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Canine H3N8 influenza vaccines partially protect mice against the canine H3N2 strain currently circulating in the United States

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Abstract

Influenza A viruses infect many species and cross-species transmission occurs occasionally. An equine H3N8 influenza virus began circulating in dogs in 1999 and an avian H3N2 influenza virus began circulating in dogs in 2006. The canine H3N8 (cH3N8) viral strain has become endemic in parts of the United States and there is a commercially available vaccine against this strain. The canine H3N2 (cH3N2) strain did not circulate widely in the United States until 2015. Here, we used a mouse model to determine if the cH3N8 and cH3N2 strains are antigenically related and if a commercially available cH3N8 vaccine protects animals against the cH3N2 outbreak strain. We find that the cH3N8 vaccine elicits antibodies that react to internal viral proteins and the hemagglutinin stalk region of cH3N2 viruses. These antibodies do not provide sterilizing immunity against cH3N2 infection, but these antibodies limit cH3N2 replication in the lung.

1. Introduction

Influenza A viruses infect many different species and these viruses occasionally jump from one species to another [1]. Cross-species introductions of influenza viruses can have devastating consequences because there is often a low level of immunity against the viral strain that enters the new population. An equine H3N8 influenza A virus strain was introduced into dogs around 1999 [1, 2]. This H3N8 virus (herein referred to as canine H3N8 (cH3N8)) was first identified in racing greyhounds and has subsequently become endemic in pet and shelter dog populations in the United States, with seropositive dogs

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Conflict of interest statement

The authors have no conflict of interests.

found in at least 19 states across the country [3]. While most cH3N8 infections are mild or asymptomatic, some dogs experience severe, even fatal, illness [2]. A cH3N8 vaccine consisting of inactivated antigen and an aluminum-based adjuvant was licensed in 2009. In previously seronegative dogs, the cH3N8 vaccine elicits 1:40 hemagglutination inhibition (HAI) titers and prevents severe disease and prolonged nasal virus shedding caused by cH3N8 infections [4].

A novel H3N2 strain was detected in dogs in South Korea in 2006-2007 [5]. Phylogenetic analyses revealed that this new canine H3N2 (cH3N2) strain was likely introduced from an avian influenza reservoir [5]. The cH3N2 did not circulate widely in the United States until 2015. In the spring of 2015, cH3N2 caused a large outbreak in dogs in the Midwestern United States and has since spread into at least 30 states (https://ahdc.vet.cornell.edu/docs/CIV_Monitoring_2016-07-11.pdf). Although cH3N8 and cH3N2 both possess H3 hemagglutinin (HA), it is unknown if the commercially available cH3N8 vaccine protects against cH3N2 infection. Recently, two cH3N2 vaccines were conditionally licensed (https://www.aphis.usda.gov/animal_health/vet_biologics/publications/CurrentProdCodeBook.pdf). Here, we used a murine model to determine if the commercially available cH3N8 and conditionally licensed cH3N2 vaccines elicit antibodies that react to and prevent replication of the new cH3N2 virus.

2. Materials and methods

2.1 Virus and vaccine

A cH3N2 influenza isolate (A/canine/Illinois/12191/2015 (H3N2)) was obtained from the National Veterinary Services Laboratory (Ames, Iowa) and propagated in Madin-Darby canine kidney (MDCK) cells. The titer of the infectious cH3N2 stock used in this study was determined by TCID₅₀ (tissue culture infectious dose) assay using MDCK cells. The virus was titrated in quadruplicate and the TCID₅₀ value was calculated using the Reed & Muench calculator. We obtained cH3N8 influenza vaccine (Merck) and cH3N2 influenza vaccine (Merck) from the Ryan Veterinary Hospital of the University of Pennsylvania.

2.2 cH3N2 and cH3N8 sequence comparisons

Amino acid sequences for cH3N2 (A/canine/Illinois/12191/2015 (H3N2), KT002536.1) and cH3N8 (A/canine/Florida/78592-2/2006 (H3N8), CY067398.1) HAs were aligned using protein BLAST. Amino acid differences were modeled on the HA from A/Hong Kong/1/1968 (H3N2) (4FNK; PDB) using PyMol. Potential N-linked glycosylation sites in the amino acid sequences of the cH3N2 and cH3N8 HAs were identified using NetNGlyc (<http://www.cbs.dtu.dk/services/NetNGlyc/>).

2.3 Murine vaccinations and viral challenge

All experiments were performed at the Wistar Institute according to protocols approved by the Wistar Institute Institutional Animal Care and Use Committee. 6 week old female BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were immunized intraperitoneally (i.p.) with 100 μ l of cH3N8 or cH3N2 vaccine. Mice were immunized i.p. again with the same vaccine dose 21 days later. Sera were collected 14 days

following the second vaccination. Mice were intranasally infected with 10^5 TCID₅₀ of cH3N2 in 50 μ l under isoflurane anesthesia 14 days following the second vaccination. We euthanized mice 3 days later and quantified virus titers in lung homogenates by TCID₅₀ assays.

2.4 Characterization of Ab responses

Abs in sera from vaccinated mice were quantified by enzyme-linked immunosorbent assay (ELISA). cH3N2 vaccine (0.4 HAU/ μ l), cH3N8 vaccine (0.4 HAU/ μ l) or recombinant cH3 from cH3N2 virus (5 μ g/ml; Influenza Reagent Resource, FR-1478) were diluted in PBS and adsorbed on Immulon 4HBX plates (Thermo Scientific, Waltham, MA) overnight at 4 °C. For chimeric HA ELISAs, recombinant chimeric HA protein was diluted in bicarbonate buffer to 2 μ g/ml and adsorbed on Immulon 4HBX plates overnight at 4 °C. Plates were blocked for 2 hr with 3% BSA in PBS at room temperature and then washed with distilled water. Sera were serially diluted in 1% BSA and incubated on the plates for 2 hrs at room temperature. Plates were washed with distilled water and anti-mouse IgG conjugated to alkaline phosphatase diluted in 1% BSA (Southern Biotech, Birmingham, AL) was incubated for 1 hr at room temperature. Plates were washed with distilled water and PNPP (p-nitrophenyl phosphate; Thermo Scientific, Waltham, MA) was added and optical densities were measured using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

3. Results

3.1 cH3N8 and cH3N2 viruses possess dramatically different HA globular heads

Phylogenetic analyses have shown that the HA and NA genes of avian-origin cH3N2 and equine-origin cH3N8 viruses are very different [6, 7]. Most vaccines target the HA protein, which consists of a variable globular head domain and a more conserved stalk domain. Amino acid sequence comparisons between the HAs of the prototypical A/canine/Illinois/12191/2015 cH3N2 strain (Genebank: KT002536.1) and the A/canine/Florida/78592-2/2006 cH3N8 strain (Genebank:CY067398.1) revealed major differences in the HA globular head (Fig. 1A,B). These differences are distributed across all five antigenic sites of H3 [8]. The HA stalk domains of these viruses are more conserved compared to the HA globular head domain, although there are also some differences in this region of HA as well (Fig. 1A). Analysis of potential glycosylation sites revealed that cH3N2 and cH3N8 HAs differ substantially in predicted N-linked glycosylation patterns. Although the HAs share three potential glycosylation sites (at residues 22, 165, and 285), they each possess several unique potential glycosylation sites (at residues 81 and 154 in cH3N2 and residues 38 and 63 in cH3N8).

3.2 cH3N8 vaccine generates cross-reactive antibodies in mice

A commercial inactivated cH3N8 vaccine has been available since 2009 (Center for Veterinary Biologics Notice No. 09-13). We completed a series of experiments in mice to determine if this commercial vaccine elicits antibodies that react to the new cH3N2 virus. We vaccinated BALB/c mice i.p. with 100 μ l of undiluted cH3N8 vaccine and then we boosted mice with the same dose of vaccine 21 days later. As a control, we vaccinated some

mice twice with a new conditionally licensed cH3N2 vaccine. We collected sera 35 days after the initial vaccination (14 days after the boost) and completed ELISA assays with plates coated with cH3N2 virus to assess antibody reactivity. As expected, antibodies in sera from cH3N2 vaccinated mice bound strongly to cH3N2 virus (Fig 1C). Surprisingly, the cH3N8 vaccine elicited an antibody response that reacted strongly to cH3N2 virus (Fig 1C), despite the observation that cH3N8 and cH3N2 possess dramatically different HA glycoproteins (Fig A,B). Similarly, both the cH3N2 and cH3N8 vaccines elicited antibodies that reacted to ELISA plates coated with cH3N8 (Fig 1D).

Influenza vaccine preparations can contain high amounts of internal virus proteins, such as nucleoprotein (NP), in addition to HA and NA glycoproteins [9]. Our initial ELISA analyses using plates coated with cH3N2 and cH3N8 virus do not discriminate between antibodies that bind to glycoproteins or internal viral proteins. For this reason, we completed additional experiments with ELISA plates coated with recombinant HA. As expected, antibodies in sera from cH3N2-vaccinated animals bound strongly to HA recombinant protein from cH3N2 virus (Fig 2A). However, antibodies in sera from cH3N8-vaccinated animals bound only weakly to HA recombinant protein from cH3N2 virus (Fig 2A). We next tested if cH3N8 vaccination elicits antibodies that react to the HA stalk region of cH3N2 viruses since the HA stalk domain of cH3N2 and cH3N8 viruses are fairly conserved (Fig. 1A). Both cH3N8 and cH3N2 vaccination elicited antibodies that bound to ELISA plates coated with chimeric HAs [10] consisting of an H5 head domain and an H3 stalk domain (Fig 2B). Antibodies elicited by these vaccinations did not bind to ELISA plates coated with chimeric HAs consisting of an H5 head domain and an H1 stalk domain (Fig 2C), indicating that the antibodies binding to the chimeric H5/H3 protein were specific for the H3 stalk domain. Collectively, our data indicate that cH3N2 vaccines elicit antibodies that recognize internal viral proteins and epitopes on the HA head and stalk domain of cH3N2 viruses. In contrast, cH3N8 vaccines elicit antibodies that recognize internal viral proteins and epitopes on the HA stalk domain of cH3N2 viruses but not epitopes on the HA head domain of cH3N2 viruses.

3.3 cH3N8 vaccine partially protects against cH3N2 infection in vivo

Antibodies that recognize conserved areas of the HA stalk can limit viral replication by preventing viral fusion [11] and antibodies against NP can limit viral replication through antibody-dependent cellular cytotoxicity (ADCC) mechanisms [12-14]. Although cH3N2 viruses replicate in mice (Fig 3), cH3N2 viral infection does not cause morbidity or mortality in mice (data not shown). Therefore, we could not complete survival vaccine efficacy studies with cH3N2 viruses. Instead, to determine if antibodies elicited against the cH3N8 vaccine limit cH3N2 viral replication, we infected vaccinated mice with 10^5 TCID₅₀ of cH3N2 virus and we quantified viral loads in lungs 3 days post infection. As a control, we also infected naïve mice. As expected, cH3N2 viral titers were lower in mice vaccinated with cH3N2 virus compared to unvaccinated mice (Fig 3). Only 3 of 9 mice vaccinated with cH3N2 had detectable lung viral titers following cH3N2 infection (Fig 3). Conversely, despite the observation that the cH3N8 vaccine elicited antibodies that react to cH3N2 (Figs 1-2), 7 of 8 cH3N8-vaccinated animals had viral titers in the lung following cH3N2 infection (Fig. 3). Differences in the number of animals productively infected with cH3N2 were

statistically significant when comparing cH3N2 versus cH3N8 vaccinated groups (Fisher's exact tests, $P < 0.05$). Notably, although almost all cH3N8-vaccinated mice had detectable cH3N2 virus in the lungs, the titers were $\sim 1.5 \log_{10}$ lower than viral titers of unvaccinated mice ($P=0.001$, one-way ANOVA with Tukey's multiple comparisons test). Therefore, cH3N8 vaccines elicit antibodies in mice that do not provide sterilizing immunity against cH3N2 infection, but these antibodies limit cH3N2 replication in the lung.

4. Discussion

There is often a delay in generating vaccines against new pandemic influenza virus subtypes, and therefore it is important to evaluate if existing vaccines confer some level of cross-protection. Although there is an approved cH3N8 vaccine for dogs, it is unknown if this vaccine elicits antibodies that prevent cH3N2 virus infection. This information is important, since the new cH3N2 strain has been detected in at least 30 states within the United States of America over the past year (https://ahdc.vet.cornell.edu/docs/CIV_Monitoring_2015-10-15.pdf). Here, we completed a series of experiments to determine if the current cH3N8 vaccine elicits antibodies that bind to and neutralize cH3N2 viruses in mice. We find that the cH3N8 vaccine elicits antibodies that bind to cH3N2 virus. These antibodies do not prevent cH3N2 infection but they do limit viral replication in the lung.

One limitation of our studies is that we evaluated the cH3N8 vaccine in mice, instead of dogs. However, mice are very useful models to study antibody responses against human and non-human influenza viruses. For example, classical HA antigenic sites have been defined using the mouse model [15] and antibodies directed against the HA globular head, HA stalk, and NP can protect mice from influenza virus infection *in vivo* [11, 12]. Although our studies show that cH3N2 viruses do not cause morbidity and mortality in mice, these viruses do replicate in the murine airway. We chose not to mouse-adapt the cH3N2 virus for our studies, since viral adaption to mice can lead to mutations in antigenic sites of HA [16-18]. Since cH3N2 viruses replicate in the airways of mice, we were able to quantify cH3N2 lung viral titers as a surrogate for protection in these studies. Lung viral titers have previously been used as a measure of disease severity during influenza infection in mice [19, 20].

Our data suggest that cH3N8 vaccines elicit antibodies that recognize internal viral proteins and the HA stalk of cH3N2 viruses. While our study shows that the cH3N8 vaccine does not provide sterilizing immunity against the cH3N2 strain in mice, antibodies elicited by this vaccine might be sufficient to protect dogs from severe disease. Our data indicate that although cH3N8 vaccines may provide some protection against cH3N2 virus, conditionally licensed cH3N2 vaccines should be considered in addition to cH3N8 vaccines in areas of the world where cH3N8 and cH3N2 viruses have spread widely.

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Highlights

- canine H3N8 vaccines elicit antibodies that react strongly to canine H3N2 viruses
- Abs elicited by canine H3N8 vaccines bind to the HA stalk region of canine H3N2
- Canine H3N8 vaccines partially protect mice against canine H3N2 infection

Figure 1. cH3N8 and cH3N2 vaccines elicit cross-reactive Abs

(A,B) HA residues that differ between cH3N2 (A/canine/Illinois/12191/2015, KT002536.1) and cH3N8 (A/canine/Florida/78592-2/2006, CY067398.1) are shown in black. These residue differences are shown on the HA of the A/Hong Kong/1/1968 H3N2 virus (Protein Data Bank: 4FNK) since an H3 canine crystal structure is not available. (C,D) Mice were immunized twice with the cH3N8 vaccine or cH3N2 vaccine. Sera were collected 14 days after the second immunization and ELISAs were completed to quantify levels of (C) cH3N2-reactive IgG and (D) cH3N8-reactive IgG. For these assays, ELISA plates were

coated with either cH3N2 or cH3N8 vaccine. Data shown are mean $\pm$ SEM and include sera collected over 2 independent experiments. N=4 mice per group for each experiment.

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Figure 2. Abs elicited by cH3N8 vaccine bind to stalk region of cH3N2 HA

Mice were immunized twice with the cH3N8 vaccine or cH3N2 vaccine and sera were collected 14 days after the second immunization. (A) ELISAs were completed to quantify levels of IgG that bound to the HA from cH3N2. For these assays, recombinant HA from cH3N2 was used to coat ELISA plates. HA-reactive IgG levels were higher in mice vaccinated with cH3N2 vaccine compared to mice vaccinated with cH3N8 vaccine ($p < 0.0001$, one-way ANOVA with Tukey's multiple comparison test of area under the curve). (B-C) Similar experiments were completed with ELISA plates coated with chimeric HAs

with an H5 globular head and an H3 stalk or H1 stalk. (B) H3 stalk-reactive IgG levels were not different in mice vaccinated with cH3N2 or cH3N8 vaccines. (C) Antibodies elicited by either vaccination bound weakly to the H1 stalk. Data shown are mean $\pm$ SEM and include sera collected over 2 independent experiments. N=4 mice per group for each experiment.

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lung titers 3 days after cH3N2 infection

Figure 3. cH3N8 vaccine partially protects mice against cH3N2 virus infection

Mice immunized twice with cH3N2 or cH3N8 vaccine were challenged with 10^5 TCID₅₀ of cH3N2 virus 14 days after the second immunization. As a control, unvaccinated mice were also infected. Lungs were removed 3 days later and viral titers were quantified by a TCID₅₀ assay. Shown are lung titers of individual mice (each circle represents an individual mouse) and geometric mean titer (line).