A broadly neutralizing human monoclonal antibody directed against a novel conserved epitope on the influenza virus H3 hemagglutinin globular head

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Abstract

Most neutralizing antibodies elicited during influenza infection or vaccination target immunodominant, variable epitopes on the globular head region of hemagglutinin (HA), which leads to narrow strain protection. In this study, we describe the properties of a unique anti-HA monoclonal antibody, D1-8, that was derived from human B-cells and exhibits potent, broad neutralizing activity across antigenically diverse influenza H3 subtype viruses. Based on selection of escape variants, we show that D1-8 targets a novel epitope on the globular head region of the influenza HA protein. The HA residues implicated in D1-8 binding are highly conserved among H3N2 viruses, and are located proximal to antigenic site D. We demonstrate that the potent in vitro antiviral activity of D1-8 translates into protective activity in mouse models of influenza infection. Furthermore, D1-8 exhibits superior therapeutic survival benefit in influenza infected mice compared to the neuraminidase inhibitor, oseltamivir, when treatment is started late in infection. The present study suggests the potential application of this monoclonal antibody for the therapeutic treatment of H3N2 influenza infection.

Importance

Recently a few globular head targeting mAbs have been discovered that exhibit activity against different subtypes of influenza subtypes, such as H1; however, none of the previously described mAbs showed broadly neutralizing activity against diverse H3 viruses. In this study, we describe a human mAb, D1-8, that exhibits potent, broad neutralizing activity against antigenically diverse H3 subtype viruses. The genotypic
analysis of escape mutants revealed a unique putative epitope region in the globular head of H3 HA that is comprised of highly conserved residues and is distinct from the receptor binding site. Furthermore, we demonstrate that D1-8 exhibits superior therapeutic efficacy in influenza infected mice compared to the neuraminidase inhibitor, oseltamivir, when treatment is started late in infection. In addition to describing a novel anti-globular head of H3 HA mAb with potent broadly neutralizing activity, our study suggests the potential of D1-8 for therapeutic treatment of seasonal influenza H3 infection.
Introduction

Seasonal influenza infection results in ~200,000-500,000 deaths each year, particularly in young children, immunocompromised patients and the elderly (1, 2). During influenza pandemics mortality rates can be even higher (3). Currently, vaccination remains the most effective means to prevent morbidity and mortality caused by influenza virus infection. However, vaccination is less effective in the elderly, which is the population at the highest risk for complications from influenza infection (4-6). In addition, antigenic drift can reduce the effectiveness of seasonal influenza vaccines, particularly if the vaccine strains are not well matched with circulating strains. Antiviral drugs (e.g., oseltamivir and zanamivir) are effective at reducing the duration of symptoms and complications due to influenza infections; however, they are most effective when administered early in infection (i.e., within 48 hours after symptom onset) (7, 8).

Although providing some benefit, such neuraminidase (NA) inhibitors are less effective at the time of hospitalization, which often occurs >48 hours after symptom onset (8). In addition, the effectiveness of current NA inhibitors may be further limited by the emergence of drug resistant variants of influenza (9). Therefore, there remains a significant unmet medical need for new therapies to treat influenza, particularly ones that are effective in high risk populations (e.g., the elderly) at the time of hospitalization.

Recently, a number of preclinical studies in animal models have demonstrated that anti-influenza HA monoclonal antibodies have protective activity when administrated late in infection, suggesting the potential utility of these agents for treating severe influenza infection (10-17).
There are three types of influenza viruses, A, B and C; however, only influenza A and B viruses cause disease in humans. Influenza A viruses are further classified into subtypes based on the homology of the HA or NA proteins, which are the major glycoproteins expressed on the surface of influenza virions. Influenza type A contains 17 HA subtypes, which are further divided into two major phylogenetic groups: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16 and H17 subtypes) and group 2 (H3, H4, H7, H10, H14 and H15 subtypes). Currently, only H1N1, H3N2 and influenza B viruses are circulating in humans and the majority of influenza hospitalizations in the United States are typically associated with influenza A infection, more specifically with H3N2 infection (6).

Most neutralizing antibodies elicited by infection or vaccination recognize HA. The mature HA protein is present on virions as a trimer and is comprised of two disulfide linked subunits, HA1 & HA2. The HA1 subunit forms the globular head region which contains the sialic acid receptor binding site (RBS). The HA2 subunit and a portion of HA1 form a stalk structure, which drives membrane fusion (18-21). Neutralizing antibodies generated in response to infection or vaccination typically target the globular head. Five distinct antigenic sites have been characterized in the H1 or H3 globular head regions and are designated either as Sa, Sb, Ca1, Ca2 and Cb, or as sites A through E (22-23). Given that these antigenic sites exhibit considerable sequence variability, antibodies generated during vaccination are usually strain specific. In contrast, the RBS on the globular head is relatively well conserved and consists of a shallow pocket located at the apex of HA1 that is surrounded by HA sequences referred to as the 130-loop, the 150-
loop, the 190-helix and the 220-loop (24). Recently, a number of broad-spectrum mAbs targeting the RBS along with adjacent regions in the globular head of HA have been identified (25-31). These mAbs exhibit some cross-reactivity with one or more influenza H3 and/or H1 subtypes. However, few of these previously described mAbs targeting the globular head of HA have demonstrated neutralizing activity across large and diverse panels of either group 1 or group 2 influenza A viruses (29).

In this study, we demonstrate that a previously described anti-HA mAb, D1-8 (32), exhibits potent, broad neutralizing activity across a diverse panel of H3 influenza strains. Based on the selection of resistant virus in vitro, we show that D1-8 recognizes a novel, conserved epitope proximal to antigenic site D on the globular head domain of H3 HA. D1-8 protects mice from lethal challenge with a representative H3 influenza strain when administered prophylactically or therapeutically. Furthermore, D1-8 exhibits superior protective activity in our therapeutic mouse model of H3 influenza infection when compared to oseltamivir, particularly when administered late in infection.
Materials and Methods

Cells, viruses and antigens

Madin Darby Canine Kidney (MDCK) cells were obtained from European Collection of Cell Cultures (ECACC) and were used for all cell based assays. Monolayer cultures of MDCK cells were maintained in minimal essential medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen) plus 2 mM L-glutamine with 1× penicillin/streptomycin. Wild type influenza strains were obtained from Centers for Disease Control (CDC) or purchased from American Tissue Culture Collection (ATCC) as indicated. Cold adapted (ca) live attenuated influenza vaccine viruses were generated by either classical reassortment or by reverse genetics (33). All viruses were propagated in embryonated chicken eggs and virus titers were determined by 50% tissue culture infectious dose (TCID$_{50}$/ml). The H3N2 viruses: A/Perth/16/09, A/Wisconsin/67/05, A/California/7/04, A/Wyoming/03/03, A/Panama/2007/99, A/Sydney/5/97, A/Argentina/3779/94, A/Shangdong/9/93, A/Los Angeles/2/87, A/Victoria/3/75, A/Hong Kong/8/68 (ATCC VR-544), swine-origin A/Minnesota/11/2010, swine-origin A/Indiana/10/2011. The live attenuated H7 strains were generated by reverse genetics using HA and NA genes of A/Netherlands/219/03 (H7N7) and a low pathogenicity (LP) A/chicken/British Columbia/CN-6/2004 (H7N3) respectively, with the six internal proteins genes of ca)A/Ann Arbor/6/60 (H2N2) virus (34). A reassortant H3 virus (rHK68) containing the H3 HA from A/HK/8/68 (H3N2) with N165S mutation (glycosylation site) and the remaining 7 gene segments from A/PR8/34 (H1N1) was produced by reverse genetics (33). The reassortant virus
containing antibody resistant mutation in HA was generated by reverse genetics as described previously (33, 35).

Cloning, expression and purification of recombinant antibody D1-8
To generate a plasmid that yields high level of expression of the target gene, VH and Vk genes were amplified from the original expression plasmid (32) and sequentially cloned into in-house pOE vector. The sequence was confirmed and the plasmid was then transfected into the 293F cells using 293fectin (Invitrogen). The antibody was purified from supernatant using MabSelect Protein column (GE Healthcare).

Cloning, expression and purification of recombinant HA proteins.
The HA expression vector was constructed as described previously (36). The HA cDNAs corresponding to amino acid residues 11-329 (HA1) and 1-176 (HA2) of the ectodomain of HA0 (A/Perth/16/09 (H3N2), A/California/7/09 (H1N1) or A/Netherlands/219/03(H7N7) were cloned into a plasmid vector under the control of the CMV promoter. The trimerization domain, thrombin cleavage site and His-tag were added to the C-terminus of the HA gene. The HA protein was purified from transient transfected 293F cell culture supernatant using a 5 ml Ni-NTA Superflow column (Qiagen) equilibrated with PBS.

Kd determination (Affinity Measurement)
Affinity measurements were performed using a ForteBio Octet QK 384 Kinetic Analyzer (Menlo Park, CA) using 384 slanted-well plates. All reagents were diluted in Octet.
Kinetics Buffer. His-tagged HA was immobilized onto anti-his sensors at 10 μg/mL. Anti-HA mAb association/disassociation was then monitored in 2-fold dilutions from 100 nM and a well without mAb was used as a control. Association and disassociation Δ nm raw data were corrected for any drift in the zero mAb controls, and then exported to GraphPad Prism (San Diego, CA) for affinity curve fitting. Data were fit using Global Association / Disassociation fitting with an imposed limit of $k_{off} > 5 \times 10^{-6}$ sec$^{-1}$.

Western Blot analysis of mAb D1-8 under reducing and nonreducing conditions.

Purified trimeric HA protein of A/Perth/09 H3 was evaluated for its reactivity with D1-8 by Western blotting under reducing and nonreducing conditions. The purified trimeric HA protein at 0.5 μg/well was heated at 70° C for 10 minutes in NuPAGE® LDS sample buffer (Invitrogen) in the presence or absence of 50mM dithiothreitol (DTT). Reduced and nonreduced preparations were subjected to electrophoresis in a 4 – 12% Bis-Tris acrylamide gel in MES SDS electrophoresis buffer (Invitrogen). The separated proteins were transferred to nitrocellulose and blocked for 1 hour (hr) in 5% powdered milk v/v in PBS containing 0.1% Triton-X 100 at room temperature. The blot was probed with D1-8 mAb at concentration of 3.0μg/ml mAb followed by addition of HRP-conjugated anti-human secondary antibody. After incubation followed by several washing, the detection reagent (LumiGLO KPL) was added to the blot and the blot was exposed to X-ray film.

Microneutralization assay

The microneutralization assay was modified from a previously described accelerated viral inhibition assay using neuraminidase activity (NA) as a read-out (37). Briefly, 100
TCID<sub>50</sub> virus was added to two-fold dilutions of antibody in a 96-well plate, after 30 minutes incubation at room temperature, 4x10<sup>4</sup> cells/well were added to the plate. After incubation at 33°C 5% CO<sub>2</sub> incubator for approximately 40 hr, the NA activity was measured by adding a fluorescently labeled substrate, methylumbelliferyl-N-acetyl neuraminic acid (MU-NANA) (Sigma) to each well and incubated at 37°C for 1 hr. Virus replication represented by NA activity was quantified by reading fluorescence in Fluorometer Envision (PerkinElmer). The neutralization titer (50% inhibitory concentration [IC<sub>50</sub>]) is expressed as the final antibody concentration that reduced the fluorescence signal by 50% compared to cell control wells.

Hemagglutination inhibition (HAI) assay

HAI assay was performed as described previously (38). In brief, the antibodies were serially diluted with PBS in 96-well V-bottom plates and 4 HA units of virus (as determined by incubation with 0.5% chicken red blood cells (RBCs) in the absence of antibody) was added to the well. After 30-60 min incubation at room temperature, 0.5% chicken RBCs (Lampire Biological Laboratories) suspended in PBS was added to each well. After an additional 30 min incubation at room temperature, the HAI titers or minimum effective concentration were defined as the reciprocal of the highest antibody dilution that completely inhibited hemagglutination of 0.5% chicken RBCs.

Selection of monoclonal antibody resistant mutants (MARMs)

All of the MARMs were selected by incubating the H3N2 influenza viruses A/Wyoming/3/2003 (WY03), A/HongKong/1/1968 (HK68), and A/Perth/16/2009.
(Perth09) under increasing concentrations of D1-8 mAb. One hundred plaque forming units (PFU) of each virus was mixed with 1x IC₅₀ of mAb D1-8 and incubated for 1 hr at room temperature. The virus and antibody mixture was added onto confluent MDCK cell monolayers in 12-well tissue culture plates. After 1 hr of adsorption, the cell monolayers were washed with PBS and culture medium containing 1x IC₅₀ of mAb D1-8 were added to the wells. The plates were incubated at 33°C for 3-7 days and observed daily for signs of cytopathic effects (CPE). Once the infected cells exhibited more than 50% CPE, the culture supernatants were harvested and used to infect fresh MDCK cells as described above. The selection process was repeated four more times with increasing mAb D1-8 concentrations (5x, 10x, 20x and 100x IC₅₀, respectively) in the medium. The viruses from 5 rounds of passage were biologically cloned by plaque assay, and plaques were individually propagated in MDCK cells. The HA gene was amplified by RT-PCR and sequenced to determine the amino acid changes in the HA.

**Evaluation of mAb for its prophylactic and therapeutic protective activity in mice**

All animal study protocols were approved and conducted in accordance with MedImmune’s Institutional Animal Care and Use Committee and subsequently performed in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) certified facility. Six-to-eight weeks old BALB/c mice (Harlan Laboratories) were used in the studies. The experiments were conducted in accordance with the protocol approved by MedImmune’s Institutional Animal Care and Use Committee. Mice were weighed on the day or one day before virus challenge and monitored daily for 14 days for weight loss and survival (mice with body weight loss ≥
229 25% were euthanized). To determine the prophylactic efficacy of the mAb, mice in
230 groups of 10 were administered intraperitoneally with D1-8 at doses of 0.1, 0.25, 0.5, 1, 5
231 and 15 mg/kg, or with a human irrelevant isotype control IgG at 15 mg/kg in 100μl
232 volumes. Four hours after dosing, mice were inoculated intranasally with 10 times the
233 fifty percent mouse lethal dose (10 MLD50) of 7:1 A/PR/8: A/HK/8/68 HA reassortant
234 (rHK68) in a 0.05 ml volume. All mice were weighed and monitored daily for any signs
235 of morbidity and mortality for two weeks. For the therapeutic efficacy study, mice were
236 infected intranasally with 3 MLD50 of rHK68. At 4, 24, 48, 72 or 96 hr post infection,
237 groups of mice were given intraperitoneal injections of 30 mg/kg of D1-8 or isotype
238 control mAb. To assess virus load in the lungs, five mice from each group were
239 euthanized at 96 hr post infection. Whole lungs were homogenized in 10% w/v sterile
240 L15 medium (Invitrogen). Virus titer in lung homogenates was determined by TCID50
241 measured by fluorescence based neuraminidase activity assay as described above and
242 previously (34). To compare therapeutic efficacy of D1-8 with oseltamivir, antibody-
243 treated groups received a single intraperitoneal injection of 2.5 mg/kg D1-8 or 30mg/kg
244 isotype control mAb on day 4 after infection. Oseltamivir-treated groups of mice were
245 treated with saline as vehicle control or oseltamivir dosed at 25 mg/kg orally (po) twice
246 daily (BID) starting 96hr post virus inoculation for 5 days (BID x 5). Mice were observed
247 and weighed daily as described through day 14.
Results

D1-8 exhibits broad neutralizing activity against H3 viruses.

In this study, we characterized a human mAb D1-8, which was isolated by molecular cloning of IgG-expressing plasmablasts from a healthy flu vaccine recipient (32). Previously, D1-8 was demonstrated to exhibit high affinity binding to the H3N2 vaccine strain (A/Wisconsin/67/2005) with a $K_d$ value of 286 nM. To determine if D1-8 recognizes other genetically diverse influenza A strains, the binding of full-length D1-8 (D1-8 IgG1; bivalent) or the D1-8 antigen binding domain (D1-8 Fab; monovalent) to recombinant trimeric HA proteins (rHA) derived from A/Perth/16/09 (H3N2), A/California/7/09 (H1N1) or A/Netherlands/219/03(H7N7) was interrogated and $K_d$ values were determined as described in Materials and Methods. D1-8 IgG bound to H3N2 rHA protein with a measured $K_d$ value of <100pM. In contrast, the monovalent D1-8 Fab exhibited a $K_d$ value that was five orders of magnitude greater than the bivalent D1-8 IgG (10.9 $\mu$M). Binding to H7 and H1 rHA proteins was not detected with either the D1-8 IgG or Fab. These data suggest that D1-8 binds specifically to H3N2 HA and demonstrates that the strong binding exhibited by this antibody is dependent on avidity.

To further characterize the functional activity of D1-8, hemagglutination inhibition (HAI) assays and virus neutralization assays were performed using a diverse group of H3N2 influenza strains originally isolated over a span of 40 years. D1-8 demonstrated HAI activity against 11/13 antigenically diverse H3N2 strains tested with IC$_{50}$ values ranging from 0.2– 12.5 ug/mL (Table 1). Alternatively, D1-8 exhibited potent neutralizing activity against all 13 H3N2 strains with a median IC$_{50}$ of 0.053 $\mu$g/ml (mean 0.18 ±0.06
µg/ml and range 0.01-0.66 µg/ml) (Table 1). Thus, D1-8 exhibited 7-150-fold greater potency in neutralization assays than in HAI assays when comparing the same virus strains. Consistent with the binding data, D1-8 did not exhibit antiviral activity against H1 and H7 influenza strains (data not shown). Antibodies that exhibit HAI activity typically target the globular head region of the HA. Therefore, these data suggest that D1-8 may recognize a conserved epitope on the globular head of the influenza H3 HA.

Epitope identification by selection of monoclonal antibody resistant mutants (MARMs).

Influenza H3N2 viruses, A/Hong Kong/1/68 (HK68), A/Wyoming/03/2003 (WY03), and A/Perth/16/2009 (Perth09), were propagated in the presence of increasing concentrations of D1-8 by serial passage. Potential escape mutants were plaque purified and their cognate HA genes were subjected to sequence analysis to identify putative amino acid substitutions that conferred resistance to D1-8. When WY03 was propagated in the presence of D1-8 at concentrations up to 20 x IC50, single amino acid substitutions at HA residues 240 (G240E) or 207 (K207N) were identified. Similarly, when HK68 was propagated in the presence of D1-8 concentrations up to 20 x IC50, a single amino acid substitution was selected at HA residue 207 (R207N) (Table 2). Serial passage of these WY03 or HK68 variants at higher D1-8 concentrations (up to 100x IC50) resulted in the selection of additional amino acid substitutions at HA residues 171 or 173 in combination with the G240E substitution in the case of WY03 (G240E, N171D/E, or G240E, K173T) or at HA position 172 in combination with the R207N substitution in the case of HK68 (R207N, D172G). Interestingly, no amino acid substitutions were detected in the HA of Perth09 propagated
in the presence of D1-8 at concentrations up to 20x IC50; however, when this virus was subsequently passaged at higher concentrations of D1-8 (100x IC50), the N171K single amino substitution was identified in the HA protein (Table 2). To evaluate the effect of these amino acid substitutions on D1-8 susceptibility, recombinant A/Wyoming/3/2003 (rWY03) viruses encoding either individual mutations or combinations of mutations were generated and evaluated in HAI and neutralization assays. As shown in Table 3, the N171E/K/D and K173T substitutions conferred >2,000-fold and >70,000-fold reductions in susceptibility to D1-8 in HAI and neutralization assays, respectively. Similar changes in susceptibility were observed for the N171K substitution when introduced into either the rHK68 or rPerth09 viruses (data not shown). In contrast, the single amino acid changes E172G, K207N or G240E resulted in more modest 3-80 fold reductions in susceptibility to D1-8 in HAI assays or 59-854 fold reductions in susceptibility to D1-8 neutralization. However, when two of these mutations, K207N and E172G, were introduced in combination, the resulting virus was highly resistant to D1-8, with calculated IC50 values for HAI and neutralization that were >2,000-fold and >70,000-fold higher, respectively, than those measured with wild type virus (Table 3). The amino acid changes K207N or K173T resulted in the introduction of a potential N-linked glycosylation site at Asn207 or Asn171 (NXS/T), respectively. This observation suggests that the HA variants may be glycosylated at Asn207 or Asn171 and that this glycosylation may interfere with D1-8 binding. Additional amino acid substitutions (T48K, N165D/S, I406L or A476T) were identified in the HA proteins of selected MARMs; however, such
substitutions did not alter the D1-8 susceptibility of recombinant viruses encoding such substitutions (data not shown).

Although not continuous in linear sequence, all resistance-associated substitutions cluster together and are located proximal to antigenic site D on the x-ray crystal structure of H3 HA (18, 21-22) (Fig. 1A). These structural observations suggest that D1-8 recognizes a conformational epitope on the globular head of H3 HA. Consistent with D1-8 recognizing a structural epitope, the antibody binds HA only under non-reducing, non-denaturing conditions in Western analysis (Fig. 1B).

To investigate the conservation of the proposed D1-8 epitope among recently circulating as well as historical H3N2 isolates, we analyzed 7432 unique HA amino acid sequences derived from H3N2 strains isolated over the past 44 years (from the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) database). The amino acid residues Asn at HA position 171, Lys or Arg at position 207 and Gly at position 240 are conserved in >99% of H3N2 HA sequences analyzed (Table 4). 90% of the H3N2 sequences analyzed contained either an Asp or Glu at position 172. The amino acid changes N171E, K207N and G240E did not affect viral replication kinetics when evaluated in vitro as compared to the wild-type recombinant parental virus, rWY03. Alternatively, the amino acid substitution, N171K, resulted in a >1 log10 reduction in peak viral titer in vitro when compared to the parental virus (data not shown). In addition, HK68 recombinant variants encoding either the K207N or G240E substitution were
evaluated in vivo. Consistent with in vitro results, the in vivo 50% lethal doses of the K207N and G240E variants were comparable to the parental rHK68 virus (data not shown).

D1-8 protected mice from lethal challenge with H3 Influenza virus when administered prophylactically or therapeutically

To determine whether potent in vitro neutralizing activity would translate into efficacy in vivo, D1-8 was evaluated in a mouse model of H3N2 influenza infection. BALB/c mice were treated with D1-8 either before (prophylactically) or after (therapeutically) challenge with a lethal dose of mouse-adapted reassortant virus (rHK68) as described in Materials and Methods. In the prophylaxis study, D1-8 conferred protection in a dose dependent manner, providing 100% protection in animals that received D1-8 at a dose of 0.5mg/kg or greater, and 50% or 10% protection in animals that received 0.25mg/kg or 0.1mg/kg, respectively (Fig. 2A). As expected, none of the mice that received the isotype control mAb at 15mg/kg survived the challenge infection. When rHK68 infected mice were treated therapeutically with a single dose of 30 mg/kg, D1-8 provided 90-100% protection in all treated animals even when administered as late as 96 h after infection (Fig. 2B). This was remarkable, given that mice treated 96 h after infection had already lost >15% of their body weight (data not shown). In addition to surviving the infection, all mice in the D1-8 treatment groups regained body weight and showed a reversal of clinical signs of infection by the end of the observation period (data not shown). In contrast, the mice in the control group succumbed to infection by 9 days post infection (Fig. 2B).
To determine whether D1-8 reduced viral load in the lower respiratory tract, mice were infected with rHK68, treated with D1-8 mAb at the indicated times, and sacrificed 4 days after infection. Viral load in the lungs was determined by measuring TCID$_{50}$. As shown in Fig. 2C, treatment of mice either on the day of infection (day 0) or 1 or 2 days after infection resulted in an ~100-fold reduction in viral titer in the lungs when compared to the control group of mice. Mice treated with D1-8 3 days after infection (i.e., treated with mAb for <24 hr) exhibited an ~5-fold reduction in viral titer in the lungs when compared to the control. Taken together, these results indicate that D1-8 significantly reduces viral replication in the lungs of infected mice and this correlated with animal survival.

**D1-8 is superior to oseltamivir in protecting mice from lethal challenge with H3 influenza virus**

Oseltamivir administered at a dose of 75 mg twice daily (BID) for 5 days is currently standard-of-care for the treatment of influenza virus infections. This clinical dose is approximately equivalent to a dose of 12.5mg/kg BID in mice, based on the body surface area normalization method (39). Our preliminary study showed that therapeutic treatment of influenza infected mice with D1-8 administered 4 days post-infection at concentrations as low as 2.5mg/kg provided ≥80% protection from lethality (data not shown). To demonstrate the potential utility of antibody therapy late in infection, we compared the therapeutic efficacy of D1-8 when administered as a single dose (2.5mg/kg) 4 days after infection with oseltamivir dosed at 25mg/kg BID (total dose of 50mg/kg/day) for 5 days, initiated on the 4th day after infection. The oseltamivir dose is equivalent to twice the...
dose typically administered in the clinic and represents a typical efficacious dose used in mouse models of influenza infection (40). As shown in Figs. 3A & 3B, a single dose of D1-8 resulted in body weight recovery and survival for >80% of treated animals, while limited body weight recovery was observed in the oseltamivir treated animals and only 20% survived the rHK68 infection. These data show that D1-8 mAb treatment was more effective than oseltamivir in protecting mice late in infection.

Discussion

Several mAbs have been described that neutralize H3 viruses and some also exhibit cross-reactivity with other subtypes of influenza (e.g., H1 strains) (13, 15, 17, 26, 27, 30, 31). Some of these previously described mAbs target the globular head region of HA (26, 27, 30, 31) while others target the more conserved stalk structure (13, 15, 17). However, only a few have demonstrated antiviral activity against diverse H3 viruses (>5 viruses) and all such mAbs target the HA stalk structure (13, 15, 17). We show that D1-8 (32) binds to H3N2 HA proteins and potently neutralizes all viruses tested from a diverse panel of H3N2 strains originally isolated over a 40 year period. However, D1-8 did not exhibit binding activity against H1 or H7 HA proteins nor did D1-8 show antiviral activity against H1 or H7 influenza strains, indicating specificity for H3 influenza. In addition, D1-8 exhibited HAI activity, a characteristic typically associated with binding to the globular head region of HA. The antigenic site of D1-8 was further confirmed by the genotypic analysis of escape mutants, which revealed a putative epitope region in the globular head of H3 HA that is unique and comprised of highly conserved residues.
To our knowledge, D1-8 is the first broadly neutralizing mAb described that binds to a conserved epitope on the globular head of HA that appears to be distinct from the RBS. Crystallographic and/or resistant virus studies have shown that previously described broadly neutralizing mAbs that target the HA globular head typically make key interactions with conserved residues overlapping the RBS (29-31). For example, the H1-specific mAb, CH65 (29), interacts with the RBS as well as proximal residues in antigenic sites Sa, Sb and CA2. Alternatively, the mouse mAb S139/1, which neutralizes multiple influenza A subtypes (27, 31), binds to highly conserved residues in the RBS and antigenic sites A, B and D (H3 designation) (31). Another mAb, C05 (30), contains an unusual HCDR3 (24 residues in length) that mediates the majority of its interactions with HA by insertion into the RBS. In contrast, D1-8 appears to interact differently with the globular head of HA when compared to these other well-characterized mAbs. D1-8 neutralization escape mutations localize proximal to HA antigenic site D that overlaps with the region of interface between monomers (22), rather than sequences directly surrounding the RBS. The HA residues implicated in D1-8 binding are well conserved across a large number of H3N2 strains, which is consistent with the broad-spectrum H3N2 antiviral activity observed for D1-8. Given that D1-8 was derived directly from the B-cells of a vaccinated individual (32), these observations provide proof-of-concept that antibody responses to conserved epitopes on the influenza H3 HA globular head outside the RBS can be generated in humans.
Consistent with its potent antiviral activity \textit{in vitro}, D1-8 exhibits significant prophylactic and therapeutic activity in mouse models of influenza infection. D1-8 protected mice from lethal H3 influenza infection when administered as late as 4 days after infection. More impressively, D1-8 exhibited superior efficacy when compared to clinically relevant doses of oseltamivir when treatment was initiated late infection (4 days) in mice. Such therapeutic efficacy in preclinical models has been demonstrated previously for other mAbs directed against influenza HA (10, 12, 13, 17), suggesting that anti-HA mAbs may be effective therapeutics for treating influenza infection. These preclinical data also raise the possibility the anti-HA mAbs such as D1-8 may be more effective than oseltamivir either alone or in combination with oseltamivir, particularly late in infection (17, 41). Anti-HA mAbs have been shown to mediate antiviral activity via multiple mechanisms in preclinical models \textit{(in vitro and in vivo)}, including direct virus neutralization as well as mechanisms dependent on mAb Fc effector function (e.g., antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity) (13). It is possible that the additional antiviral mechanisms mediated by anti-HA mAbs could extend the utility of influenza therapies into patient populations with a high unmet medical need, such as hospitalized patients with more advanced infection.
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Figure Legends

Figure 1. Characteristics of the putative D1-8 antigenic site. (A) D1-8 epitope illustrated on a space filling-model of the trimeric membrane-distal globular head of H3 HA. The H3 HA antigenic sites are colored pink (site A), light blue (site B), light purple (site C), tan (site D) and light green (site E). The D1-8 mAb escape mutations are labeled by amino acid letter and residue numbers. The residues 171 (dark purple), 172 (dark green), 173 (yellow), 207 (red) and 240 (dark blue) are proximal to the antigenic site D that is adjacent to the receptor binding pocket. (B) Western blot analysis of the D1-8 /H3 HA interaction. Top panel: The recombinant trimeric HA of A/Perth/09 proteins were disrupted in 2% SDS without DTT (-) or with 100mM DTT (+), and then analyzed by Western blotting with mAb D1-8. Bottom panel: a Coomassie blue stained duplicate gel to show equal loading of HA proteins.

Figure 2. Percent survival of mice in a model of influenza infection following prophylactic or therapeutic administration of D1-8 (A) BALB/c mice were treated with D1-8 at the indicated concentrations or a control mAb and then infected with rHK68 4 hrs later. Percent survival vs time is presented. (B) BALB/c mice were infected with...
rHK68 and treated with D1-8 (30 mg/kg) 4 hrs (d0), 1 (d1), 2 (d2), 3 (d3) or 4 (d4) days after infection. Control mice were treated with an irrelevant control mAb 4 hrs prior to infection. Percent survival vs time is presented. (C) Mice were infected as described in the therapeutic efficacy study (B), sacrificed 4 days after infection and virus titers in lungs were measured using the TCID50 method (**, \( P < 0.01 \) versus control mice, Mann-Whitney test).

Figure 3. Therapeutic efficacy of D1-8 when compared to oseltamivir in an influenza mouse model late in infection. Mice received a single intraperitoneal injection of D1-8 (2.5 mg/kg) 4 days after infection or 25 mg/kg oseltamivir, given orally twice a day (50 mg/kg/day) for 5 days with dosing initiated 4 days after infection. Mice were monitored for 14 days for body weight loss (A) and survival (B). Mean change in body weight is expressed as a percentage of the baseline body weight. Error bars represent 95% confidence intervals.

REFERENCES


antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. Science 333:850-856


antigenic sites and residues mediating human receptor specificity in H1N1 viruses. PLoS Pathog. 12 : e1003067.


to simultaneously quantify hemagglutinin and neuraminidase-inhibiting antibody responses. Vaccine 28:790-797.


Mean log10 TCID50/g of tissue

Administration on the indicated days post infection
Table 1. D1-8 Hemagglutination inhibition (HAI) and neutralization (Neut) activity against representative influenza H3N2 virus strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>HAI activity*</th>
<th>Neut IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Hong Kong/8/1968</td>
<td>1.56 µg/ml</td>
<td>0.03±0.02 µg/ml</td>
</tr>
<tr>
<td>A/Victoria/3/1975</td>
<td>6.25 µg/ml</td>
<td>0.04±0.003 µg/ml</td>
</tr>
<tr>
<td>A/Los Angeles/2/1987</td>
<td>6.25 µg/ml</td>
<td>0.10±0.05 µg/ml</td>
</tr>
<tr>
<td>A/Shangdong/9/1993</td>
<td>12.5 µg/ml</td>
<td>0.66±0.15 µg/ml</td>
</tr>
<tr>
<td>A/Argentina/3779/1994</td>
<td>12.5 µg/ml</td>
<td>0.27±0.12 µg/ml</td>
</tr>
<tr>
<td>ca A/Sydney/5/1997</td>
<td>1.56 µg/ml</td>
<td>0.05±0.03 µg/ml</td>
</tr>
<tr>
<td>ca A/Panama/2007/1999</td>
<td>1.56 µg/ml</td>
<td>0.08±0.03 µg/ml</td>
</tr>
<tr>
<td>A/Wyoming/03/2003</td>
<td>0.4 µg/ml</td>
<td>0.03±0.01 µg/ml</td>
</tr>
<tr>
<td>ca A/California/7/2004</td>
<td>0.78 µg/ml</td>
<td>0.04±0.01 µg/ml</td>
</tr>
<tr>
<td>ca A/Wisconsin/67/2005</td>
<td>0.2 µg/ml</td>
<td>0.03±0.003 µg/ml</td>
</tr>
<tr>
<td>ca A/Perth/16/2009</td>
<td>0.2 µg/ml</td>
<td>0.01±0.007 µg/ml</td>
</tr>
<tr>
<td>A/Minnesota/11/2010</td>
<td>&gt;50 µg/ml</td>
<td>0.33±0.14 µg/ml</td>
</tr>
<tr>
<td>A/Indiana/10/2011</td>
<td>&gt;50 µg/ml</td>
<td>0.63±0.13 µg/ml</td>
</tr>
</tbody>
</table>

ca: cold-adapted

* HAI titers are expressed as the lowest concentrations of purified D1-8 that completely inhibited hemagglutination

Table 2. Amino acid changes in the H3 HA of D1-8 mAb resistant mutants

<table>
<thead>
<tr>
<th>H3N2 Virus</th>
<th>HA Amino Acid Substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 x IC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>A/WY/3/2003</td>
<td>K207N</td>
</tr>
<tr>
<td></td>
<td>G240E</td>
</tr>
<tr>
<td></td>
<td>G240E, N171E</td>
</tr>
<tr>
<td></td>
<td>G240E, K173T</td>
</tr>
<tr>
<td>A/HK/1-5/1968</td>
<td>R207N</td>
</tr>
<tr>
<td>A/Perth/16/2009</td>
<td>None</td>
</tr>
</tbody>
</table>

*: The concentration of D1-8 mAb at 20 xIC<sub>50</sub> was achieved after 3 passages, while 100 x IC<sub>50</sub> was reached after 2 more passages.
Table 3. Susceptibility of rWY03 variants to D1-8 Neutralization (Neut) or HAI

<table>
<thead>
<tr>
<th>H3 variants</th>
<th>IC50 µg/ml</th>
<th>Fold changes relative to wild type rWY03</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAI</td>
<td>Neut</td>
</tr>
<tr>
<td>rWY03</td>
<td>0.55</td>
<td>0.017</td>
</tr>
<tr>
<td>rWY03_N171K</td>
<td>&gt;1200</td>
<td>&gt;1200</td>
</tr>
<tr>
<td>rWY03_N171E</td>
<td>&gt;1200</td>
<td>&gt;1200</td>
</tr>
<tr>
<td>rWY03_N171D</td>
<td>&gt;1200</td>
<td>&gt;1200</td>
</tr>
<tr>
<td>rWY03_E172G</td>
<td>29</td>
<td>4.49</td>
</tr>
<tr>
<td>rWY03_K173T</td>
<td>&gt;1200</td>
<td>&gt;1200</td>
</tr>
<tr>
<td>rWY03_K207N</td>
<td>1.65</td>
<td>1.54</td>
</tr>
<tr>
<td>rWY03_G240E</td>
<td>44</td>
<td>14.51</td>
</tr>
<tr>
<td>rWY03_E172G/K207N</td>
<td>&gt;1200</td>
<td>&gt;1200</td>
</tr>
</tbody>
</table>

Table 4. Conservation of H3N2 HA Residues Substituted in D1-8 MARMsa

<table>
<thead>
<tr>
<th>Residues in D1-8 MARMs</th>
<th>Residues (%) observed in circulating influenza H3N2 strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>N171K/E/D</td>
<td>N (99.64%) T (0.24%) S (0.12%)</td>
</tr>
<tr>
<td>E172 G</td>
<td>E (77.9%) D (12.1%) G (9.5%) K (0.5%)</td>
</tr>
<tr>
<td>K173T</td>
<td>K (67.7%) Q (18.4%) N (9.3%) E (4.4%) R (0.2%)</td>
</tr>
<tr>
<td>K207N</td>
<td>K (96.2%) R (3.6%) Q (0.2%)</td>
</tr>
<tr>
<td>G 240E</td>
<td>G (100.0%)</td>
</tr>
</tbody>
</table>

a A total of 7432 human H3N2 HA protein sequences form the Influenza Research Database from 1968 to 2011 were analyzed.