

In vitro assessment of the allergenicity of a novel influenza vaccine produced in dog kidney cells in individuals with dog allergy

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Background: An inactivated influenza vaccine produced in canine kidney cells (MDCK 33016-PF) contains no egg proteins and may be used to immunize egg-allergic patients. Although no major dog allergens were identified in MDCK 33016-PF cells, minor dog allergens might be present and cause reactions in dog-allergic individuals.

Objective: To evaluate the allergenicity of the inactivated influenza vaccine produced in cell culture in a mediator release assay.

Methods: Rat basophil leukemia (RBL) cells transfected with human IgE receptor-1 were sensitized with sera from dog-allergic adults with positive skin prick test reactions to dog extract and detectable dog dander IgE and were stimulated with serial dilutions of vaccine and dog dander extract. N-hexosaminidase release (NHR) was used as a marker of RBL cell degranulation. Western blots were performed, and UniCAP was used to measure dog-specific IgE antibody levels.

Results: The median (interquartile range) level of dog dander IgE was 8.31 kU_A/L (1.895–14.5 kU_A/L) and of dog epithelium IgE was 3.19 kU_A/L (0.835–6.27 kU_A/L). Median (range) maximum NHR (at the first 10-fold dilution) was 0% (0%–1.4%) to vaccine and 10.2% (0%–35.9%) to dog dander ($P < .001$). In an egg-allergic control subject, the maximum NHR to a vaccine cultured in chick embryo and containing egg protein was 10.2%. IgE antibodies in pooled sera did not bind to vaccine on immunoblots but produced strong binding to dog dander and epithelium extracts. Serum from an egg-allergic control subject strongly bound embryonated egg-derived vaccine.

Conclusion: An influenza vaccine produced in continuous canine kidney cells did not trigger degranulation in RBL cells passively sensitized with human anti-dog IgE.

Ann Allergy Asthma Immunol. 2010;104:426–433.

INTRODUCTION

Influenza is a cause of considerable morbidity and mortality, particularly in vulnerable populations, such as children, the elderly, and people with underlying diseases, including asthma and chronic respiratory conditions.^{1,2} During epidemics, the attack rates may exceed 40% in preschool children and 30% in school-aged children.^{1–4} The 2008 Centers for Disease Control and Prevention's Advisory Committee on Immunization Practices⁴ recommends routine annual vaccination of all children younger than 18 years. The current H1N1 pandemic, in which hospitalization rates are highest in

children younger than 5 years, underscores the public health need for influenza vaccines that can be given to all children, including those who may be egg allergic.^{5,6}

Because all US-licensed influenza vaccines are derived from the infected allantoic fluid of embryonated chicken eggs and, therefore, contain measurable quantities of egg protein allergens, they are relatively contraindicated in persons with egg allergy.^{6,7} Various adverse allergic events have been reported in patients with egg allergy immunized with inactivated influenza vaccines, including death due to anaphylaxis in a child known to be egg allergic.^{7–16}

An inactivated influenza vaccine produced in cell cultures that is noninferior with respect to efficacy and safety compared with an egg-derived vaccine is licensed in Europe and is under investigational status in the United States.^{17,18} The vaccine, produced in a proprietary continuous cell culture line of Madin-Darby canine kidney cells (MDCK 33016-PF), contains no egg proteins and, therefore, offers egg-allergic persons a new option for influenza prevention.¹⁹

However, the safety of the vaccine in persons with hypersensitivity to dog proteins is a hypothetical concern. It has been shown that the 3 canine proteins most commonly associated with hypersensitivity reactions—Can f 1, Can f 2, and Can f 3—are not expressed in MDCK 33016-PF cells (data on file, Novartis Vaccines). Moreover, no Can f 1 has

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Disclosures: Authors have nothing to disclose.

Funding Sources: This study was supported in part by grant AI 059318, R from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Dr Nowak-Węgrzyn), and in part by Nutricia Foundation and Novartis Vaccines (Dr Bencharitiwong).

Received for publication September 1, 2009; Received in revised form February 10, 2010; Accepted for publication March 16, 2010.

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doi:10.1016/j.anai.2010.03.012

been detected in the finished vaccine using a commercial Can f 1 antigen detection enzyme-linked immunosorbent assay. However, further in vitro assays to confirm the absence of other (potentially undescribed) canine antigens in the vaccine that could result in hypersensitivity reactions in dog-allergic patients would provide additional reassurance of its safe use in these patients.

Assays with 2H3 rat basophil leukemia (RBL) cells transfected with human high-affinity receptor for IgE antibody (FcεRI) that are passively sensitized with human specific IgE antibody from allergic subjects' sera have been used in several studies to date.^{20–23} Mediator release assay is an in vitro approximation of the functional activity of the allergen specific IgE antibodies produced by allergic individuals with respect to their ability to cross-link receptors on the surface of RBL cells and to cause cell activation and degranulation.^{23–25} We sought to determine the allergenicity of the novel influenza vaccine produced in canine kidney cells in individuals with dog allergy through use of a mediator release assay.

METHODS

Participants

Participants were recruited via informational flyers posted throughout the Mount Sinai Medical Center and via listserv emails sent to the Mount Sinai Medical School and the Mount Sinai Medical Center in New York. The study was approved by the Mount Sinai institutional review board, and written informed consent was obtained before enrollment.

Eligibility criteria included age 14 to 60 years, a history of immediate (onset within 2 hours of exposure) allergic symptoms on exposure to dog, a positive skin prick test (SPT) reaction to dog allergen extract, and a serum IgE antibody level to dog dander of at least 0.35 kU_A/L (UniCap; Phadia, Portage, Michigan). Exclusion criteria included age younger than 14 years or older than 60 years, a negative SPT reaction, an undetectable serum IgE antibody level to dog dander, and symptomatic asthma, extensive skin rash, or febrile illness at the time of SPT.

Allergy Evaluation

Participants who responded to the advertisements were contacted by telephone and were screened for age eligibility and convincing symptoms of immediate hypersensitivity on exposure to dogs, such as sneezing, rhinorrhea, nasal congestion, nasal pruritus, ocular pruritus, ocular discharge, ocular injection, cough, wheezing, shortness of breath, and cutaneous symptoms. Individuals who met all the inclusion criteria were scheduled for a study visit for SPT and possible venipuncture.

Skin Prick Testing

The SPTs were performed using a sterile smallpox needle (Hollister-Stier Laboratories LLC, Spokane, Washington), commercially available dog allergen extracts (dog hair dander, 1:100 wt/vol, from Hollister-Stier Laboratories LLC; and

dog epithelium mix breed, 1:20 wt/vol, from Greer Laboratories Inc, Lenoir, North Carolina), glycerinated saline control as a negative control, and histamine base, 1 mg/mL (histamine phosphate, 2.75 mg/mL), as a positive control. Results were recorded at 10 minutes; wheal and erythema were traced with a ballpoint pen and were transferred to a piece of paper using adhesive tape. The size of the skin test was calculated as a mean of the longest diameter and its longest orthogonal. A positive skin test reaction was considered to be a mean wheal diameter of 3 mm greater than that produced by saline. Participants with positive skin test reactions had blood samples drawn for measurement of specific IgE antibody levels to dog dander and epithelium.

Measurement of Serum IgE Levels to Dog Dander and Dog Epithelium

Levels of specific IgE antibody to dog dander and to dog epithelium were measured using the UniCap System (Phadia) (range, <0.35 [undetectable] to >100 kU_A/L). Sera from participants with dog dander IgE levels of at least 0.35 kU_A/L were used in the passive sensitization assay.

Immunoblots With Sera From Participants Allergic to Dog

Dog epithelium and dog dander powder were obtained from Greer Laboratories Inc and were diluted in distilled water at 1:10 wt/vol. Protein contents in influenza vaccine grown in the MDCK cells (Optaflu vaccine, lots 018011A and 019021A; Novartis Vaccines), lysed MDCK cells (a gift from Dr. Lisa Satlin, Mount Sinai School of Medicine), dog epithelium and dog dander extract (Greer Laboratories Inc), and egg-derived influenza vaccine (Fluvirin, lot 8111; Novartis Vaccines) were determined using Bradford assay. Aliquots were loaded on gels at 2 μg/mL per well and were applied to sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis (gradient 4%–12% gels) (Nu-Page Novex Bis–Tris System; Invitrogen, Carlsbad, California) following the protocol of the manufacturer. The resolved proteins were transferred to Immobilon-P transfer membranes (Millipore, Bedford, Massachusetts) for immunoblotting and then were stained with Amido Black 10B (Bio-Rad Laboratories, Hercules, California) for total protein analyses. For molecular weight determination, SeeBlue Plus2 Prestained Standard (Invitrogen) was used.

For the detection of IgE binding to the separated proteins, immunolabeling was performed using a pool made of equal parts of 10 patients' sera with detectable dog dander and epithelium specific IgE (median [range], 34.5 kU_A/L [<0.5 to >100 kU_A/L] and 20 kU_A/L [10.1 to >100 kU_A/L], respectively). Sera from an individual egg-allergic participant with an egg white specific IgE level greater than 100 kU_A/L and from a nonatopic participant with undetectable IgE antibody levels (<0.35 kU_A/L) for dog epithelium, dog dander, and egg white proteins were used as controls. Participants' sera were adjusted, based on specific IgE levels, to gain clear binding by diluting 1:10 to 1:30 in phosphate-buffered saline

(PBS)–Tween plus 1% bovine serum albumin and 10% normal goat serum and were incubated with immunoblots with gentle agitation at room temperature. After 1 hour, immunoblots were briefly rinsed with 1X PBS and then were labeled with ¹²⁵I-goat anti-human IgE (DiaMed, Windham, Maine) diluted 1:5 to 1:7 in PBS–Tween plus 1% bovine serum albumin with 0.8 μCi/mL of total activity of ¹²⁵I as per the manufacturer. ¹²⁵I-conjugated antibodies were used because of high sensitivity for detecting antigen specific IgE. After gentle agitation for 1 hour at room temperature and washing with 1X PBS, immunoblots were mounted on filter paper and exposed to Kodak BioMax Imaging Film (Kodak, Rochester, NY) for 4 to 6 days.

Mediator Release Assay

The RBL-2H3 cell line transfected with human FcεRI and the protocol for the assay were kindly provided by Dr. S. Vieths. The RBL cells were transfected with all 3 chains of human FcεRI, but expression of only the α chain was confirmed. The RBL cell cultures were maintained as a monolayer in modified Eagle's medium, 15% RPMI with 10% fetal calf serum, and G418 sulfate (pH 7.4 in 20 mM Hepes; Akcros Chemicals Inc, New Brunswick, New Jersey). Passive sensitization was performed by incubating RBL cells with individual patient serum and normal (nonatopic) human serum control at a final dilution of 1:40 at 37°C in 5% carbon dioxide for 18 to 20 hours in 96-well tissue culture plates (BD Falcon; BD Biosciences, Bedford, Massachusetts); each serum sample was run as a duplicate. Sensitized cells were stimulated with 100 μL per well of the 10-fold serial dilutions of influenza vaccine grown in MDCK cells and dog dander extract in a release buffer with D₂O (Akcros) for 1 hour at 37°C in 5% carbon dioxide. Controls were run without allergens and without serum to measure spontaneous release and with allergens but without serum to estimate non-IgE-mediated release. Rabbit IgG anti-human polyclonal IgE (Bethyl Laboratories Inc, Montgomery, Texas) was used as a positive control for IgE-mediated degranulation. N-hexosaminidase is released from the cells at the same rate as histamine; for practical reasons, N-hexosaminidase release (NHR) into in the supernatant was measured as a marker for mediator release. Total mediator release was obtained by lysing the cells with 1% Triton X-100 (Sigma-Aldrich Chemical Co, St Louis, Missouri). To determine NHR, 30 μL of supernatant was gently mixed with 50 μL of P-nitrophenyl-N-acetyl-β-D-glucosaminide (pH 4.5) (Sigma-Aldrich Chemical Co). After 1 hour of incubation at 37°C in 5% carbon dioxide, the reaction was terminated by adding 100 μL of 0.2M glycine solution (pH 10.7), and absorbance at 405 nm was measured. Results are expressed as the percentage of release from cells sensitized with serum minus spontaneous release, which was then divided by total release.

Sample Size and Statistical Analysis

We calculated the sample size (n=30) based on a model used for the estimation of hypoallergenicity of amino acid-based

infant formula in children with cow's milk allergy.²⁶ The model assumes that if none of the 30 sera from individuals allergic to dogs induces a response in the passive sensitization assay with influenza vaccine grown in the MDCK cells as an allergen, we will be able to state with 95% confidence that sera from at least 90% of dog-allergic participants will not induce a response to influenza vaccine grown in the MDCK cells, thereby fulfilling the criterion of hypoallergenicity in the passive sensitization assay. The response is defined as NHR of at least 5% to at least 1 antigen dilution and detectable NHR to at least 3 consecutive antigen dilutions, consistent with generation of a dose-response curve. A paired *t* test or Wilcoxon signed rank test was used for comparing responses to different allergens using SigmaStat 3.5 (Systat Software Inc, Chicago, Illinois). Dichotomous variables were analyzed using the χ^2 test.

RESULTS

Characteristics of Study Participants

During 6 months, 41 individuals were screened with SPT, and those with a positive SPT result underwent phlebotomy. Characteristics of the 30 individuals whose sera were used for further testing in the passive sensitization assay are given in Table 1. All 30 participants had detectable dog dander IgE antibody, and 20 had detectable dog epithelium IgE antibody. Except for 1 participant, dog dander IgE level was higher than dog epithelium IgE level. Five participants had positive SPT reactions to dog extract but undetectable serum specific IgE antibody levels to dog dander and dog epithelium. Therefore, in the mediator release assay, we chose to use dog dander as an allergen to which responses to influenza vaccine grown in the MDCK cells would be compared.

Table 1. Characteristics of 30 Dog-Allergic Participants

Sex, F/M, No.	20/10
Age, median (IQR), y	36 (23–58)
Atopy, No.	
Allergic rhinitis	17
Asthma	16
Food allergy	5
Atopic dermatitis	4
Symptoms on dog exposure, No.	
Rhinitis	21
Ocular	17
Lower respiratory	14
Cutaneous (rash, urticaria)	10
Dog extract SPT wheal, median (IQR), mm	4 (4–6) ^a
Dog dander IgE, median (IQR), kU _A /L	8.31 (1.895–14.5)
Participants with level <0.35 kU _A /L, No.	0
Dog epithelium IgE, median (IQR), kU _A /L	3.19 (0.835–6.27)
Participants with level <0.35 kU _A /L, No. (%)	10 (33)

Abbreviations: IQR, interquartile range (25%–75%); SPT, skin prick test.

^a Three participants had negative SPT results to dog epithelium extract but positive SPT results to dog hair and dander extract (wheals: 3, 5, and 14 mm).

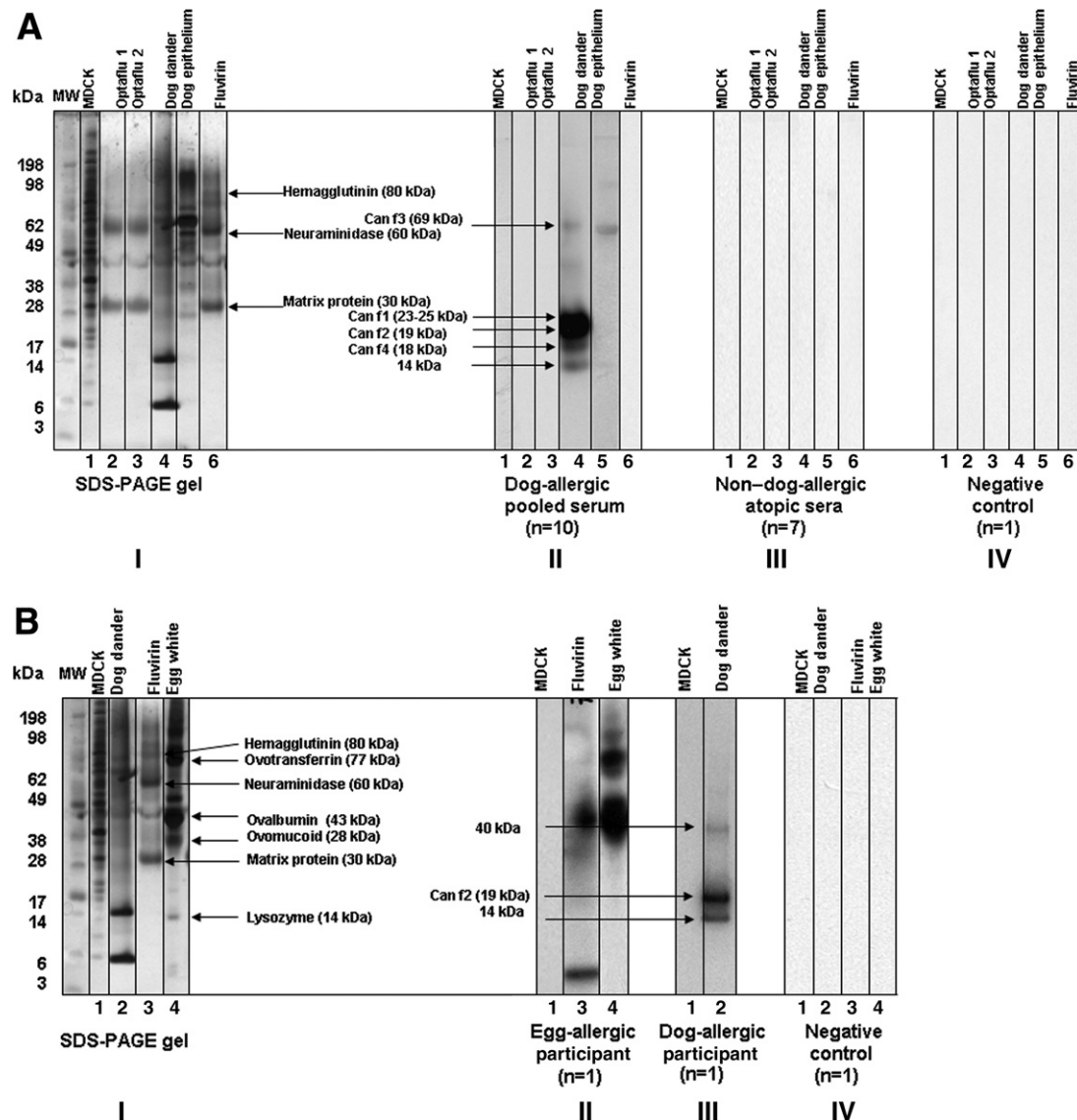


Figure 1. Protein detection by electrophoresis and immunoblotting; the influenza vaccine grown in the MDCK cells and lyzed MDCK cells are not immunolabeled by the sera from the dog-allergic participants. A, I shows silver stain sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, with a total protein concentration of 2 μ g per lane; II, immunolabeling with 10 dog-allergic participants' pooled serum (mean dog dander specific IgE level, 34.5 kU_A/L; and mean dog epithelium specific IgE level, 20 kU_A/L); III, immunolabeling with 7 non-dog-allergic atopic participants' pooled serum (mean dog dander specific IgE level, 7.7 kU_A/L; and mean dog epithelium specific IgE level, 5.8 kU_A/L); and IV, immunolabeling with a negative nonatopic control serum (dog dander, dog epithelium, and egg white specific IgE levels, <0.35 kU_A/L [undetectable]). Lane 1 shows MDCK cells lyzed; lane 2, influenza vaccine grown in the MDCK cells, Optafu lot 018011A; lane 3, influenza vaccine grown in the MDCK cells, Optafu lot 019021A; lane 4, dog dander extract; lane 5, dog epithelium extract; and lane 6, egg-derived influenza vaccine, Fluvirin lot 8111. B, I shows silver stain SDS-PAGE gel, with a total protein concentration of 2 μ g per lane; II, immunolabeling with an egg-allergic participant's serum (egg white specific IgE level >100 kU_A/L); III, immunolabeling with a dog-allergic participant's serum (dog dander and dog epithelium specific IgE levels >100 kU_A/L); and IV, immunolabeling with a negative control serum (egg white specific IgE level <0.35 kU_A/L). Lane 1 shows MDCK cells, lyzed; lane 2, dog dander extract; lane 3, egg-derived influenza vaccine, Fluvirin lot 8111; and lane 4, egg white powder (Just Whites; Deb-El Food Products, Elizabeth, New Jersey). MW indicates molecular weight.

Protein Electrophoresis and Immunoblotting

Protein concentrations in undiluted samples were as follows: dog dander extract, 0.26 mg/mL; dog epithelium extract, 0.94 mg/mL; influenza vaccine grown in the MDCK cells lot

018011A, 0.21 mg/mL; influenza vaccine grown in the MDCK cells lot 019021A, 0.19 mg/mL; egg white extract, 32 μ g/mL; and egg-derived vaccine lot 8111, 0.11 mg/mL. Protein electrophoresis identified several protein bands in

influenza vaccine grown in the MDCK cells, dog epithelium, and dog dander extract (Fig 1). There was no immunolabeling of protein bands in influenza vaccine grown in the MDCK cells and lysed MDCK cells by serum pools from dog-allergic individuals and by a negative control serum with undetectable (<0.35 kU_A/L) levels of IgE antibody to dog dander and dog epithelium (Fig 1A). There was strong immunolabeling of a protein band at the ovalbumin molecular weight (approximately 43 kDa) in the egg-based vaccine Fluvirin by IgE in the serum of an egg-allergic individual with an egg white IgE antibody level greater than 100 kU_A/L (Fig 1B).

Mediator Release Assay

Passive sensitization was performed with 30 individual dog-allergic participants' sera and serial dilutions of influenza vaccine grown in the MDCK cells and dog dander extract as

an allergen and was compared with the release observed when passive sensitization was performed with negative control sera from 12 individuals with undetectable IgE antibody levels to dog dander and dog epithelium (Fig 2). In dog-allergic participants, the response to influenza vaccine grown in the MDCK cells was comparable with that of negative controls, whereas the response to dog dander was significantly higher ($P < .001$) (Fig 2). We chose to compare maximal NHR to 2 allergens at 10^{-1} dilution because of the non-IgE-mediated (in the absence of human serum) mediator release with the concentrated dog dander extract (data not shown). None of the sera gave NHR greater than 5% or any detectable release at 3 consecutive dilutions of the influenza vaccine grown in the MDCK cells. In addition, no release was induced by MDCK lysate in the experiment in which a pooled serum sample from 10 dog-allergic individuals was used to

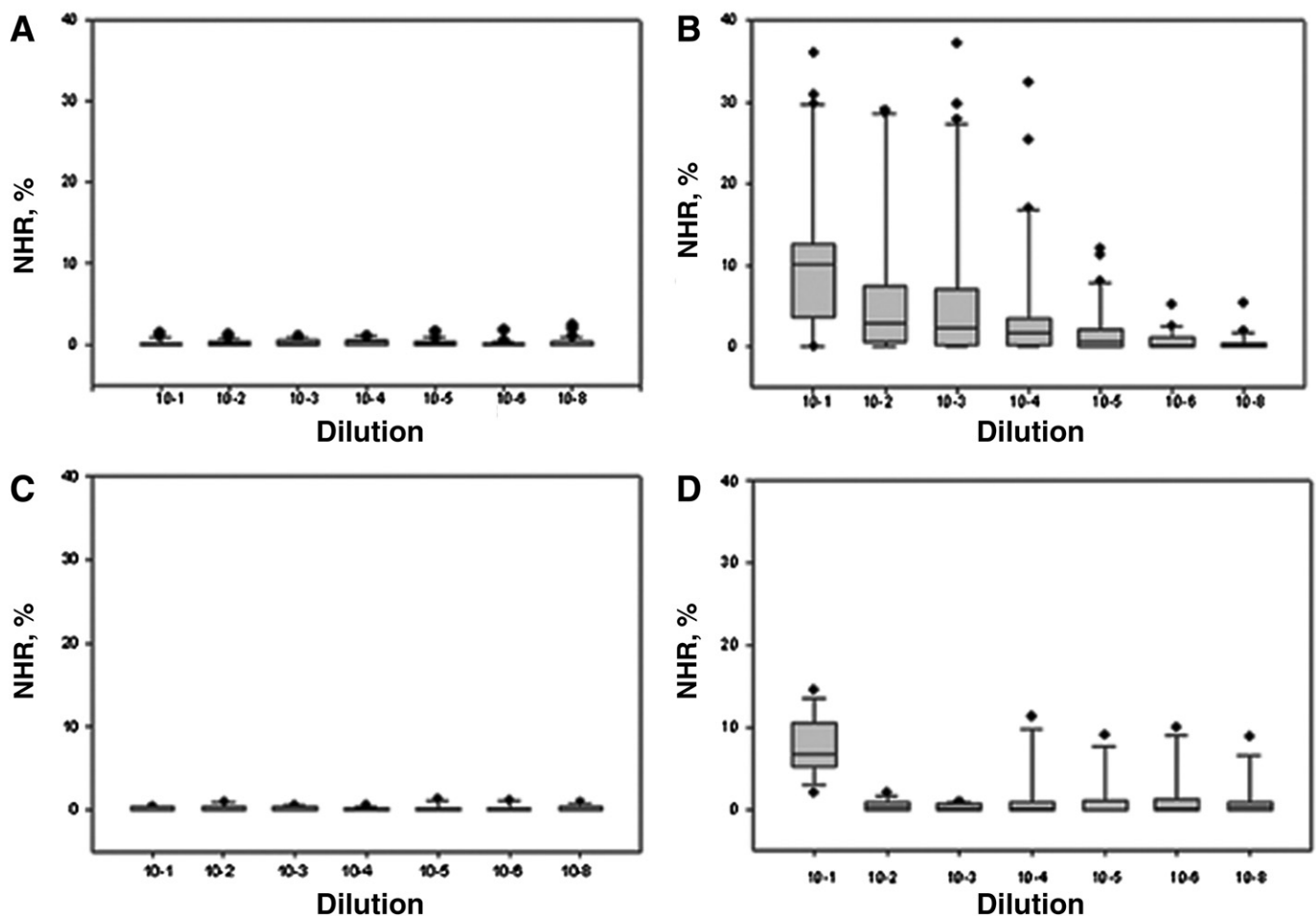


Figure 2. Influenza vaccine grown in the MDCK cells does not induce mediator release in the rat basophil leukemia cells passively sensitized with sera from dog-allergic individuals. A, Influenza vaccine grown in the MDCK cells with dog-allergic sera ($n=30$). B, Dog dander with dog-allergic sera ($n=30$). C, Influenza vaccine grown in the MDCK cells with dog-non-allergic atopic sera ($n=12$). D, Dog dander with dog-non-allergic atopic sera (dog dander IgE levels <0.35 kU_A/L) ($n=12$). The solid horizontal line represents the median; the lower and upper boundaries of the box represent 25% and 75%, respectively. The error bars represent 10% and 90%, and the circles represent outliers. NHR indicates N-hexosaminidase release.

sensitize the RBL cells (data not shown). To demonstrate the ability of the mediator release assay to detect the amount of allergens in the vaccine preparations, we chose to show the responses of the 2 individual dog-allergic participants, each with a serum dog dander specific IgE antibody level greater than 100 kU_A/L. Sera from these participants gave strong release when stimulated with dog dander but gave no release when stimulated with influenza vaccine grown in the MDCK cells. In contrast, passive sensitization performed with serum from an egg-allergic individual with an egg white specific IgE level greater than 100 kU_A/L and egg-derived influenza vaccine as an allergen detected mediator release, demonstrating the capacity of the RBL cell-based mediator release assay to detect a very low level of the relevant allergen (Fig 3).

DISCUSSION

We chose to evaluate the potential allergenicity of a novel influenza vaccine for individuals with dog allergy by using a bioassay in which the RBL-2H3 cells transfected with human high-affinity FcεRI are passively sensitized with human IgE and triggered to release mediators on exposure to the allergen. The RBL-2H3 cells are stable transfectants that can be maintained in culture for long periods.²⁰ The RBL cells have the functional characteristics of mast cells regarding IgE-induced mediator release, but in contrast to wild-type mast cells, cross-linking of IgG via FcεRI on RBL-2H3 cells fails to induce any detectable mediator release.²¹ The RBL cell-based mediator assay was reported to detect very low levels of allergen, eg, cat allergen Fel d 1 gave detectable release at 10 pg/mL.²¹

The RBL cell-based assay was previously reported to estimate the potency of tree and grass pollen, house dust mite, cat dander, and peanut extracts.^{20–23} Sera from individuals with high allergen specific IgE antibody concentrations were used for passive sensitization (eg, birch specific IgE from 41.7 to >100 kU_A/L), and maximal release ranged generally from 7.1% to 44.0% for birch pollen.²¹ The assay was sensitive and had a low coefficient of variation: 15% for NHR and 25% for a dose eliciting 50% maximal response, which made it suitable for detecting differences between batches of allergen extracts.²³

The negative results of this bioassay in individuals documented to have dog protein allergy is substantiated by safety observations in clinical trials of the novel vaccine. Of the more than 9,000 clinical trial participants who have received the vaccine, it is likely that hundreds were dog allergic given the prevalence of dog allergy in the general population. No acute hypersensitivity event was reported in any of the exposed participants (Novartis Vaccines, unpublished data, 2010).

Not only are rates of seasonal influenza infection highest in children, but rates of influenza-associated hospitalization are substantially higher in infants and young children and actually exceed those in adults older than 65 years who are acknowledged to be at high risk for influenza and its complications.^{1–4,27–30} Each year, an estimated average of 92 influenza-related deaths occur in US residents younger than 5 years.^{4,30} In the current pandemic, an even greater burden of severe morbidity and death has fallen on young children, with 117 deaths attributed to the pandemic H1N1 strain as of

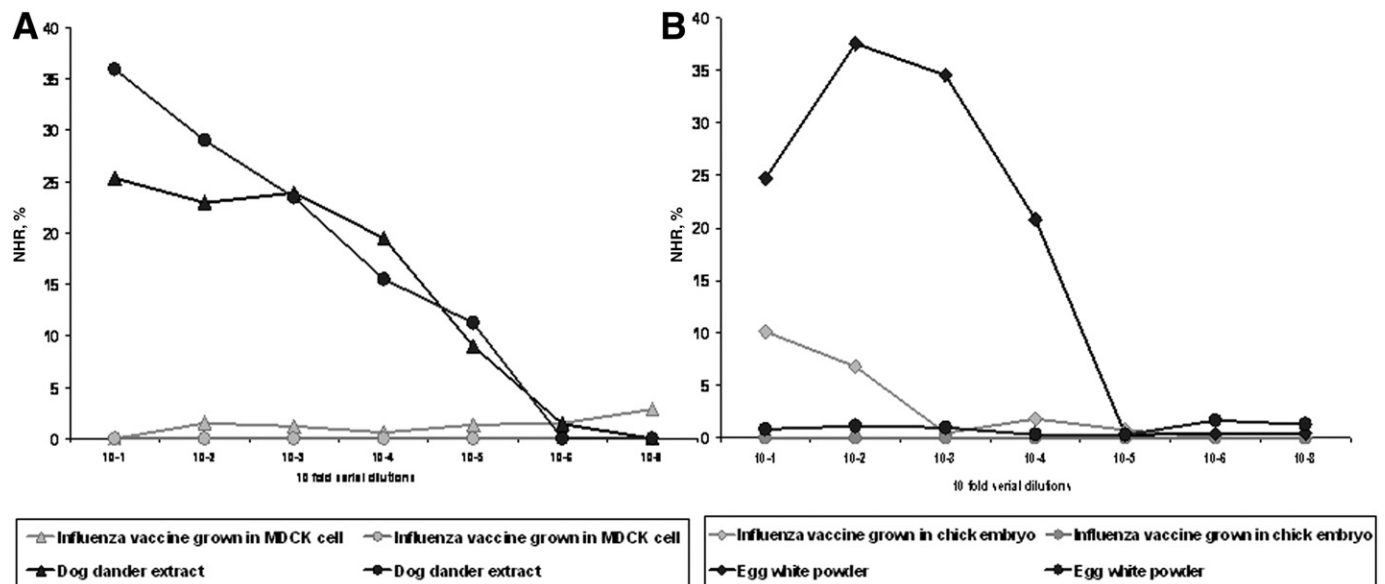


Figure 3. Comparison of mediator release by sera from participants with high levels of allergen specific IgE antibody and with different influenza vaccines. A, No mediator release with influenza vaccine grown in MDCK cells in 2 participants with high dog dander specific IgE antibody levels (>100 kU_A/L) and strong mediator release with dog dander. B, Detectable mediator release with egg-derived influenza vaccine in a participant with a high egg white specific IgE antibody level (>100 kU_A/L) and strong mediator release with egg white (squares). Circles indicate the control; NHR, N-hexosaminidase release.

October 24, 2009, at a point before seasonal influenza transmission has begun in most years, and with 5 or more months of transmission ahead.^{30,31} The age-specific cumulative hospitalization rate has been highest in children younger than 5 years, with a rate of approximately 40 per 100,000, approximately 4-fold higher than that in adults older than 65 years.^{6,31}

Egg allergy, although uncommon in adults, is estimated to occur in 2% to 3% of children younger than 2 years, with a significantly higher prevalence in individuals with atopic dermatitis and other atopic conditions.^{32–36} Therefore, many children who are recommended to receive an annual influenza vaccination cannot avail themselves of the vaccine. They may choose to undergo a time-consuming desensitization procedure or forego vaccination and rely on antiviral treatment. However, therapy must be initiated within 24 to 48 hours of onset of illness, and even then, antivirals reduce the duration of illness by approximately 1 day.^{37–39} Moreover, antiviral therapy with oseltamavir seems less effective for influenza B virus, which affects children disproportionately, and for the H1N1 subtype; resistance to oseltamavir now has increased to a point where the drug no longer is useful.^{40–45}

The ongoing pandemic of novel influenza A (H1N1) has drawn attention to vulnerabilities of current manufacturing platforms and attendant delays in vaccine production. Cell culture–based production potentially could begin more quickly. Cell culture technology significantly reduces lead times and provides greater flexibility of production, which is particularly advantageous in a pandemic situation, when the demand for vaccine will increase without notice. The demand for vaccine also has underscored the need to provide equitable access so that vulnerable members of society are not excluded. In the first wave of the pandemic, children have been at highest risk for hospitalization, and, indeed, the National Pandemic Plan prioritizes children younger than 3 years in the highest tier to be vaccinated.⁴⁶ The availability of a cell culture–derived alternative to the current egg-based influenza vaccine will give egg-allergic children access to a potentially lifesaving intervention.

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