Intranasal Immunization of Ferrets with Commercial Trivalent Influenza Vaccines Formulated in a Nanoemulsion-Based Adjuvant

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Abstract

NB-1008 is a surfactant-stabilized soybean oil-in-water nanoemulsion (NE) adjuvant with influenza antigen incorporated into the NE by simple mixing. Intranasal administration of the antigen with NE adjuvant efficiently produces both mucosal and serum antibody responses as well as a robust cellular Th1 immune response. To demonstrate the adjuvant effect of the W805EC NE, a killed commercial influenza vaccine for intramuscular administration (Fluzone® or Fluvirin®), were mixed with the W805EC NE adjuvant and administered intranasally to naïve ferrets. After a single intranasal immunization, the adjuvanted influenza vaccine elicited elevated serum hemagglutination inhibition (HAI) geometric mean titers (GMT) ranging between 196 and 905 for the three HA antigens present in the vaccine, approximately 19 to 90-fold higher at 1/50th of the standard intramuscular commercial non-adjuvanted influenza vaccine dose. Seroconversion rates of 67% - 100% were achieved against each of the three viral strains present. The adjuvanted nasal influenza vaccine also produced significant cross immunity to five other H3N2 influenza strains not present in the vaccine, and produced sterile immunity after challenge with homologous live virus. No safety issues were observed in the 249 ferrets receiving the adjuvanted influenza vaccine. These findings demonstrate the ability of W805EC NE to adjuvant influenza vaccine when administered nasally and provide the basis to study the intranasal W805EC-adjuvanted influenza vaccine in humans.

Key words:

Influenza vaccine, nanoemulsion adjuvant, nasal vaccination, ferrets, Hemagglutination inhibition
Introducing Nanoemulsion Adjuvanted Influenza Vaccine in Ferrets

Introduction

Influenza illness is an important respiratory infection with particularly serious complications in children, the elderly, and immunocompromised subjects (22). Influenza illness is caused by influenza virus types A and B that undergo frequent mutations and re-assortments in their antigenic surface proteins, hemagglutinin (HA) and neuraminidase (NA). The resultant antigenic drift necessitates the production of new influenza vaccines annually, whereas major antigenic shift poses the threat of pandemic influenza (5, 16). Three major influenza epidemics have occurred in the 20th century; there were approximately 40 million deaths in the 1918-1919 pandemic flu outbreak and about one million deaths each in the 1957 and 1968-69 outbreaks (17, 29). Additionally, more than 420,000 cases were reported to be caused by the 2009 swine flu (H1N1) outbreak (32). In addition to the annual threat posed by the seasonal influenza strains, avian influenza strains are now posing a potential threat to the human population (18). All of these facts argue for improved approaches to preventing influenza infections.

Active immunization with influenza vaccines is a mainstay for preventing influenza illness. The first commercial influenza vaccines were highly effective whole virus-inactivated influenza preparations (27), but issues involving reactogenicity led to the development of subvirion (split) influenza vaccines. These vaccines are safer and efficacious (60-90% efficacy rates) (11, 12). However, the efficacy of these subvirion vaccines is much lower in at-risk populations, such as children, the elderly, and immunocompromised subjects (21). A live attenuated influenza vaccine (LAIV), FluMist®, is approved for intranasal use in humans in the age range of 2-49 years (24) and generates protective immunity (13) for drifted influenza strains (2, 3). However, the administration of LAIV to high risk populations including the immunocompromised must be carefully weighed in terms of risk-benefit. Nasal administration of killed influenza vaccine that is...
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protective via mucosal and systemic immune responses would offer significant advantages over
currently available injectable and intranasal influenza vaccines. It avoids the use of needles, local
side effects and logistical storage and distribution problems particularly in developing countries.

We have developed an oil-in-water nanoemulsion (NE)-based adjuvant, W805EC, composed of
pharmaceutical grade surfactants, soybean oil and ethanol for intra-nasal administration.
W805EC NE contains droplets approximately 400 nm in diameter and has inherent antimicrobial
activity (23). Studies with prototype NE formulations showed that the NE can inactivate whole
influenza virus and induce an immune response after nasal administration that protected mice
from homologous influenza virus challenge (25). Similar protective immune responses were
observed using the NE with either whole vaccinia virus (VV) (7) or purified antigens such as
recombinant anthrax protective antigen (8). Immunization with recombinant HIV gp120 and NE
adjuvant produced a Th1 immune response and neutralizing antibodies in mice (9). Recently, a
more optimized nanoemulsion formulation, W805EC NE was combined with hepatitis B surface
antigen and produced a robust immune response when administered intranasally in mice without
evidence of inflammation in the nasal cavity or key body organs, including the brain, in four
animal species (23). These data suggest broad adjuvant activity and an acceptable safety profile
for the W805EC-adjuvant. NE adjuvant activity resulting in mucosal, systemic, as well as Th1
and Th17 cellular immunity and protection against challenge was also seen in mice immunized
with influenza virus inactivated with β-propiolactone (20).

In the current studies, we extensively investigated the immune response of naïve ferrets
immunized intranasally with NB-1008 vaccines prepared by mixing W805EC-adjuvant with
commercial trivalent inactivated influenza vaccines Fluzone® and Fluvirin®. The immune
response produced by NB-1008 was compared with both intranasally and intramuscularly
administered non-adjuvanted flu vaccine. IM adjuvanted vaccine was not tested since the NE was not optimized for parenteral administration.

**Materials and Methods**

**Nanoemulsion Adjuvant:** W_{80}SEC-adjuvant was prepared by NanoBio Corporation (Ann Arbor, MI). The NE is an oil-in-water emulsion manufactured by high speed emulsification and the 60% NE contains 3.55% Tween 80, 4.04% ethanol, 37.67% Soybean oil, 0.64% cetylpyridinium chloride and 54.1% water. The mean NE particle diameter is approximately 400 nm.

**Fluzone® and Fluvirin® Vaccines:** Fluzone® (Sanofi-Pasteur) and Fluvirin® (Novartis) trivalent influenza vaccines for 2007-2008 season and Fluzone® 2008-2009 seasonal vaccine were obtained from a commercial supplier for use in these ferret studies. Each 0.5 mL contained a total of 45 µg of HA (15 µg from 3 distinct influenza strains). The 2007-2008 influenza vaccines contained A/Solomon Islands/3/2006 (H1N1) (referred to as A/Solomon Islands), A/Wisconsin/67/2005 (H3N2) (referred to as A/Wisconsin), and B/Malaysia/2506/2004 (referred to as B/Malaysia). The 2008-2009 influenza vaccine contained A/Brisbane/59/2007(H1N1) (referred to as A/Brisbane/59); A/Uruguay/716/2007 (H3N2), an A/Brisbane/10/2007-like strain (H3N2) (referred to as A/Brisbane/10); and B/Florida/4/2006 (referred to as B/Florida).

Fluzone® is a subunit vaccine prepared in eggs and inactivated using formaldehyde. The virus is purified, and then disrupted using a non-ionic surfactant to produce the split virus. The split virus is then purified and suspended in phosphate buffer saline (PBS). Fluvirin® is a subunit vaccine prepared in eggs and inactivated by β-propiolactone. Hemagglutinin and neuraminidase surface antigens are purified and suspended in PBS.
Nanoemulsion Adjuvanted Influenza Vaccine Preparation (NB-1008): The $W_{80}5EC$-adjuvanted influenza vaccine formulations were prepared by mixing PBS (Hyclone) with different volumes of Fluzone® or Fluvirin® to achieve the final desired total HA antigen concentration. $W_{80}5EC$ NE was added to the mixture to achieve a final adjuvant concentration of 5%, 10% or 20%. The vaccine formulation was then vortexed for 10 seconds. The vaccine was stored at 4°C until administered intranasally to the naïve ferrets; usually within 24 hours after preparation.

Nanoemulsion-Adjuvanted Influenza Vaccine Stability: NB-1008 was filled and stored in 2 mL glass vials either at 4°C or at room temperature (RT) for up to 2 weeks. Vaccine stability was assessed for visual appearance (absence of separation), pH, particle size and zeta potential. Separation was assessed visually and pH was measured using a pH meter. Particle size and zeta potential of NB-1008 were measured using a Malvern Zeta Sizer (Model ZS3600).

The amount of each individual antigen present in the Fluzone® influenza vaccine (2008-2009 formulation) after mixing with 5% or 20% NE adjuvant was determined using single radial immune assay (SRID) following storage for 8, 24 and 48 hours at 4°C. These studies were performed at Retroscreen Virology labs (London, UK) using a standard protocol (28) (33).

Hemagglutination Inhibition (HAI) Titer Determination: Ferret sera were treated with receptor destroying enzyme (RDE-Tx) according to established procedures (31). RDE-Tx sera were serially two-fold diluted into microtiter plates containing V-shaped wells. An equal volume of virus adjusted to 8 hemagglutination units (HAU) per 50µL was added to each well. The plates were covered and incubated at RT for 30 minutes followed by addition of 0.5% Turkey red blood cells (TRBC). The plates were mixed by agitation, covered and the TRBC were allowed to settle for 30 minutes at RT. The HAI titer was determined by the reciprocal dilution
of the last well which contained non-agglutinated TRBC. Positive and negative serum controls were included for each plate. Samples were run in singlet in two independent assays. Results were compared and samples with greater than 2-fold differences in titer were repeated in a third assay. The viruses used in the assays corresponded to the viruses present in the administered vaccines (see Fluzone® and Fluvirin® vaccines subsection above). Viruses used in the assay were provided by the CDC (Atlanta, GA) and amplified at Southern Research Institute (SRI) (Birmingham, Alabama) in embryonated eggs. Viruses were titrated to determine its HAU for use in the assay. Additional H3N2 viruses tested for cross-reactivity included A/Panama/07/99, A/New York/55/04, A/California/7/04, A/Wellington/1/04, and A/Wyoming/3/03.

**Viral Load Determination:** Frozen nasal washes from the ferrets were thawed and clarified by centrifugation. Frozen ferret lung and turbinate samples were re-suspended in PBS-Ab [phosphate buffered saline supplemented with antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin)] to make a 10% w/v suspension and homogenized. Ten day old Specific Pathogen Free (SPF) embryonated chicken eggs (S&G Poultry, Clanton AL) were inoculated in triplicate with serially diluted tissue homogenates, 0.1 mL/egg, and incubated for 48 hours at 33°C without CO₂. Virus growth was assessed by determination of HA positivity of allantoic fluid using 0.5% TRBC. The 50% endpoint was determined by the method of Reed and Muench (26) from egg dilutions testing positive for HA activity in TRBC. Results were expressed as Egg Infectious dose 50 (EID₅₀)/mL.

**Preparation and Characterization of Influenza Virus for Challenge:** Influenza A/Wisconsin, was obtained from the CDC (Atlanta, GA), amplified at SRI in embryonated eggs, and used to challenge ferrets. The virus had a 50% egg infectious dose of $1 \times 10^{11.25}$ EID₅₀/mL. For challenge, the virus was adjusted to $10^6$ EID₅₀/mL.
Transmission Electron Micrographs and Sectioning Technique: Twenty mL of the NE adjuvant alone or with Fluzone® was fixed with 1% (w/v) osmium tetroxide solution. The fixed preparations were mixed with histogel in 1:10 ratio to form a solid mass. The solid mixture of was sliced into thin 1mm slices and rinsed with double distilled deionizer water. The cross-sectioned samples were dehydrated with ascending concentrations (30%, 50%, 70%, 90%, 100%) of component A of the Durcupan® kit (Fluka, EM #14020) in double distilled deionizer water. These samples were transferred into embedding solution (mixture of components A, B, C and D) of the Durcupan® kit. The embedded samples were sectioned to a 75 nm thickness and placed on 300 mesh carbon-coated copper grid. The sections on the grids were stained with saturated uranyl acetate in distilled and deionizer water (pH 7) for 10 minutes followed by lead citrate for 5 minutes. The samples were viewed with a Philips CM-100 TEM equipped with a computer controlled compustage, a high resolution (2K x 2K) digital camera and digitally imaged and captured using X-Stream imaging software (SEM Tech Solutions, Inc., North Billerica, MA).

Ferrets: Ferret experiments were performed at SRI. Approximately 5-8 month-old naïve, castrated and descented male Fitch ferrets (Mustela putorius furo) were purchased from Triple F Farms (Sayre, PA). Ferret sera were negative for influenza HAI to the seasonal antigens prior to vaccination. Ferrets were quarantined for 4-7 days in animal biosafety level-3 (ABSL-3) facilities. All procedures were performed in accordance with laboratory animal care and use guidelines. Ferrets were implanted with a temperature transponder (BioMedic Data Systems Inc. Seaford Delaware) and housed in pairs prior to treatment. Ferrets were anesthetized with intramuscular injection of 25 mg/kg ketamine, 0.05 mg/kg atropine and 2 mg/kg xylazine (KAX) prior to blood collection, treatment administration and challenge. For HAI analysis, 1 mL blood
was collected from the anterior vena cava using a 23G 1 inch needle connected to a 1cc

Experimental Design of Ferret Studies: Three ferret studies were performed to determine the
immunogenicity of the NE-adjuvanted influenza vaccine, the dose response of both NE adjuvant
total HA and vaccine volume, cross protection against strains not present in the vaccine and
protection against challenge with live influenza virus. Ferret safety was assessed by cage side
observation during the course of the studies. The three experiments are summarized in Table 1.

Ferret Study 1 This study was designed to examine the immune response in ferrets following
one and two intranasal doses of NE-adjuvanted influenza vaccine. Ferrets were vaccinated on
days 1 and 28. Total HA antigen doses of 7.5, 22.5 and 36 µg mixed with 20% W805EC NE were
assessed. The HA antigen used in this study was from commercially available influenza
vaccines, either 2007-2008 Fluzone® or Fluvirin®. Control arms included non-adjuvanted 36 µg
total HA administered intranasally and non-adjuvanted 45 µg total HA administered
intramuscularly. HAI titers to A/Solomon Islands, A/Wisconsin and B/Malaysia contained in the
2007-2008 formulation were evaluated in ferret sera drawn on days 27 and 48 post-initial
vaccination.

Post-Immunization Viral Challenge: Ferrets that received the smallest dose of influenza HA Ag
(7.5µg) adjuvanted with W805EC NE were challenged intranasally on day 49 with 1 mL
inoculum containing 10⁷ EID₅₀ of A/Wisconsin. Ferrets were observed weekly prior to the
challenge and daily following the challenge, for clinical signs, body weight and temperature until
euthanized on day 63. Nasal washes were collected on days 1-6 post-challenge (days 50-55)
from 6 of the 10 ferrets receiving viral challenge. Lungs and nasal turbinates were collected from
the remaining 4 ferrets (out of 10) on day 4 post-challenge (day 53). These samples were cultured and viral load was determined to ascertain if the NE-adjuvanted vaccines prevented viral multiplication in the ferrets.

**Ferret Study 2:** This study was performed to further assess the antigen-sparing properties and cross-reactivity following a single intranasal immunization of NB-1008. Ferrets received low antigen doses (0.9, 3 or 7.5 µg total HA/dose) mixed with 20% W80.5EC NE. Control animals were administered an intramuscular dose of 45 µg total HA. Fluzone®2007-2008 seasonal vaccine was used. Sera were collected for HAI analysis on day 27.

Ferrets were challenged intranasally on day 28 using 1 mL inoculum containing 10⁷ EID₅₀ of A/Wisconsin. Nasal washes were collected from the ferrets on days 1-6 post-challenge (days 29-34). Nasal washes were cultured for determination of viral load. No lung tissues or nasal turbinates were collected. Ferrets were euthanized on day 42.

**Ferret Study 3:** This study was performed to assess the effect of nanoemulsion concentration and dosing volume on immune response. Ferrets were vaccinated on days 1 and 28. Adjuvanted vaccine doses were 3µg HA in 5%, 10% or 20% NE in 200 µL volume or 6µg HA in 10% NE in 100µL or 12µg HA in 20%NE in either 200 or 500µL volumes. Control ferrets were administered 45µg HA intramuscularly. Fluzone®2008-2009 season vaccine was used. Sera were collected for specific HAI titers on days 27 and 48. Viral challenge was not performed in this study. Ferrets were observed weekly for their clinical signs, body weight and temperature and euthanized on day 48.
**Statistical Analysis:** Mann Whitney U analysis has been used to analyze HAI titers between the ferret groups that received IN vaccine with and without NE adjuvant. Significance was based on \( p \leq 0.05 \).

**Results:**

**Immunogenicity of W\(_{80}\)5EC-adjuvanted Vaccine:**

High HAI titers and seroconversion rates were consistently seen against the three strains present in the vaccine (A/Solomon Islands, A/Wisconsin and B/Malaysia) four weeks after a single intranasal vaccination with either W\(_{80}\)5EC-adjuvanted Fluvirin\(^\circledR\) (Fig 1) or W\(_{80}\)5EC-adjuvanted Fluzone\(^\circledR\) (Fig 2) in naïve ferrets. Intranasal administration of the NE adjuvanted vaccines had higher seroconversion rates (100\%) compared to intramuscular administration of the commercially available Fluvirin\(^\circledR\) (22\% - 56\%) or Fluzone\(^\circledR\) (67\% - 89\%) vaccine.

Notably, 100\% of ferrets vaccinated either intranasally with Fluvirin\(^\circledR\) mixed with 20\%W\(_{80}\)5EC NE or Fluzone\(^\circledR\) mixed with 20\%W\(_{80}\)5EC NE (NB-1008) responded with HAI titers >240. A single intranasal vaccination of NB-1008 (7.5 µg total HA antigen per dose) in naïve male ferrets resulted in geometric mean titers (GMT) >2,200 for A/Wisconsin. This is a >220-fold increase from baseline and a 32-fold increase compared to the response after Fluvirin\(^\circledR\) IM (Fig 1), and an >8-fold increase when compared to Fluzone\(^\circledR\) IM (Fig 2). Increases in HAI titers relative to the baseline were also observed with A/Solomon Islands strain (>100-fold) and B/Malaysia strain (≥ 25-fold) with both W\(_{80}\)5EC adjuvanted vaccines. The adjuvanted vaccine had 45-fold higher HAI titer levels against A/Solomon Islands when compared with the titer achieved after Fluvirin IM and more than a 15-fold increase in HAI titers compared to Fluzone\(^\circledR\) IM. For B/Malaysia...
there was a >11-fold increase in HAI titers when compared to Fluvirin® IM (Fig 1) and a > 9 fold increase in titers compared to Fluzone® IM (Fig 2).

The titers achieved with adjuvanted-NE vaccines were statistically significantly different (p ≤ 0.05) when compared with non-adjuvanted influenza vaccine administered intranasally except for the following: A/Wisconsin strain (Fluvirin) at 22.5µg HA (p=0.1), B/Malaysia strain (Fluvirin) at 7.5µg HA (p=0.08) and A/Wisconsin (Fluzone) at 7.5 µg and 22.5 µg total HA antigen doses (P = 0.6 or 0.4 respectively) (Fig 2)

A subsequent study was performed to identify the minimum antigen dose required to elicit seroconversion using the W80EC-adjuvanted Fluzone® vaccine. In this study, the ferrets received a single vaccination using lower concentrations of HA antigen (0.9 – 7.5 µg total HA antigen). The control group was vaccinated IM with 0.5 ml (45 µg total HA) of the commercial vaccine. Protective antibody titers (GMT ≥40) and seroconversion rates against all three strains present in the 2007-2008 Fluzone® vaccine were achieved using antigen concentrations as low as 0.9 µg total HA (Fig 3). Greater variability in the percentage of seroconversion was noted for ferrets vaccinated with the lowest HA antigen dose. Seroconversion was not achieved with any strain following intramuscular vaccination using a significantly higher total HA antigen concentrations than the nasal adjuvanted vaccine.

**Dose Response**

The effect of NE concentrations was evaluated. Each formulation studied contained a total of 3 µg total HA antigen and the NE concentration in each formulation ranged from 5% to 20% in a total volume of 200 µL. The control arm was 0.5 ml of the trivalent commercial influenza vaccine (45µg total) HA IM. For all three strains, the antibody response in the ferrets increased with increasing NE concentration; 17-33% seroconversion was achieved with 5%NE, 0-83%
with 10% NE, and 67-100% with 20% NE, indicating that there is a dose response. The antibody response was markedly higher than that elicited with the IM vaccine which contained 15-fold higher total antigen content (Fig 4).

Another dose response compared the effect of volume administered on immunogenicity. Ferrets received 100, 200 or 500µL of influenza W805EC -adjuvanted vaccine. The 200 and 500 µL vaccine doses contained 12 µg of total HA antigen mixed with 20% W805EC NE. Due to antigen concentration constraints, the 100µL vaccine dose contained only 6µg of total HA and was mixed with 10% W805EC NE. The immune response increased with increasing dose volume and the response was greater than IM vaccination. The use of either 200 or 500 µL volume resulted in 100% seroconversion in all the strains (Fig 5). Thus, a dose response relationship exists with respect to NE concentration and vaccine volume.

Cross-Reactivity Titers to H3N2 Influenza Strains: The cross-reactivity to 5 different H3N2 strains, other than A/Wisconsin, was assessed in 2 different studies. Ferrets were primed on day 1 and boosted on day 28 with NB-1008 vaccine containing doses ranging from 7.5 and 36 µg total HA antigen/dose. Ferrets were tested on days 27 and 48 for cross-reactivity to five antigen-shifted H3N2 strains. Significantly higher cross-reactive titers were achieved with NB-1008 vaccination compared to intramuscular vaccination of Fluzone (Data not shown). The degree of response appeared to be strain-dependent; higher antibody titers were observed against A/California, A/New York and A/Wyoming strains. The NB-1008 vaccines elicited HAI titers 25- to 720-fold higher than baseline. Seroconversion rates of over 90% were achieved with these strains compared to seroconversion rates ranging between 40 – 90% with A/Wellington and A/Panama strains (Data not shown). Of note, there was a dose-sparing effect achieved with the NB-1008 intranasal vaccines. Intramuscular vaccination with 45 µg total HA of Fluzone® had...
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significantly lower rates of seroconversion (56%) compared to the seroconversion rates attained with intranasal vaccination of NB-1008 with a 6-fold lower dose.

In another study, cross-reactivity was observed with A/California, A/New York strains and to a lesser extent A/Wyoming in ferrets receiving a single IN vaccination of NB-1008 (Fig 6). A $\geq 50\%$ seroconversion rate was seen with the A/New York strain with the NB-1008 formulation containing the lowest total HA concentration (0.9 $\mu$g total antigen). A single IN vaccination at this dose was not sufficient to seroconvert the ferrets against the A/Wellington and A/Panama strains. Cross-reacting antibodies were not produced against any of the tested type A (H3N2) strain following a single intramuscular vaccination with the commercial vaccine (Fig 6).

**Protective Efficacy of NB-1008 Vaccine:** Pilot studies in naïve ferrets showed that exposure to large doses of A/Wisconsin influenza virus is not fatal to the ferrets (unpublished data). Naïve ferrets challenged intranasally with $10^7$ EID$_{50}$ of A/Wisconsin virus were assessed for the presence of influenza virus in their nasal washings, nasal turbinates and lungs 2-6 days following challenge. No changes in body weight or temperature in the ferrets were seen following intranasal viral challenge. In study 1, no viral load was detected on days 1 - 6 post challenge in the nasal washes from ferrets immunized with NB-1008. Control animals, receiving IM vaccine in the pilot study, had significant levels of virus ($>10^4$ EID$_{50}$/mL) in their nasal washes for up to 6 days following challenge. Viral load in the nasal turbinates and lungs of a subgroup of challenged ferrets (4 out of 10) was determined 4 days following challenge; no influenza virus was detectable in the ferrets vaccinated with NE. Low level viral concentrations were detected in the lungs of the control ferrets 5 days after challenge (30-160 EID$_{50}$/mL), but higher level of the challenge virus were found in the nasal turbinates ($>2x10^5$ EID$_{50}$/mL). Study 2, showed a low viral titer in relation to the dose response in the initial days following the challenge; however, on
day 6 post-challenge, ferrets receiving NB-1008 had no virus in their nasal washes compared to 
$>10^3$ EID50/mL in the IM control group. These findings indicate that the ferrets vaccinated 
intranasally with NB-1008 developed sterilizing immunity.

**Stability of NB-1008 Vaccine:** The stability of NB-1008 vaccine was assessed for 2 weeks at 
room temperature and at 2-8°C to confirm that the vaccine formulation was stable at the time of 
vaccination. All the experimental vaccine formulations administered to the ferrets were prepared 
one day before vaccination and stored at 2-8°C. NB-1008 vaccines stored at room temperature 
and at 2-8°C were stable through the course of the study. There was no change in pH, particle 
size, zeta potential, or any visual signs of separation or settling. Potency testing for HA in NB-
1008 was carried out using SRID 48 hours after vaccine mixing. HA antigens of A/Brisbane/59, 
A/Brisbane/10, and B/Florida were found to be stable when stored at 2-8°C for 48 hours in the 
presence of the NE (Table 2), and were within the accepted variability of the assay. The vaccine 
formulations containing 20% W$_{80}$5EC NE showed a slight decrease in HA potency reading at all 
time points (not shown). This was attributed to the NE altering the radial diffusion pattern of the 
antigens in the immunogels.

**Safety of NB-1008 Vaccine in Ferrets:** The safety of the W$_{80}$5EC-adjuvanted vaccine was 
assessed in 249 ferrets using cage side observation and weekly recording of body weight and 
body temperature. Intranasal administration of the W$_{80}$5EC NE vaccine was well tolerated 
without treatment-related clinical abnormalities. This finding was consistent with the lack of 
local or systemic side effects in four other animal species (23).

**Electron Micrographs:**
Cross sectioned TEM of 20% W$_{80}$5EC NE showed NE droplets with a uniform inner core 
material. NE vaccine containing 30µg of HA shows discrete antigen materials/particles inside
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The oil core of the droplets that represent the Fluzone® antigens. Since the antigen is incorporated in the core, and is surrounded by the core material, it is protected from staining by the electron dense stain. This leads to a white counter staining effect in the core. The localization of the antigen within the core shields the antigen-sensitive protein subunits in the emulsion, and may protect the antigen from degradation, and thus enhancing stability. There are very few Fluzone® particles outside of the NE particles that were stained dark in color (Fig 7).

**Discussion**

The non-inflammatory oil-in-water NE- adjuvant, W₈₀EC mixed with commercial influenza vaccines and administered intranasally was well-tolerated and significantly enhanced the humoral response in ferrets demonstrating antigen-sparing when compared to IM non-adjuvanted influenza vaccine.

The disease burden of seasonal and pandemic influenza continues to warrant the development of novel, improved influenza vaccines (14). The efficacy of live-attenuated mucosal vaccines and inactivated parenteral influenza vaccines is similar in adults (6) and better in children (4, 10).

The risk-benefit ratio of live attenuated vaccine use in high risk populations including children less than two years of age, the elderly, and immunocompromised subjects makes the development of more effective inactivated influenza vaccine administered intranasally desirable (6).

Mucosal vaccination also offers the advantage of mimicking the route of entry of natural infection, including the stimulation of mucosal as well as systemic immune responses (15).
Thus, the primary benefit from IN versus IM administration is the induction of nasal secretory antibody, an important defense against respiratory infections (1).

The use of adjuvants in human prophylactic vaccines has been challenged by the need to enhance immune responses without significant reactogenicity (19). The mucosal adjuvant $W_{805EC}$ NE is composed of water, oil, surfactant, and ethanol, components that are categorized by the Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS).

Previously, we studied the immune response of $W_{805EC}$-adjuvanted influenza vaccines in mice. $W_{805EC}$ adjuvant was used to either inactivate live A/Puerto Rico/8/34 (H1N1) influenza virus or mixed with $\beta$-propiolactone-inactivated virus (20). $W_{805EC}$-adjuvanted vaccine induced a Th17 response that was present in addition to a significantly enhanced Th1-bias (20). In other studies, when a NE adjuvant prototype was combined with purified antigens such as recombinant anthrax protective antigen (8) and HIV gp120, it elicited a Th1 systemic response with neutralizing serum antibodies and mucosal IgA when administered intranasally in mice or guinea pigs (9). $W_{805EC}$ NE combined with hepatitis B surface antigen administered intranasally produced an enhanced immune response and caused no inflammation in the nasal cavity or histopathological changes in key organs, or abnormal laboratory findings in safety studies performed in mice, rats, guinea pigs and dogs (23). These data suggest broad adjuvant activity for the $W_{805EC}$ NE and an acceptable safety profile.

In the current study, experience with the $W_{805EC}$ adjuvant was extended to ferrets, the recommended preclinical model for influenza (30). The adjuvant was tested with two different split-commercial vaccines (Fluvirin® and Fluzone®) and was compatible with either preparation, augmenting antibodies to all strains in the trivalent vaccines. Enhanced immunogenicity correlates with superior immunoprotection with the $W_{805EC}$ adjuvant. The nasal nanoemulsion
adjuvanted vaccine was proved superior to nasal and muscular immunization with non-
adjuvanted flu vaccines. NEs were not optimized for IM vaccination. The adjuvanted vaccine
was dose-sparing, using up to 50-fold less HA antigen than IM non-adjuvanted vaccines. The
nasal adjuvanted vaccine resulted in greater seroconversion rates after a single vaccination with
HAI GMT as high as 7,000, while the non-adjuvanted commercial vaccines achieving HAI GMT
less than 300. Such high antibody titers after a single immunization are consistent with systemic
immune responses to NE-based adjuvants combined with other antigens (7-9, 23, 25). In
addition, W80.5EC-adjuvanted vaccine resulted in the production of antibodies against
heterologous influenza strains not present in the vaccine and sterilization of the nasal secretions
and turbinates in ferrets following challenge with live virus. The adjuvanted vaccine stabilized
the HA antigen to degradation normally seen at room temperature in the absence of adjuvant;
NE-adjuvanted vaccine was stable when stored at room temperature for up to 2 weeks. Potency
studies using SRID demonstrated the stability of the influenza antigen for up to 2 days. Antigen
stability could be explained by the interaction between the NE and the protein antigen where the
antigen appeared to be embedded in the oil droplets thereby shielding immunoprotective antigen
epitopes. There were no abnormal clinical signs and no deaths and changes in body weight gain
or temperature in 249 ferrets comprising this study. Thus, W80.5EC continues to maintain a good
safety profile.

The data indicate the possibility that W80.5EC NE-adjuvanted influenza vaccines may represent a
breakthrough in vaccinology. The adjuvant is non-inflammatory, safe in animals, allows for
adjuvanticity as defined by dose-sparing and an improved immune response with a given dose of
antigen, avoids needles, elicits cross-protection against influenza strains which are not present in
the vaccine, and remains stable for weeks without refrigeration. The high titers produced in naïve
ferrets also suggest that the adjuvant might be useful for immunization of high risk populations such as children and elderly where immunity is not fully mature or is impaired. These data support the development of the W80SEC adjuvant for use in humans.

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References


24 Nanoemulsion Adjuvanted Influenza Vaccine in Ferrets


Figure 1: HAI titers against A/Solomon Islands, A/Wisconsin and B/Malaysia viruses following vaccination of naïve ferrets with NE adjuvanted Fluvirin®. Ferrets received 500µL vaccine containing HA doses ranged between 7.5 and 36µg. Sera were collected on day 27 following a single intranasal vaccination on day 1. The numbers above the bars represent the seroconversion rates. There was a statistically significant difference (*) between NE adjuvanted vaccine and nasal non-adjuvanted vaccine for all the strains with all the doses (p≤0.05) with the following exceptions; A/Wisconsin strain at 22.5µg HA (p=0.1) and B/Malaysia strain at 7.5µg HA (p=0.08).

Figure 2: HAI titers against A/Solomon Islands, A/Wisconsin and B/Malaysia viruses following vaccination of naïve ferrets with NE adjuvanted Fluzone®. Ferrets received 500µL vaccine containing HA doses ranged between 7.5 and 36µg. Sera were collected on day 27 following a single intranasal vaccination on day 1. The numbers above the bars represent the seroconversion rates. There was a statistically significant difference (*) between nasal NE adjuvanted vaccine and nasal non-adjuvanted vaccine for the A/Solomon Islands and B/Malaysia strains with all the doses administered (p≤0.006). For the A/Wisconsin strain, a statistically significant difference was only observed between the 36µg adjuvanted and non-adjuvanted Fluzone (p=0.004). There was no significant difference between the 7.5µg (p=0.6) or 22.5 µg (p=0.4) adjuvanted vaccine and the 36µg non-adjuvanted vaccine.

Figure 3: HAI titers against A/Solomon Islands, A/Wisconsin and B/Malaysia viruses following vaccination of naïve ferrets with NE adjuvanted Fluzone®. Ferrets received 500µL vaccine containing HA doses ranged between 0.9 and 7.5µg. Sera were collected on day 27 following a single intranasal vaccination on day 1. The numbers above the bars represent the seroconversion rates.
Figure 4: Effect of NE adjuvant concentration on immune response. HAI titers against A/Brisbane 59, A/Brisbane 10 and B/Florida following single intranasal vaccination with varying NE concentrations mixed with 3µg total HA antigen in 200µL total volume. Control ferrets received 500µL IM. Sera were collected on day 27 following a single intranasal vaccination on day 1. The numbers above the bars represent the seroconversion rates.

Figure 5: Effect of volume of NE vaccine on immune response. HAI titers against A/Brisbane 59, A/Brisbane 10 and B/Florida following single intranasal vaccination with varying NE adjuvanted vaccine containing 10% or 20% NE mixed with 6 or 12 µg total HA antigen. The 6 µg dose was formulated in 100µL volume and the 12 µg dose was formulated in 200µL dose. Control ferrets received 500µL IM. Sera were collected on day 27 following a single intranasal vaccination on day 1. The numbers above the bars represent the seroconversion rates.

Figure 6: Cross protection: HAI titers and percentage seroconversion against several influenza A/H3N2 viruses following intranasal vaccination with different doses of W805EC adjuvanted commercial vaccine (Fluzone®). Ferrets received 500µL vaccine containing HA doses ranged between 0.9 and 7.5µg. Sera were collected on day 27 following a single intranasal vaccination on day 1.

Figure 7: TEM shows cross section images of the 20% W805EC NE with and without 30µg total HA. The panel on the right illustrates that the HA antigens are located in the oil droplets. The darkly stained antigens are located outside of the NE particles.
Table 1: Comparison of the different ferret studies.

<table>
<thead>
<tr>
<th></th>
<th>Ferret Study 1</th>
<th>Ferret Study 2</th>
<th>Ferret Study 3</th>
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<tbody>
<tr>
<td>Number of ferrets/arm</td>
<td>9-10</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Number of Doses</td>
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<td>1</td>
<td>2</td>
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<tr>
<td>HA Dose - Total HA</td>
<td>7.5, 22.5 and 36 µg HA</td>
<td>0.9, 3 and 7.5 µg HA</td>
<td>3, 6, 12 µg HA</td>
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<td>Dosing Schedule</td>
<td>Day 1 and Day 28</td>
<td>Day 1</td>
<td>Day 1 and Day 28</td>
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<tr>
<td>Vaccine volume</td>
<td>500µL</td>
<td>500µL</td>
<td>100, 200 and 500µL</td>
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<td>NE Adjuvant</td>
<td>20% W&lt;sub&gt;80&lt;/sub&gt;SEC</td>
<td>20% W&lt;sub&gt;80&lt;/sub&gt;SEC</td>
<td>5, 10 and 20% W&lt;sub&gt;80&lt;/sub&gt;SEC</td>
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<tr>
<td>Controls - Total HA</td>
<td>36µg IN and 45µg IM</td>
<td>45µg IM</td>
<td>45µg IM</td>
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<td>Antibody test time</td>
<td>D27 and D48</td>
<td>Day 27</td>
<td>D27 and D48</td>
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<tr>
<td>Cross reactivity</td>
<td>Day 48 – 5 antigens</td>
<td>Day 27 – 5 antigens</td>
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<td>Challenge arm</td>
<td>7.5 µg HA</td>
<td>0.9, 3 and 7.5 µg HA</td>
<td>ND</td>
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<tr>
<td>Challenge time</td>
<td>Day 49</td>
<td>Day 28</td>
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<td>Nasal viral shedding</td>
<td>Days 50-55</td>
<td>Days 29-34</td>
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<td>Lungs and Nasal turbinate viral shedding</td>
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<td>Euthanasia</td>
<td>Day 63</td>
<td>Day 42</td>
<td>Day 48</td>
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Table 2: Potency of HA antigens in NE adjuvanted vaccines using SRID assay after storage for 48 hours at 4°C.

<table>
<thead>
<tr>
<th></th>
<th>A/Brisbane/59/2007 (H1N1) µg/ml</th>
<th>A/Brisbane/10/2007 (H3N2) µg/ml</th>
<th>B/Florida/4/2006 µg/ml</th>
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<tbody>
<tr>
<td></td>
<td>0 Hours</td>
<td>48 Hours</td>
<td>0 Hours</td>
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<tr>
<td>Fluzone®</td>
<td>20±1.7</td>
<td>22±3.1</td>
<td>24±1.5</td>
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<td>Fluzone® + 5% W₅₀₅EC</td>
<td>19±1.7</td>
<td>18±1.7</td>
<td>20±3.5</td>
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<tr>
<td>Fluzone® + 5% W₅₀₅EC</td>
<td>17±1.2</td>
<td>16±0.6</td>
<td>18±0.6</td>
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The diagram shows the HA GMT (hemagglutination inhibition geometric mean titer) for different strains and doses of influenza vaccine. The categories represented are:

- A/Brisbane 59/1H1N1
- A/Brisbane 10/H3N2
- B/Florida

The doses and HA concentrations are:

- 100 μL - 10% NE - 6 μg HA (IN)
- 200 μL - 20% NE - 12 μg HA (IN)
- 500 μL - 20% NE - 12 μg HA (IN)
- 500 μL - 45 μg (IM Fluzone)

The titers are indicated as percentages: 0%, 17%, 50%, and 100%.
20% Nanoemulsion

20% NE + 30µg Fluzone

Magnification 155,000X