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

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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, which undergo frequent mutations and reassortments in their antigenic surface proteins, hemagglutinin (HA) and neuraminidase (NA). The resultant antigenic drift necessitates the production of new influenza vaccines annually, whereas the possibility of a major antigenic shift poses the threat of pandemic influenza (5, 16). Three major influenza epidemics occurred in the 20th century; there were approximately 40 million deaths in the 1918–1919 pandemic flu outbreak and about 1 million deaths each in the 1957 and 1968–1969 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 seasonal influenza virus strains, avian influenza virus strains are now posing a potential threat to the human population (18). All of these facts argue for improved approaches to preventing influenza virus 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 virus preparations (27), but issues involving reactogenicity led to the development of subvirion (split) influenza vaccines. These vaccines are safer and are efficacious (60 to 90% efficacy rates) (11, 12). However, the efficacy of these subvirion vaccines is much lower for at-risk populations, such as children, the elderly, and immunocompromised subjects (21). A live attenuated influenza vaccine (LAIV), FluMist, is approved for intranasal (i.n.) use in humans in the age range of 2 to 49 years (24) and generates protective immunity (13) against drifted influenza virus strains (2, 3). However, the administration of LAIV to high-risk populations, including the immunocompromised, must be weighed carefully in terms of risk and benefit. Nasal administration of a killed influenza vaccine that is protective via mucosal and systemic immune responses would offer significant advantages over currently available injectable and intranasal influenza vaccines. This would avoid 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 intranasal administration. W805EC NE contains droplets of approximately 400 nm in diameter and has inherent antimicrobial activity (23). Studies with a prototype NE formulations showed that the NE can inactivate whole influenza virus and induce an immune response after nasal administration that protects 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 virus surface antigen and produced a robust immune response when administered intranasally, 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 WmSEC adjuvant. NE adjuvant activity resulting in mucosal, systemic, and Th1 and Th17 cellular immunity and in protection against challenge was also seen in mice immunized with influenza virus inactivated with β-propiolactone (20).

In the current study, we extensively investigated the immune responses of naïve ferrets immunized intranasally with NB-1008 vaccines prepared by mixing WmSEC adjuvant with the commercial trivalent inactivated influenza vaccines Fluzone and Fluvirin. The immune response produced by NB-1008 was compared with those produced by both intramuscularly and intra-muscularly (i.m.) administered nonadjuvanted flu vaccines. Intramuscularly adjuvanted vaccine was not tested, since the NE was not optimized for parenteral administration.

MATERIALS AND METHODS

Nanoemulsion adjuvant. WmSEC adjuvant was prepared by NanoBio Corporation (Ann Arbor, MI). The NE is an oil-in-water emulsion manufactured by high-speed emulsification, and 60% NE contains 3.55% Tween 80, 4.04% etha-nol, 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 the 2007–2008 season and the Fluzone 2008–2009 seasonal vaccine were obtained from a commercial supplier for use in these ferret studies. Each 0.5-mL vaccine contained a total of 45 μg of HA (15 μg each from 3 distinct influenza virus 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/Uru-guay/716/2007 (H3N2), an A/Brisbane/10/2007-like strain (H3N2) (referred to as A/Brisbane/10/07); and B/Malaysia/31/2006 (referred to as B/Malaysia). Fluzone was a subunit vaccine prepared in eggs and inactivated using formaldehyde. The virus is purified and then disrupted using a nonionic surfactant to produce the split virus. The split virus is then purified and suspended in phosphate-buffered saline (PBS). Fluvirin is a subunit vaccine prepared in eggs and inactivated by β-propiolactone. Hemagglutinin and neuraminidase surface antigens are then purified and suspended in PBS.

Nanoemulsion-adjuvanted influenza vaccine preparation (NB-1008). The WmSEC-adjuvanted influenza vaccine formulations were prepared by mixing PBS (HyClone) with different volumes of Fluzone or Fluvirin to achieve the final concentration of 5% egg infectious doses (EID50/ml).

Transmission electron micrographs and sectioning technique. Twenty milliliters of the NE adjuvant alone or with Fluzone was fixed with 1% (wt/vol) osmium tetroxide solution. The fixed samples were washed with distilled water. The samples were dehydrated with an increasing series of ethanol concentrations (30%, 50%, 70%, 90%, and 100%) and rinsed with double-distilled deionized water. The fixed samples were dehydrated with ascending concentrations (30%, 50%, 70%, 90%, and 100%) of component A of a Durcupan kit (Fluka) in double-distilled deionized water. These samples were transferred to embedding solution (a mixture of components A, B, C, and D of a Durcupan kit). The embedded samples were sectioned to a 75-nm thickness and placed on 300-mesh carbon-coated copper grids. The sections on the grids were stained with saturated uranyl acetate in distilled and deionized water (pH 7.0) for 10 min, followed by lead citrate for 5 min. The samples were viewed with a Philips CM-100 transmission electron microscope equipped with a computer-controlled stage and a high-resolution (2,000 × 2,000) digital camera and were digitally imaged and captured using X-Stream imaging software (SEM Tech Solutions, Inc., North Billerica, MA).

Ferrets. Ferret experiments were performed at SRL. Approximately 5- to 8-month-old naïve, castrated, and descanted male Fitch ferrets (Mustela putorius furo) were purchased from Triple F Farms (Sayre, PA). Ferret sera were negative for influenza virus HAI for the seasonal antigens prior to vaccination. Ferrets were quarantined for 4 to 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 (Bio-Medic Data Systems Inc., Salford, DE) and were housed in pairs prior to vaccination. Ferrets were aerosolized and intramuscularly vaccinated with ketamine/kg of body weight, 0.05 mg/kg atropine, and 2 mg/kg xylazine (KAX) prior to blood collection, treatment administration, and challenge. For HAI analysis, 1 ml of blood was collected from the anterior vena cava by use of a 23G 1-in. needle connected to a 1-mL tuberculin syringe.

Experimental design of ferret studies. Three ferret studies were performed to determine the immunogenicity of the NE-adjuvanted influenza vaccine, the dose-response relationships of both NE-adjuvanted 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 observations during the course of the studies. The three experiments are summarized in Table 1.

(ii) Ferret study 2. The first 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% WmSEC NE were assessed. The HA antigen used in this study was from commercially available influenza vaccines, either 2007–2008 Fluzone or Fluvirin. Control groups included 36 μg non-adjuvanted total HA administered intranasally and 45 μg nonadjuvanted total HA administered intramuscularly. HAI titers in response 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 after initial vaccination.

For postimmunization viral challenge, ferrets that received the smallest dose of influenza HA (7.5 μg) adjuvanted with WmSEC 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 they were euthanized on day 63. Nasal washes were collected on days 1 to 6 postchallenge (days 50 to 55) from 6 of the 10 ferrets receiving viral challenge. Lungs and nasal turbinates were collected from the remaining 4 ferrets (of the total of 10) on day 4 postchallenge (day 53). These samples were cultured, and viral loads were determined to ascertain if the NE-adjuvanted vaccines prevented viral multiplication in the ferrets.

(ii) Ferret study 2. The second study was performed to further assess the antigen-sparing properties of NB-1008 and the cross-reactivity following single intranasal immunization with NB-1008. Ferrets received low antigen doses (0.9, 3, and 7.5 μg total HA dose) mixed with 20% W805EC NE. Control animals were administered an intramuscular dose of 45 μg total HA. The 2007–2008 Fluzone 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 10^7 EID_{50} of A/Wisconsin. Nasal washes were collected from the ferrets on days 1 to 6 postchallenge (days 29 to 34). Nasal washes were cultured for determination of viral loads. No lung tissues or nasal turbinates were collected. Ferrets were euthanized on day 42.

(iii) Ferret study 3. The third study was performed to assess the effects of nanoemulsion concentration and dosing volume on the immune response. Ferrets were vaccinated on days 1 and 28. Adjuvanted vaccine doses were 3 μg HA in 5%, 10%, or 20% NE in a 200-μl volume, 6 μg HA in 10% NE in a 100-μl volume, and 12 μg HA in 20% NE in either a 200- or 500-μl volume. Control ferrets were administered 45 μg HA intramuscularly. The 2008–2009 Fluzone seasonal vaccine was used. Sera were collected for determination of specific HAI titer on days 27 and 48. Viral challenge was not performed in this study. Ferrets were observed weekly for clinical signs, body weight, and temperature and were euthanized on day 48.

Statistical analysis. Mann–Whitney U analysis was used to analyze differences in HAI titers between the groups that received i.n. vaccine with and without NE adjuvant. Significance was based on P values of ≥0.05.

RESULTS

Immunogenicity of W805EC-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) 4 weeks after a single intranasal vaccination of naïve ferrets with either W805EC-adjuvanted Fluvirin (Fig. 1) or W805EC-adjuvanted Fluzone (Fig. 2). Intranasal administration of the NE-adjuvanted vaccines had a higher seroconversion rate (100%) than intramuscular administration of the commercially available Fluvirin (22% to 56%) or Fluzone (67% to 89%) vaccine.

Notably, 100% of ferrets vaccinated intranasally with either Fluvirin mixed with 20% W805EC NE or Fluzone mixed with 20% W805EC NE (NB-1008) responded with HAI titers of >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 (GMTs) of >2,200 for A/Wisconsin. This is a >220-fold increase from baseline, a 32-fold increase compared to the response after i.m. Fluvirin (Fig. 1), and a >8-fold increase compared to that after i.m. Fluzone (Fig. 2). Increases in HAI titers relative to the baseline were also observed with the A/Solomon Islands strain (>100-fold) and the B/Malaysia strain (>25-fold) for both W805EC-adjuvanted vaccines. The adjuvanted vaccine gave 45-fold higher HAI titer levels against A/Solomon Islands than the titer achieved after i.m. Fluvirin and a >15-fold increase in HAI titer compared to that with i.m. Fluzone. For B/Malaysia, there was a >11-fold increase in HAI titer compared to that with i.m. Fluvirin (Fig. 1) and a >9-fold increase in titer compared to that with i.m. Fluzone (Fig. 2).

The titers achieved with NE-adjuvanted vaccines were statistically significantly different (P ≤ 0.05) from those with non-adjuvanted influenza vaccine administered intranasally, except for the following: A/Wisconsin strain (Fluvirin) at 22.5 μg HA (P = 0.1), B/Malaysia strain (Fluvirin) at 7.5 μg HA (P = 0.08) (Fig. 1), and A/Wisconsin (Fluzone) at 7.5 μg and 22.5 μg total HA antigen (P = 0.6 and 0.4, respectively) (Fig. 2).

A subsequent study was performed to identify the minimum antigen dose required to elicit seroconversion using the W805EC-adjuvanted Fluzone vaccine. In this study, the ferrets received a single vaccination with a lower concentration of HA antigen (0.9 to 7.5 μg total HA antigen). The control group was vaccinated i.m. 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 concentration than that in the nasal adjuvanted vaccine.

Dose-response relationships. The effect of NE concentration 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 received 0.5 ml of the trivalent commercial influenza vaccine (45 μg total HA) i.m. For all three strains, the antibody response in the ferrets increased with increasing NE concentrations; 17 to 33% seroconversion was achieved with 5% NE, 0 to 83% seroconversion was achieved with 10% NE, and 67 to 100% seroconversion was achieved with 20% NE, indicating that there is a dose response. The antibody response was markedly higher than that elicited with the i.m. vaccine, which contained a 15-fold higher total antigen content (Fig. 4).

Another dose-response study compared the effect of the volume administered on immunogenicity. Ferrets received 100, 200, or 500 μl of WNV5EC-adjuvanted influenza vaccine. The 200- and 500-μl vaccine doses contained 12 μg of total HA antigen mixed with 20% WNV5EC NE. Due to antigen concentration constraints, the 100-μl vaccine dose contained only 6 μg of total HA and was mixed with 10% WNV5EC NE. The immune response increased with increasing dose volumes, and the response was greater than that with i.m. vaccination. The use of either a 200- or 500-μl volume resulted in 100% seroconversion in all strains (Fig. 5). Thus, a dose-response relationship exists with respect to NE concentration and vaccine volume.

**Cross-reactivity titers to H3N2 influenza virus strains.** 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 to 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 than with intramuscular vaccination with Fluzone (data not shown). The degree of response appeared to be strain dependent: higher antibody titers were observed against the A/California, A/New York, and A/Wyoming strains. The NB-1008 vaccines elicited HAI titers that were 25- to 720-fold higher than baseline. Seroconversion rates of over 90% were achieved with these strains, compared to seroconversion rates ranging from 40 to 90% with the 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 resulted in significantly lower rates of seroconversion (56%) than the seroconversion rates attained by intranasal vaccination of NB-1008 with a 6-fold lower dose.

In another study, cross-reactivity was observed with the A/California and A/New York strains and, to a lesser extent, with A/Wyoming in ferrets receiving a single i.n. vaccination with NB-1008 (Fig. 6). A seroconversion rate of ≥50% was seen with the A/New York strain for the NB-1008 formulation containing the lowest total HA concentration (0.9 μg total antigen). A single i.n. 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) strains following a single intramuscular vaccination with the commercial vaccine (Fig. 6).
Protective efficacy of NB-1008 vaccine. Pilot studies with naïve ferrets showed that exposure to large doses of A/Wisconsin influenza virus was not fatal to 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 to 6 days following challenge. No changes in body weight or temperature were seen in the ferrets following intranasal viral challenge. In study 1, no viral load was detected on days 1 to 6 postchallenge in the nasal washes from ferrets immunized with NB-1008. Control animals receiving i.m. vaccine in the pilot study had significant levels of virus ($\geq 10^4$ EID$_{50}$/ml) in their nasal washes for up to 6 days following challenge. Viral loads in the nasal turbinates and lungs of a subgroup of challenged ferrets (4 of 10 ferrets) were determined 4 days following challenge; no influenza virus was detectable in the ferrets vaccinated with NE. Low viral concentrations were detected in the lungs of the control ferrets 5 days after challenge (30 to 160 EID$_{50}$/ml), but higher levels of the challenge virus were found in the nasal turbinates ($> 2 \times 10^3$ EID$_{50}$/ml). Study 2 showed low viral titers in relation to the dose response in the initial days following the challenge; however, on day 6 postchallenge, ferrets receiving NB-1008 had no virus in their nasal washes, compared to $>10^3$ EID$_{50}$/ml in the i.m. control group. These findings indicate that the ferrets vaccinated intranasally with NB-1008 developed sterilizing immunity.

Stability of NB-1008 vaccine. The stability of the NB-1008 vaccine was assessed for 2 weeks at room temperature and at 2 to 8°C to confirm that the vaccine formulation was stable at the time of vaccination. All of the experimental vaccine formulations administered to the ferrets were prepared 1 day before vaccination and were stored at 2 to 8°C. NB-1008 vaccines stored at room temperature and at 2 to 8°C were stable throughout the course of the study. There was no change in pH, particle size, or zeta potential or any visual signs of separation or settling. Potency testing for HA in NB-1008 was carried out using SRID assay 48 h after vaccine mixing. HA antigens of A/Brisbane/59, A/Brisbane/10, and B/Florida were found to be stable when the viruses were stored at 2 to 8°C for 48 h in the presence of NE (Table 2) and were within the accepted variability of the assay. The vaccine formulations containing 20% W805EC NE showed a slight decrease in HA potency readings 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$_{so}5$EC-adjuvanted vaccine was assessed in 249 ferrets by cage-side observation and weekly recording of body weight and body temperature. Intranasal administration of the W$_{so}5$EC 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 transmission electron micrographs of 20\% W\textsubscript{80}SEC NE showed NE droplets with a uniform inner core material. The NE-adjuvanted vaccine containing 30\%/H\textsubscript{9262}g of HA showed discrete antigen materials/particles inside the oil core of the droplets that represent the Fluzone antigens. Since the antigen is incorporated into the core and is surrounded by the core material, it is protected from staining by the electron-dense stain. This leads to a white counterstaining.

FIG. 3. HAI titers against A/Solomon Islands, A/Wisconsin, and B/Malaysia viruses following vaccination of naïve ferrets with NE-adjuvanted Fluzone. Ferrets received 500\%/H\textsubscript{9262}l vaccine containing HA doses ranging from 0.9 to 7.5\%/H\textsubscript{9262}g. Sera were collected on day 27 following a single intranasal vaccination on day 1. The numbers above the bars represent the seroconversion rates.

FIG. 4. Effect of NE adjuvant concentration on immune response. The data shown are HAI titers against A/Brisbane/59, A/Brisbane/10, and B/Florida following single intranasal vaccination with various NE concentrations mixed with 3\%/H\textsubscript{9262}g total HA antigen in a 200-\mu l total volume. Control ferrets received 500\%/H\textsubscript{9262}l i.m. Sera were collected on day 27 following a single intranasal vaccination on day 1. The numbers above the bars represent the seroconversion rates.
The localization of the antigen within the core shields the antigen-sensitive protein subunits in the emulsion and may protect the antigen from degradation, thus enhancing stability. There were very few Fluzone particles outside the NE particles that were stained darkly (Fig. 7).

**DISCUSSION**

The noninflammatory oil-in-water NE adjuvant W₈₀SEC, mixed with commercial influenza vaccines and administered intranasally, was well tolerated and significantly enhanced the hu-
moral immune response in ferrets, demonstrating antigen sparing compared to nonadjuvanted influenza vaccine administered i.m.

The disease burden of seasonal and pandemic influenza continues to warrant the development of novel, improved influenza vaccines (14). The efficacies of live attenuated mucosal vaccines and inactivated parenteral influenza vaccines are similar in adults (6) and higher in children (4, 10). The risk-benefit ratio for live attenuated vaccine use in high-risk populations, including children younger than 2 years of age, the elderly, and immunocompromised subjects, makes the development of more effective inactivated influenza vaccines for intranasal administration a desirable goal (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 of i.n. versus i.m. 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 W805EC 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 to W805EC-adjuvanted influenza vaccines in mice. W805EC adjuvant was either used to inactivate live A/Puerto Rico/8/34 (H1N1) influenza virus or mixed with β-propiolactone-inactivated virus (20). W805EC-adjuvanted vaccine induced a Th17 response that was present in addition to a significantly enhanced Th1 bias (20). In other studies, when an 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 it was administered intranasally to mice or guinea pigs (9). W805EC NE combined with hepatitis B virus surface antigen and administered intranasally produced an enhanced immune response and caused no inflammation in the nasal cavity, no histopathological changes in key organs, and no abnormal laboratory findings in safety studies performed with mice, rats, guinea pigs, and dogs (23). These data suggest a broad adjuvant activity for the W805EC NE and an acceptable safety profile for this adjuvant.

In the current study, experience with the W805EC adjuvant was extended to ferrets, the recommended preclinical model for influenza (30). The adjuvant was tested with two different commercial split vaccines (Fluvirin and Fluzone) and was compatible with either preparation, augmenting antibodies to all strains in the trivalent vaccines. Enhanced immunogenicity correlated with superior immunoprotection with the W805EC adjuvant. The nasal nanoemulsion-adjuvanted vaccine proved to be superior to nasal and muscular immunization with nonadjuvanted flu vaccines. The NE was not optimized for i.m. vaccination. The adjuvanted vaccine was dose sparing, using up to 50-fold less HA antigen than nonadjuvanted i.m. vaccines. The nasal adjuvanted vaccine re-

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FIG. 7. Transmission electron micrographs showing cross-sectional images of the 20% W805EC NE with and without 30 µg total HA. The panel on the right illustrates that HA antigens are located in oil droplets. The darkly stained antigens are located outside the NE particles.
sulted in higher seroconversion rates after a single vaccination, with HAI GMTs as high as 7,000, while the nonadjuvanted commercial vaccines achieved HAI GMTs of <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, W$_{805}$EC-adjuvanted vaccine resulted in the production of antibodies against heterologous influenza virus strains not present in the vaccine and in sterilization of the nasal secretions and turbinates in ferrets following challenge with live virus. The adjuvanted vaccine stabilized the HA antigen against the degradation normally seen at room temperature in the absence of adjuvant; NE-adjuvanted vaccine was stable at room temperature for up to 2 weeks. Potency studies using SRID assay demonstrated the stability of the influenza virus antigen for up to 2 days. Antigen stability could be explained by an 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 or changes in body weight gain or temperature in the 249 ferrets comprising this study. Thus, W$_{805}$EC continues to maintain a good safety profile.

The data indicate the possibility that W$_{805}$EC NE-adjuvanted influenza vaccines may represent a breakthrough in vaccinology. The adjuvant is noninflammatory and 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 naive ferrets also suggest that the adjuvant might be useful for immunization of high-risk populations such as children and the elderly, where immunity is not fully mature or is impaired. These data support the development of the W$_{805}$EC adjuvant for use in humans.

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