mutation on amino acid 175 from Lysine to Glutamate. The numbering of amino acid on HA starts from "ATG" and includes the signal peptide.

In order to determine the significance of the neutralization epitope of Mab 62, the protein polymorphism of H7 was studied, taking into account all H7 sequences in the NCBI database. On the 175th amino acid, Lysine and Asparagine appear in more than 99.9% of H7 AIV strains listed. Lysine is the most dominant amino acid with the frequency of 97.9% among avian H7 strains and 100% among human H7s, including strains from the recent H7N9 outbreak in eastern China. This finding suggests the potential of the Mab 62 to be used to recognize or neutralize all the H7 human strain identified so far and to be further formulated as an effective H7 AIV treatment.

3.2. Effective prophylactic immunization by a single dose of Mab 62 against a lethal challenge of H7

The prophylactic efficacy of Mab 62 was evaluated against a challenge with 5 MLD_{50} of H7N7 virus (A/Netherlands/219/03). Groups of mice (n = 5) were inoculated via either the intraperitoneal or intranasal route with different concentrations (2.5, 5 and 10 mg/kg) of Mab 62, 1 day prior to viral challenge. The negative control group of mice (treated with PBS only) showed the most rapid decline in body weight (above 25%) and died from

Table 1
Hemagglutination inhibition (HI) and virus neutralization (VN) titer of Mab 62 (100 ug/ml) against different H7 viruses. HI titer below 8 and VN titer below 20 indicated negative activity.

Virus	Subtype	HI titer	VN titer
A/Chicken/Malaysia/94	H7N1	256	640
A/Canada/rv504/04	H7N3	64	160
A/quail/Aichi/4/09	H7N6	128	160
A/duck/Hokkaido/1/10	H7N7	256	640
A/Netherlands/219/03	H7N7	256	1280
A/Shanghai/1/13	H7N9	256	640
A/Puerto Rico/8/34	H1N1	<8	<20

complications associated with infection by day 8 post challenge. All of the mice pre-treated via either route with a single dose of Mab 62 showed less than 6 % (Fig. 1A and C) loss of body weight and a 100% survival rate (Fig. 1B and D) against the lethal challenge of H7 virus. In mucosal route, immunization with 2.5 mg/kg of Mab 62 was sufficient for 100% protection against the H7 challenge with a loss in body weight of lower than 5%. The intraperitoneal dose of 5 mg/kg of Mab 62 was able to prevent significant body weight loss in challenged mice while a 6% reduction in body weight was observed in mice with the lower IP dose of 2.5 mg/kg.

3.3. Effective therapeutic immunization by intranasal administration of Mab 62

The therapeutic efficacy of Mab 62 was evaluated against a challenge with 5 MLD50 of H7N7 virus (A/Netherlands/219/03). Groups of mice ($n = 5$) were inoculated via either the intraperitoneal or intranasal route with different concentrations (5, 10 and 15 mg/kg) of Mab 62, 1 day post viral challenge. The negative control group of mice (treated with PBS only) showed the most rapid decline in body weight (above 25%) and died from complications associated with infection by day 8 post challenge. All of the mice intransally treated with a single dose of Mab 62 showed less than 10% (Fig. 2A) loss of body weight and a 100% survival rate (Fig. 2B) against the lethal challenge of H7 virus. Intranasal doses from 5 to 15 mg/kg did not present significant differences in efficacy of the treatment. The intraperitoneal dose of 5 mg/kg of Mab 62 failed to protect mice from death 10 days post challenges. In the group with a higher intraperitoneal dose of 15 mg/kg, 4 mice survived with more than 15% loss of body weight.

3.4. Therapeutic efficacy of a single dose versus two doses of Mab 62 against a higher challenge dose of H7

In order to test the therapeutic potential of Mab 62 against the higher challenge dose of 10 MLD_{50} of H7N7, the efficacy of the single nasal dose 1 day post challenge was compared with that in mice treated with double nasal doses of 10 mg/kg Mab 62 each time at different time points (1 and 2 dpi; 1 and 7 dpi). Upon the challenge with 10 MLD_{50} of H7N7 virus (A/Netherlands/219/03), the negative control group of mice (treated with PBS only) showed a more rapid decline in body weight (above 25%) than the infections with 5 MLD_{50} and died from complications associated with infection by day 6 post challenge. As shown in Fig. 3, all the mice treated intranasally with the single dose of 10 mg/kg of Mab 62 survived the infection of 10 MLD_{50} with around 13% loss in body weight. A double-nasal dose 1 and 2 days post infection successfully protected all the infected mice with a loss in body weight of less than 5%. Moreover, the group of mice that received the double dose of Mab 62 (1 and 2 dpi) regained their body weight more rapidly (within 8 days) when compared to the mice that received a single dose, which regained their body weight only by 10 days after the viral infection. No enhancement in therapeutic efficacy was observed in mice with a double nasal dose 1 and 7 days post infection. The mice in this group presented with a similar loss and recovery in body weight as the group receiving a single dose.

3.5. Therapeutics with Mab 62 via the intranasal route was more effective than intraperitoneal treatment

As differences between intranasal and intraperitoneal therapeutics were observed based on mouse mortality and body weight,

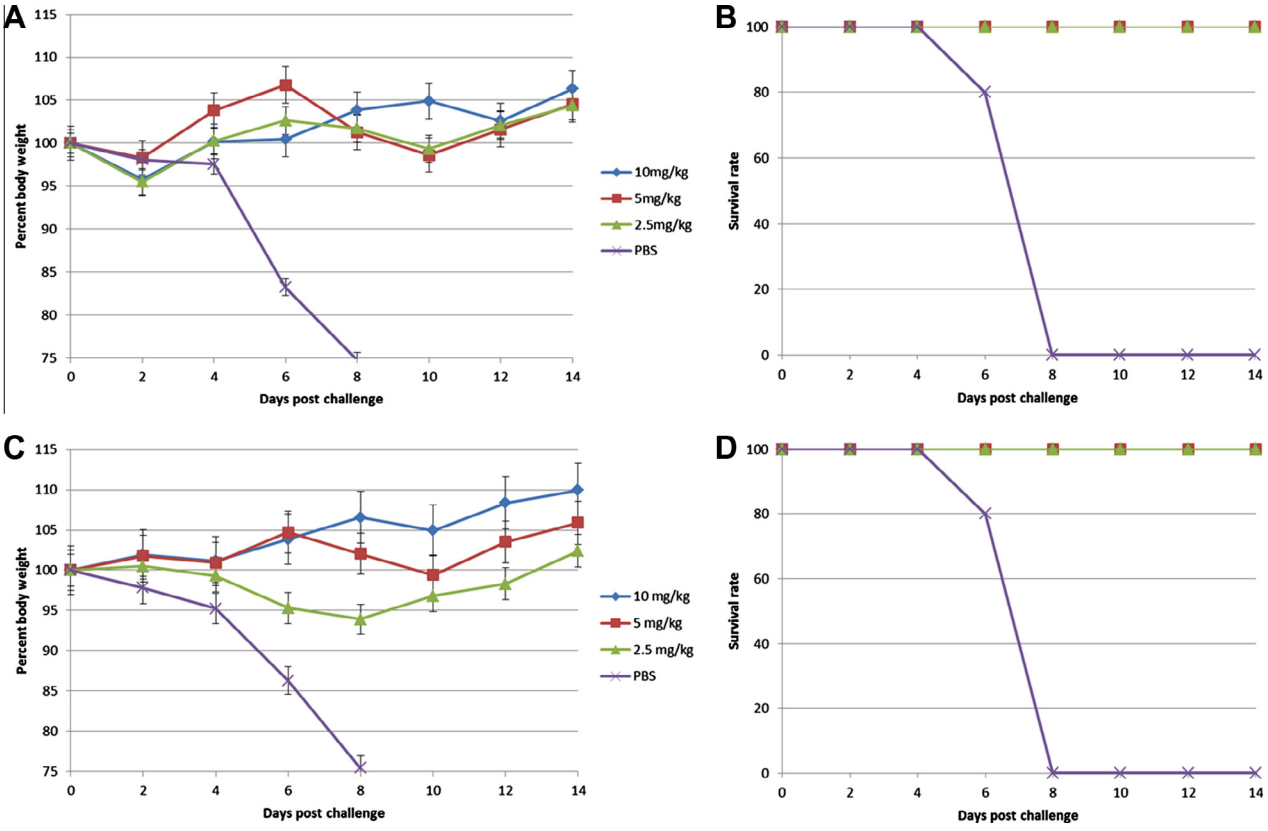

Fig. 1. Prophylactic efficacy of Mab 62 against H7 challenge in mice. (A and B) Groups of mice ($n = 5$) were pre-treated intranasally with 2.5, 5, 10 mg/kg or 0 mg/kg (PBS) of Mab 62; (C and D) Groups of mice ($n = 5$) were pre-treated intraperitoneally with 5, 10 or 0 mg/kg (PBS) of Mab 62, 1 day before challenge with 5MLD_{50} of mouse-adapted HPAI H7N7 (A/Netherlands/219/03, RG). Mice were monitored for survival and weight loss throughout a 14 days observation period. The results are expressed in terms of percent survival (C and D) and percent body weight (A and B) (at the beginning of the trial), respectively.

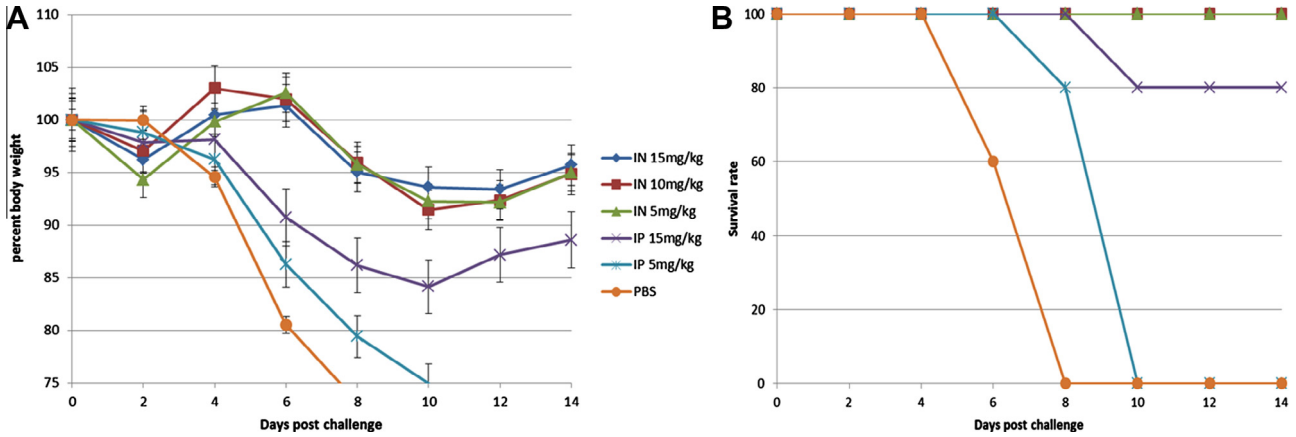

Fig. 2. Therapeutic efficacy of Mab 62 against H7 challenge in mice. Groups of mice ($n = 5$) were infected with 5MLD50 of mouse-adapted HPAI H7N7 (A/Netherlands/219/03, RG). One day post-challenge, the mice were treated via intraperitoneal route with 15 mg/kg of Mab 62, or via intranasal route with 5, 10, 15 mg/kg or 0 mg/kg (PBS) of Mab 62. Mice were monitored for survival and weight loss throughout a 14 days observation period. The results are expressed in terms of percent survival (B) and percent body weight (A) (at the beginning of the trial), respectively.

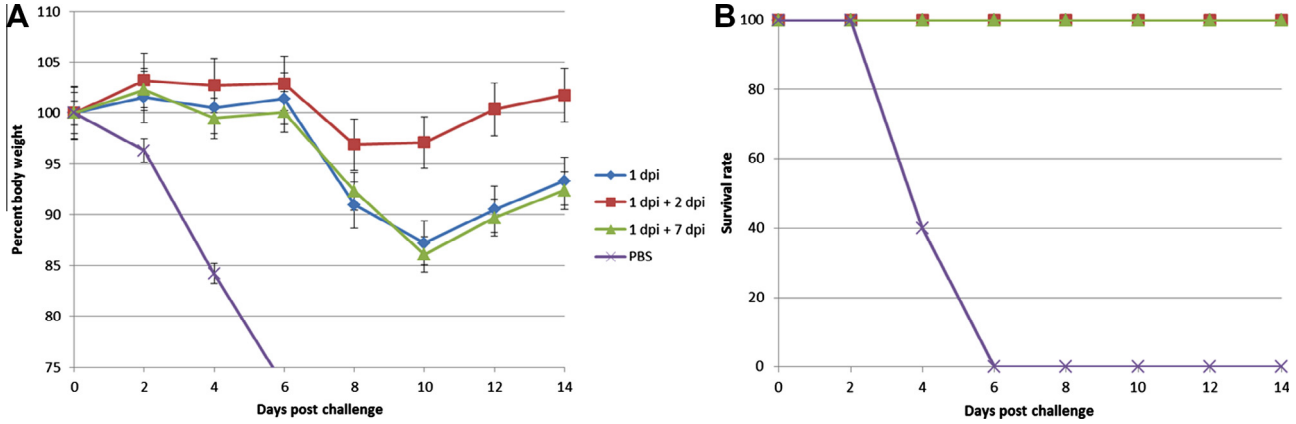

Fig. 3. Therapeutic efficacy of two doses of Mab 62 against H7 challenge in mice. Groups of mice ($n = 5$) were infected with 10MLD50 of mouse-adapted HPAI H7N7 (A/Netherlands/219/03, RG). 1, 2, or 7 days post-challenge, the mice were treated once or twice via intransal route with 10 or 0 mg/kg (PBS) of Mab 62. Mice were monitored for survival and weight loss throughout a 14 days observation period. The results are expressed in terms of percent survival (B) and percent body weight (A) (at the beginning of the trial), respectively. dpi: days post infection.

further studies were perform to confirm the better protection efficacy of intranasal administration. Histopathology studies were conducted on the lungs of mice treated intranasally or intraperitoneally with Mab 62. As shown in Fig. 4C and D, pulmonary lesions developed with the progressive infection in the late stage in H7N7 infected mice without treatment, consisting of moderate to severe necrotizing bronchitis and histiocytic alveolitis with associated pulmonary edema. Meanwhile, the uninfected mice lacked lesions in the lungs (Fig. 4E). Lungs collected on day 14 post infection, from mice treated intranasally with 15 mg/kg Mab 62 showed no lung pathology and looked similar to the uninfected control (Fig. 4A), while moderate bronchitis was present in the lungs of mice intraperitoneally treated with 15 mg/kg of Mab 62 (Fig. 4B).

Moreover, virus load in lungs was evaluated in H7 infected or treated mice. The kinetics of viral replication was determined by measuring the viral titers in the mouse lungs on days 2, 4, 6 and 14 post infections (Fig. 5). The virus titers were most elevated on day 6 after viral challenge when the highest virus titers of more than $10^{7.15}$ were detected in the infected but untreated mice which died within the following 2 days. The mice treated intranasally with a single dose of 15 mg/kg of Mab 62 showed decreasing viral loads from day 2 post infection. However those mice treated intraperitoneally with the same antibody dose showed the decrease in virus load only by day 6 post-infection. Virus titers in intraperitoneally treated mice were higher than those in the intranasal group at every time point, indicating that intranasal treatment reduced the virus load from the start of the infection. All these findings confirm that intranasal therapeutics with Mab 62 is more effective than intraperitoneal treatment against H7N7 HPAI virus.

4. Discussion

H7 HPAI virus has come into focus as a major pandemic concern because of its ability to transmit directly from domestic poultry to humans and the high mortality caused in humans (Koopmans et al., 2004; Yang et al., 2012). The most recent outbreak in Eastern China enhanced the need for effective prophylactics and therapeutics against H7 virus in humans. As reported in this study in mice, the intranasal administration of H7 Mab 62 is able to provide immediate prevention and protection against H7 virus infection, hence meeting the current need.

The relevant amino acid of the Mab 62 epitope was identified by the sequencing of escape mutants. The identified 175th amino acid on HA1 is conserved in all the H7 human strains and in more than 97.9% of avian strains. According to the newly released data,

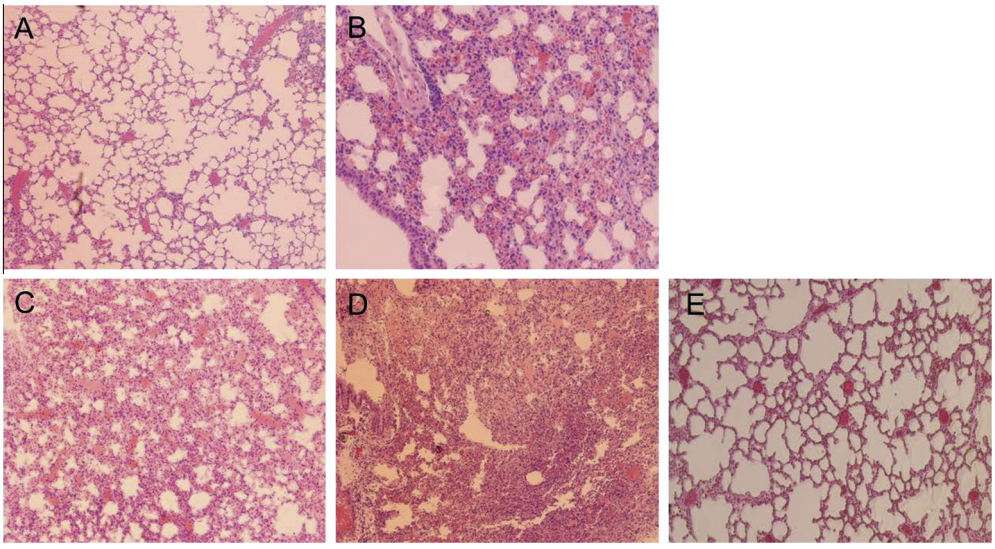

Fig. 4. Histopathology of lung tissue in passively treated mice. Photomicrographs of hematoxylin and eosin stained lungsections of mice treated with Mab 62 (15 mg/kg) either via intranasal or intraperitoneal route after post experimental viral infection with $5MLD_{50}$ of HPAI H7N7 (A/Netherlands/219/03, RG). (A) Lung section from mice intranasally treated with Mab 62 collected on 14 days postinfection. (B) Lung section from mice intraperitoneal treated with Mab 62 collected on 14 days postinfection. (C) Lung section from infected mice without treatment collected on 6 days postinfection. (D) Lung section from infected mice without treatment collected on 8 days postinfection. (E) Lung section from uninfected mice.

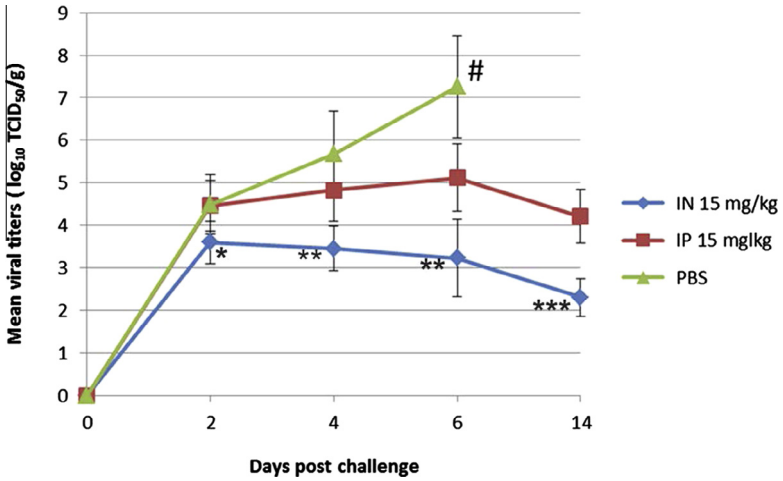

Fig. 5. Measurement of viral infectivity titers in the lungs of passively treated mice. Mice treated with Mab 62 (15 mg/kg) either via intranasal or intraperitoneal route after experimental viral infection with $5MLD_{50}$ of HPAI H7N7 (A/Netherlands/219/03, RG). Lung samples were collected on 2, 4, 6 and 14 days post infection. The results of virus load in lungs were expressed in terms of mean value of $\log_{10}$ $TCID_{50}$/g. "#" represents no survival of any animals in the group ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 5$; for points of the intranasal group as compared with the intraperitoneal counterpart).

the H7s from the recent human cases in China also carries Lysine at amino acid position 175. Therefore, it is believed and confirmed that Mab 62 is able to neutralize the H7s from this human outbreak in China. The site recognized by Mab 62 is located between the 180-helix and 140-loop of H7 HA1. The 180-helix is part of the receptor binding site (RBS) and the 140-loop contributes to the recognition of RBS (Yang et al., 2012). The amino acid position 175 is close to the H7 RBS but not within it, allowing the site to be conserved and recognized by neutralizing antibodies. Although virus evasion may occur after therapy with single Mab and after long-term use in populations, the problem can be solved by administering a pair of complementary Mabs (He et al., 2010) or a cocktail of several neutralizing Mabs (Prabakaran et al., 2009b). Besides Mab 62, a panel of H7 neutralizing Mabs has been produced, paving the way for efficacious and reliable treatment against H7 infection.

Results presented in this study revealed that intranasal therapeutics protected all the mice from a H7 lethal challenge while the treatment via the intraperitoneal route was not very successful even with a high dosage of Mab. Mice from the intranasal group recovered without any lesion in the lung while obvious lung lesions were still observed in surviving mice treated intraperitoneally. All these findings indicate that H7 HPAI treatment with monoclonal antibodies is only effective via the intranasal route. The mucosal delivery may be most useful for treatment of the upper airways, where the attachment of pathogens for respiratory diseases occurs firstly (Rose et al., 2012). Passive immunized Mab 62 in the upper airways can promptly neutralize those H7 virus particles inhaled and attaching to the mucosal surface (Gambaryan et al., 2012) when the nature prevalence of secretary antibodies has not been effectively formed yet. Previous studies indicated that passive immunized IgG antibody can neutralize newly replicated

virus after infection had been initiated (Renegar et al., 2004). The virus neutralization by Mab 62 significantly prevents or relieves further virus infection at the first stage (Kandel and Hartshorn, 2001). Other treatment strategies, such as intraperitoneal immunization, may not take effect until virus spread within the host body occurs, by which time serious symptoms and lesions from acute H7 infection have already developed (Belser et al., 2011). For the same reason, the second administration of Mab 62 on day 7 post-infection did not contribute to any enhancement of treatment, since host immune responses from the initial infection can continue to cause tissue damage (Kreijtz et al., 2011). Significant relief of body weight loss was gained from the second dosage 2 days post-infection, which further cleared the virus load in the upper airways following the first dose. It is confirmed that passive intranasal immunization of Mab is able to prevent or limit virus infection and hence to relief symptoms. Besides, as observed by studying the virus load in lungs, intranasal treatment post infection with Mab 62 can effectively reduce the initial virus titer and prevent the increase in virus load. It is important to cut down virus infectious load at the early stage of infection from H7 HPAI virus, which presents rapid replication and leads to progressive symptoms quickly (Belser et al., 2011). Although a low virus titer of below 10^3 was present in the late stage of intranasal treatment with Mab 62, mice were recovering and gaining body weight without any symptom, since hosts gradually elicit antibodies to clear low amounts of viruses from 10 days post infection (van Riet et al., 2012). Overall, a double dose of Mab within 48 h via mucosal route is hence recommended.

As known from the recent H7 outbreak in eastern China, the virus was transmitted to humans from asymptomatic birds. Most of these cases happened among people with a history of interacting with poultry birds. Illness from H7, which shows rapid replication in the host, appeared progressive and irreversible in most patients once symptoms developed. This highlights the importance of both prophylactics and therapeutics among susceptible people and suspected infected individuals against sudden viral disease outbreaks, such as H7 HPAI this time. IgG is the major antibody used in prophylactics and therapeutics (Magnusson and Stjernstrom, 1982; Marasco and Sui, 2007) because it is more readily available than other isotypes. Ease of purification, long-term stability and better neutralization efficiency make monoclonal antibodies of IgG ideal therapeutic reagents for large scale production, meeting the urgent need posed by the acute viral infectious disease like H7.

In conclusion, passive immunization of Mab 62 via the mucosal route was proven to be an effective strategy for prevention and treatment of H7 HPAI. Other advantages of intanasal passive immunization include a needle-free procedure, the ready availability of Mab without humanization and efficient scale-up capacity. This makes it an essential tool against multiple human H7 infections and their high mortality from infection, which are the major challenges currently faced in the China outbreak.

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