Evaluation of the Efficacy of a Canine Influenza Virus (H3N8) Vaccine in Dogs Following Experimental Challenge*

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CLINICAL RELEVANCE

Canine influenza virus (CIV) subtype H3N8 is an emerging pathogen with sustained horizontal transmission in the dog population in the United States. This study evaluated the efficacy of an inactivated CIV vaccine in 6- to 8-week-old beagle pups challenged with virulent CIV. One group of CIV-seronegative pups was vaccinated with two doses of a CIV vaccine 3 weeks apart; a second group of pups received adjuvanted placebo as a control. Blood samples were collected at various times to determine antibody titers. All pups were challenged with a virulent CIV isolate 13 days after the second vaccination and monitored for clinical signs of respiratory disease, virus shedding, and lung consolidation. Vaccinated pups developed hemagglutination inhibition antibody titers after vaccination. The severity of clinical signs ($P < .001$) and the magnitude and duration of virus shedding ($P < .0001$) were significantly lower in vaccinated pups compared with control pups. These results demonstrate that the CIV vaccine used in this study provides protection against virulent CIV challenge in dogs.

■ INTRODUCTION

Influenza viruses cause acute, highly contagious respiratory infections in various verte-brate species, including humans, birds, pigs, and horses.[1] Although experimental and natural infections with influenza virus (H5N1) have occurred in dogs previously, the influenza viruses were believed to be incapable of sus-

tained transmission in dogs.[2] Influenza virus A subtype H3N8 (referred to as canine influenza virus [CIV]) was first isolated in racing greyhounds suffering from respiratory disease at Florida racetracks.[3] Unlike previous influenza infections in dogs, CIV is believed to have established itself in the dog population with sustained dog-to-dog transmission.[3,4] Affected dogs exhibit two forms of clinical disease: a milder disease characterized by initial fever followed by coughing for 10 to 14 days and subsequent recovery, and a peracute disease characterized by hemorrhagic pneumonia and death. Since first being isolated, CIV has been isolated from dogs in at least nine states in the United States.[3,4] As of April 2009, at least 30 states and Washington, DC, have reported CIV-infected dogs.[a] The widespread distribu-

■ MATERIALS AND METHODS

Virus

Two CIV isolates, CIV14-06A and CIV25-06B, isolated from dogs exhibiting respiratory disease in Florida, were used in this study. The isolates were confirmed as subtype H3N8 by serotyping and polymerase chain reaction analysis. The CIV isolates used in this study were confirmed to be free of bacteria, including *Mycoplasma*, and other extraneous canine viruses.

Animals

Thirty-six CIV-seronegative beagle pups of both sexes, 6 to 8 weeks old, were enrolled in the study. The pups were group-housed in a biosafety level 2 isolation facility. Pups were blocked by litter and randomly assigned to one

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tion of H3N8 virus in US dogs suggests that this virus is different from equine viruses that occasionally cross over from horses to dogs, such as reported by Daly and associates.[5]

Recently, avian influenza virus subtype H3N2 has been isolated from the dog population in Korea and has also established itself in the canine population with sustained dog-to-dog transmission.[6,7] The presence of two subtypes of influenza potentially circulating in the worldwide dog population points to the need for efficacious vaccines to control this disease and virus spread. The objective of this study was to evaluate the efficacy of an inactivated CIV vaccine against experimental challenge with a virulent CIV subtype H3N8 in dogs.

of two treatment groups (21 vaccinates and 15 controls) using computer-generated random numbers. All pups were healthy at the time of enrollment and were CIV antibody negative (hemagglutination inhibition titer, <10) and negative for CIV shedding at the time of first vaccination. All pups were subjected to standard husbandry practices. The study was conducted with the approval of Schering-Plough Animal Health Institutional Animal Care and Use Committee.

Vaccination and Challenge

The CIV vaccine was formulated with an inactivated CIV (CIV25-06B) antigen and an aluminum-based adjuvant. An adjuvanted placebo was formulated similar to the experimental vaccine with no CIV antigen. Pups in the vaccinate group ($n = 21$) and in the control

[a]Crawford PC. Personal communication, University of Florida Veterinary Medical Center, 2009.

Assignment of Clinical Scores

Ocular Discharge
  0 = No discharge
0.5 = Serous
1.0 = Mild mucus
2.0 = Copious mucus

Nasal Discharge
  0 = No discharge
0.5 = Serous
1.0 = Mild mucus
2.0 = Copious mucus

Sneezing
0 = Absent
2 = Present

Spontaneous Cough
  0 = Absent
0.5 = Mild
1.0 = Moderate, persistent
2.0 = Severe cough
        accompanied by choking
        or retching sounds

Dyspnea
0 = Absent
2 = Present

Depression
0 = Absent
2 = Present

Body Temperature
0 = <39.5°C
2 = ≥39.5°C

group ($n = 15$) were given the CIV vaccine and the placebo, respectively, by the SC route. A second dose of vaccine or placebo was administered to the pups 3 weeks (day 21) after the first vaccination. Thirteen days after the second vaccination (day 34), all dogs were challenged with approximately $10^{7.5}$ TCID$_{50}$ [50% tissue culture infective dose] of a heterologous CIV isolate (CIV14-06A) per dog by aerosolization.

Samples

Blood samples were collected before the first and second vaccinations (days −1 and 20, respectively), before challenge (day 33), and on the day of necropsy (day 44). Nasal swabs were collected in Dulbecco's modified eagle's medium supplemented with gentamicin and amphotericin B before the first vaccination (day −1), before challenge (day 33), and then daily for 10 days beginning the day after challenge (i.e., days 35 through 44). Virus isolation and titrations were performed on Madin–Darby canine kidney (MDCK) cells.[8] To screen all pups for other respiratory pathogens such as *Bordetella, Mycoplasma, Streptococcus equi* subsp *zooepidemicus,* and canine parainfluenza virus, nasal swabs were collected before the first vaccination (day −1) and before challenge (day

33) and lung swabs were collected at the time of necropsy (10 days after challenge; day 44). The swabs were cultured on MacConkey agar plates supplemented with 5% sheep blood (for *Bordetella*), *Mycoplasma* agar plates (for *Mycoplasma*), and Columbia CNA agar plates (for *S. equi*) or were titrated on dog kidney cell line for canine parainfluenza virus. Personnel testing the samples were blinded to the treatment group assignment.

Clinical Observations

Pups were observed daily for clinical signs of respiratory disease including ocular and nasal discharge, sneezing, coughing, dyspnea, depression, and abnormal body temperature. Each clinical sign was assigned a score based on the severity (see box, above).

The individuals performing clinical observations were blinded to treatment group assignment. The data were tabulated, and the average daily clinical score was calculated.

Necropsy

All pups in both the vaccine and control groups were euthanized 10 days after challenge. The percent consolidation of each lung lobe was scored and was converted into a weighted lung lesion score based on an in-

TABLE 1. Serologic Response to Canine Influenza Virus Vaccination and Challenge in Dogs			
	Geometric Mean Hemagglutination Inhibition Titer		
Treatment	*Before 1st Vaccination (Day −1)*	*Before 2nd Vaccination (Day 20)*	*Before Challenge (Day 33)*	*After Challenge (Day 44)*
Control (*n* = 15)	<10	<10	<10	512
Vaccine (*n* = 21)	<10	25	138	1,887

house lung lesion scoring system similar to the one described by Christensen et al.[9] The person scoring lung lesions was blinded to the treatment group assignment. Lung tissues were collected in buffered formalin during necropsy for histopathology and immunohistochemistry.

Immunohistochemistry

The formalin-fixed lung tissues were processed for immunohistochemistry using a standard protocol. Briefly, the deparaffinized and rehydrated tissues were treated with 2% hydrogen peroxide to quench the endogenous peroxidase activity. Nonspecific binding was blocked by incubation with normal horse serum at room temperature for 15 to 20 minutes. The tissues were then incubated at room temperature for 60 minutes with a mouse anti–CIV (H3N8) monoclonal antibody (developed by Intervet Inc.). After a brief wash, the slides were incubated with biotinylated rabbit anti–mouse secondary antibody for 30 minutes followed by washing and incubation with avidin–biotin peroxidase complex solution for 30 minutes. The slides were rinsed and incubated with 3,3′-diaminobenzidine substrate solution for 10 minutes and counterstained with Harris hematoxylin for 15 to 20 seconds. The slides were dehydrated through graded alcohols, cleared in xylene, and coverslipped with DPX mountant medium.

Hemagglutination Inhibition Assay

Serial twofold dilutions of test serum were performed in phosphate-buffered saline in V-bottomed, 96-well microtiter plates. An equal volume of virus suspension (H3N8) containing 4 to 8 hemagglutination units of CIV was added to each well containing test serum, and the plates were incubated at room temperature for 60 minutes for antigen–antibody binding to occur. Then an equal volume of 0.5% turkey red blood cell suspension in phosphate-buffered saline was added. The plates were incubated at room temperature for an additional 30 to 60 minutes, and hemagglutination inhibition (HI) results were read visually for the presence of hemagglutination. The reciprocal of the highest dilution of the serum showing HI was considered the HI titer of the test sample. All assays were performed in duplicate, and mean values were reported.

Statistical Analysis

Statistical analysis was performed using SAS version 8.2 (SAS Institute, Cary, NC).

All vaccinated pups demonstrated a substantial increase in HI antibody titer following vaccination.

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A P value < .05 was declared as statistically significant. For clinical signs, the summed scores were compared between vaccinates and controls (placebo) using Wilcoxon exact rank sum tests. For virus shedding, the mean area under the curve was compared between vaccinates and controls using analysis of variance. The number of days of virus shedding was analyzed using Wilcoxon exact rank sum tests. Median lung lesion scores for vaccinated and control groups were compared using Wilcoxon exact rank sum tests.

RESULTS

Seroconversion

All pups were CIV seronegative before the first vaccination, and all control pups remained seronegative before challenge (Table 1). All vaccinated pups demonstrated a substantial increase in HI antibody titer following vaccination. After one dose, the HI antibody titers ranged between 10 and 80, with a geometric mean titer (GMT) of 25 at day 20. The antibody titer increased by more than fivefold (GMT, 138; range, 80 to 320) after the booster vaccination, with 100% of the pups exhibiting an HI titer >40. After challenge, antibody titers in vaccinated pups increased by approximately 14-fold (GMT, 1,887), demonstrating an anamnestic response after vaccination. The antibody titers in these pups ranged between
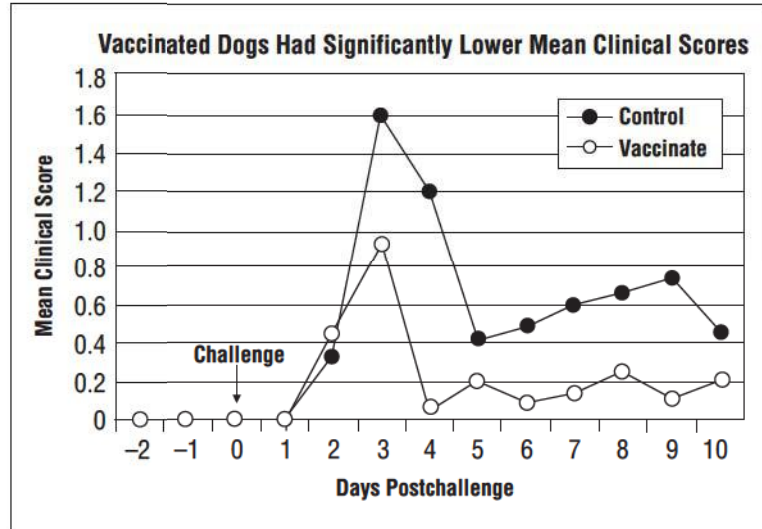

Figure 1. *Canine influenza virus–induced clinical disease after experimental infection. All pups were monitored daily from 2 days before through 10 days after challenge for clinical signs of respiratory disease (ocular and nasal discharge, sneezing, coughing, and depression). The scores for each day were summed, and an average daily score was calculated. The daily clinical scores were plotted against time. The mean clinical scores were significantly lower in the vaccinated group compared with the control group (P < .001).*
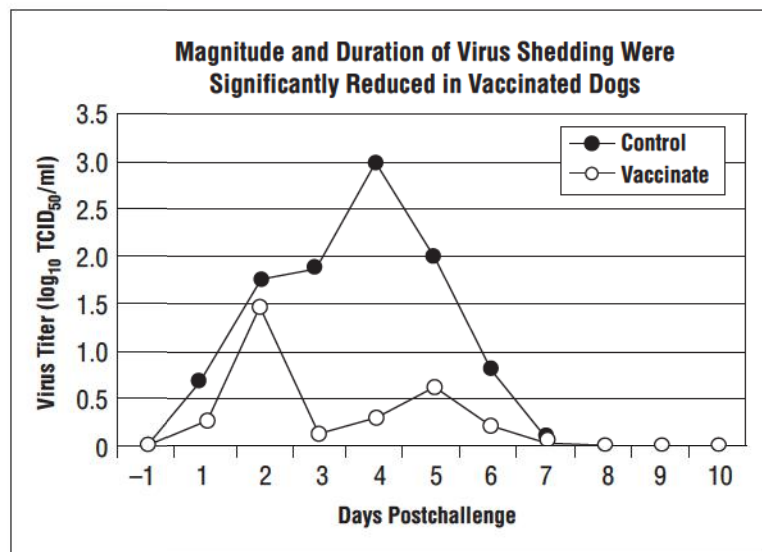

Figure 2. *Virus shedding in pups challenged with canine influenza virus. All pups were monitored for nasal virus shedding daily for 10 days after challenge. The geometric mean virus titers were calculated and plotted against time. The magnitude and the duration of virus shedding were significantly reduced in the vaccinated group compared with the control group (P < .0001).*

640 and 5,120 on day 44. The control group seroconverted following CIV challenge, with a GMT of 512 (range, 160 to 1,280).

Clinical Disease

Beginning 2 days before challenge and continuing through 10 days after challenge, all pups were monitored daily for body temperature and clinical signs including ocular and nasal discharge, sneezing, coughing, dyspnea, and depression. Pups in both the control and vaccinated groups exhibited a range of clinical signs starting 2 days after challenge (Figure 1). Coughing was the predominant sign exhibited by pups in the control group, with 93% (14 of 15) of the pups exhibiting varying degrees of coughing that lasted up to 6 days during the 10-day postchallenge observation period. On the other hand, only 33% (7 of 21) of the pups in the vaccinated group displayed a mild

shedding CIV in their nasal secretions on the first day after challenge (day 35). All the pups (100%) in the control group shed virus, with peak virus shedding 4 days after challenge followed by a precipitous drop the following day (Figure 2). In contrast, vaccinated pups showed peak virus shedding 2 days after challenge (day 36), and the vaccinated group shed significantly ($P < .0001$) lower levels of virus during the observation period compared with controls. Further, the mean number of days of shedding in the control group (5 days) was also significantly higher ($P < .0001$) compared with the vaccinated group (2 days).

Lung Lesions

Gross lung lesions were scored as percent consolidation of each lobe, and a weighted score was calculated based on the lung scoring system.[8,9] Eighty-seven percent (13 of 15) of

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The reduction in gross lung lesions in vaccinated versus control pups was statistically significant.

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cough that lasted for 1 to 2 days in six pups and 6 days in only one animal. The mean summed clinical scores were significantly higher ($P < .001$) in control pups (mean score, 6.53) compared with vaccinated pups (mean score, 2.50). None of the pups in either the control group or the vaccine group developed clinical fever, defined as a body temperature $\geq 39.5°C$.

Virus Shedding

Nasal virus shedding was monitored in all pups daily for 10 days after challenge (i.e., days 35 through 44). The virus shedding for each group, expressed as $\log_{10} TCID_{50}/ml$, was plotted against days postchallenge (Figure 2). Both the control and the vaccinated pups started

the pups in the control group exhibited varying degrees of lung consolidation, and 73% (11 of 15) of these pups had a lung lesion score of ≥ 0.8 (Figure 3). Lung lesions were characterized by consolidation (hepatization) (Figure 4). By comparison, only 28% (6 of 21) of the pups in the vaccinated group showed a mild lung consolidation, with only one pup having a score of ≥ 0.8 (Figure 3). The lung lesion scores in the control group ranged from 0 to 27.9 with a median lung score of 1.1 (mean score, 5.52), whereas lung scores in the vaccine group ranged from 0 to 2.9 with a median score of 0 (mean score, 0.18). The reduction in gross lung lesions in vaccinated versus control pups was statistically significant ($P < .0001$).

Formalin-fixed lung tissues were examined

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microscopically for evidence of histopathologic changes; tissues from 53% (8 of 15) of control pups had evidence of moderate to severe lesions including interstitial pneumonia, proliferative bronchiolitis, and tracheitis. By contrast, only 4 of 21 (19%) vaccinated pups displayed histologic signs of mild to moderate interstitial pneumonia.

Immunohistochemistry

CIV antigen was identified by the presence of brownish material produced by the staining reaction. The staining revealed that the CIV antigen was located predominantly in the cytoplasm of the alveolar and peribronchiolar macrophages and scattered diffusely within some areas containing bronchiolar epithelial cells (Figure 5). CIV antigen was detected in the lungs of 53% of the control pups and 10% of the vaccinated pups 10 days after challenge. There was a strong, positive association between the degree of gross lung lesion scores and the likelihood of detecting CIV antigen by immunohistochemistry. Together, all these data confirm that vaccination substantially reduced the risk for lung infection and lesions caused by experimental heterologous CIV challenge.

Furthermore, all dogs were negative for *Mycoplasma, S. equi* subsp *zooepidemicus,* and canine parainfluenza virus throughout the course of the study. *B. bronchiseptica* was isolated from nasal swabs of two vaccinated dogs before challenge; however, all lungs with pneumonic lesions were negative for *Bordetella bronchiseptica, Mycoplasma,* and *S. equi* subsp *zooepidemicus.*
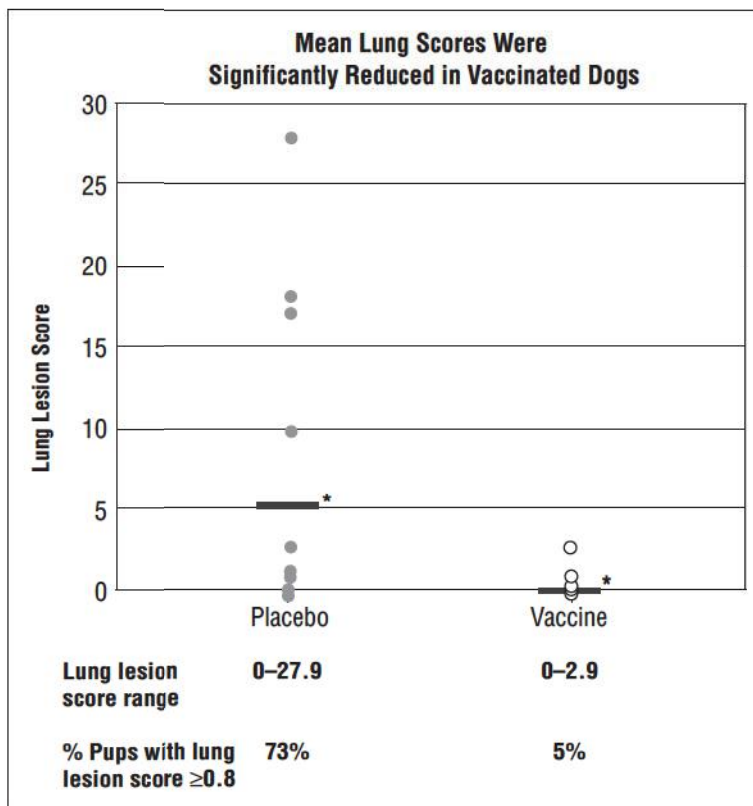

Figure 3. *Canine influenza virus–induced lung scores 10 days after challenge. Vaccinated and control pups were challenged on day 34, and lung lesion scoring was performed 10 days after challenge (day 44). Lung scores for individual dogs and the mean lung scores (horizontal lines) are illustrated. The mean lung scores were significantly reduced in the vaccinated group compared with the control group (P < .0001). *Mean lung lesion score.*

■ DISCUSSION

CIV is an emerging infectious disease of dogs; since it was first described in 2004, the disease has spread rapidly across the United States. More than 43 million households in the United States have pet dogs, and an accurate estimate of the number of dogs affected by CIV is not available.[10] However, according to an American Veterinary Medical Association (AVMA) estimate, the racing industry, boarding kennels, veterinary practices, and animal shelters together have incurred more than $5 million in losses due to CIV between 2004 and 2006.[11] In a letter to the USDA, the
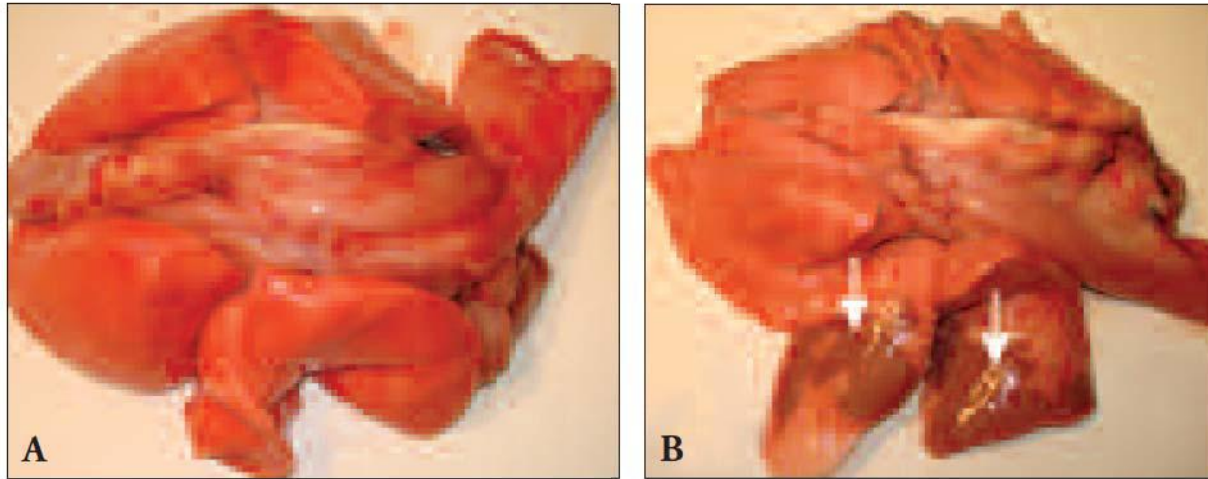
Figure 4. *Canine influenza virus–induced lung lesions in pups. (A) Lung from a vaccinated pup 10 days after challenge showing no lesions (weighted lung score, 0). (B) Lung from a control pup 10 days after challenge. Lesions were characterized by consolidation and hepatization (weighted lung score, 17.1). Arrows point to areas of consolidation and hepatization (liver-like appearance).*

AVMA emphasized the critical need for an efficacious vaccine against CIV for the promotion of animal health and welfare.[10]

Recently, we successfully developed a CIV challenge model that mimics clinical disease and lesions as reported in field cases.[8] Additionally, we have demonstrated natural transmission of CIV from infected dogs to healthy, susceptible dogs during co-housing.[12] In the study described here, we evaluated the efficacy of a CIV vaccine by challenging vaccinated pups with a virulent, heterologous CIV isolate.

Vaccines against influenza provide good protection and have been successfully used to prevent and/or reduce the severity of the disease in humans, horses, and pigs. In horses, an HI antibody titer ≥1:40 is considered to correlate with a protective immune response against influenza.[13] The CIV vaccine used in this study successfully induced detectable levels of HI antibody titers (GMT, 25) 3 weeks after the first vaccination. More than a third of the vaccinated dogs (38%) developed an HI titer ≥1:40 after a single vaccination. This is in contrast to a report by Rosas and associates[14] in which an equine

herpesvirus–vectored H3 vaccine did not induce any measurable HI antibody titer by 4 weeks after the first vaccination in dogs. The CIV vaccine used in our study increased HI antibody titers by more than fivefold 2 weeks after booster vaccination (GMT, 138), and all vaccinated dogs developed HI titers ≥1:40. These results suggest that a minimum of two doses of the vaccine are required to induce a protective response. In our study, the 14-fold increase in HI titers in vaccinated pups in response to challenge demonstrates the presence of an anamnestic response due to the vaccine in these pups.

In addition to inducing a strong immune response, an effective CIV vaccine should be able to reduce the severity of the disease in infected pups and aid in reducing the spread of infection to healthy pups. Coughing is the predominant clinical sign observed in the dogs experimentally infected with CIV.[8] The CIV vaccine used in this study was successful in alleviating the severity and duration of coughing in vaccinated pups when compared with control pups. Furthermore, the CIV vaccine also significantly reduced the magnitude and duration of virus
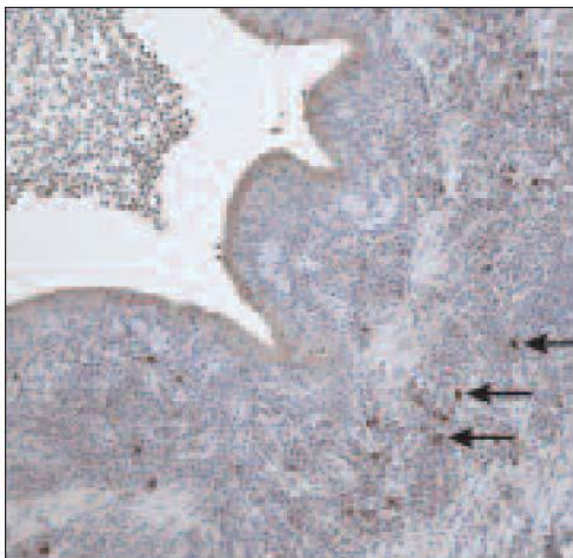
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Figure 5. *Demonstration of canine influenza virus (CIV) antigen in the lung tissue. Lung tissue from a CIV-infected control pup was stained with CIV-specific monoclonal antibody for immunohistochemistry. Arrows point to peribronchiolar macrophages containing CIV antigen (brownish intracytoplasmic material).*

shedding in vaccinated pups. This is very important because the reduced virus shedding will lower the risk of spreading the virus to healthy dogs and may help contain the infection during an outbreak.

Pneumonia and/or lung consolidation is the characteristic lesion of influenza infections. CIV causes severe lung consolidation in both natural and experimental infections.[3,8] The vaccine used in this study protected dogs against lung lesions caused by experimental challenge with virulent heterologous CIV. To our knowledge, this is the first report demonstrating protection against CIV-induced lung lesions by a CIV vaccine. Lung damage caused by CIV can lead to secondary infections, most commonly with bacteria (including *Mycoplasma*). In this study, we controlled these secondary pathogens by strict isolation procedures and ruled out their presence in lung lesions. However, with routine dog housing, it will be much more difficult to prevent secondary infections that are

set up by CIV tissue damage. Therefore, vaccination against CIV could play an important role in controlling the respiratory disease complex, which is a problem in group-housing situations such as kennels and shelters.

Currently, there are no reported field cases of CIV infection in animals other than dogs. Recently, it has been demonstrated experimentally that CIV could cause influenza-like disease in horses and cats.[15,16] In the wake of cross-species transmission of influenza viruses, particularly avian flu (H5N1) infections in dogs, cats, and humans over the past few years and more recently H1N1 influenza (swine flu) infections in humans, the control of canine influenza infections in dogs becomes more important because of the close association between dogs and humans. Vaccination of pet animals is one of the most effective ways to control the disease and reduce potential transmission to other animals. The experimental vaccine used in this study successfully reduced clinical signs, virus shedding, and lung lesions caused by CIV. The data suggest that the CIV vaccine used in this study could be successfully used to promote the health and welfare of animals by reducing the severity and spread of canine influenza.

■ **CONCLUSION**

The data presented here demonstrate that CIV subtype H3N8 causes respiratory disease in dogs and that the inactivated vaccine used in this study provides protection against CIV by reducing the severity of the clinical disease, the magnitude and duration of virus shedding, and virus-induced lung lesions in dogs.

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