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Nanoemulsion W₈₀5EC improves immune responses upon intranasal delivery of an inactivated pandemic H1N1 influenza vaccine

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Abstract

Currently available influenza vaccines provide suboptimal protection. In order to improve the quality of protective immune responses elicited following vaccination, we developed an oil-in-water nanoemulsion (NE)-based adjuvant for an intranasally-delivered inactivated influenza vaccine. Using a prime-boost vaccination regimen, we show that intranasal vaccines containing the W₈₀5EC NE elicited higher titers of serum hemagglutination inhibiting (HAI) antibody and influenza-specific IgG and IgA titers compared to vaccines that did not contain the NE. Similarly, vaccines containing the W₈₀5EC NE resulted in higher influenza-specific IgA levels in the bronchoalveolar lavage (BAL) fluid and nasal wash when compared to vaccines formulated without NE. The higher antibody titers in mice immunized with the NE-containing vaccines correlated with reduced viral loads in the lungs and nasal turbinates following a high dose viral challenge. Mice immunized with vaccines containing the W₈₀5EC NE also showed a reduction in body weight loss following challenge compared to mice immunized with equivalent vaccines produced without NE. Taken together, our results show that the W₈₀5EC NE substantially improves the magnitude of protective influenza-specific antibody responses and is a promising mucosal adjuvant for influenza vaccines and vaccines against other mucosal pathogens.

Introduction

Influenza virus represents a major human pathogen that causes significant morbidity and mortality during seasonal epidemics and occasional pandemics, as underscored by the recent

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emergence of a novel H1N1 influenza virus [1, 2]. Vaccination is one of the most proven means of preventing infection and can also limit the clinical course of influenza [3]. Influenza immunization platforms currently approved in the United States for human use include vaccines consisting of inactivated viral components and live-attenuated influenza vaccines [4-6].

Inactivated, split-virion influenza vaccines are administered by intramuscular injection and elicit a strong serum influenza strain-specific antibody response dominated by IgG [7, 8]. The inability to generate high-quality mucosal and cell-mediated immune protection is a well-recognized weakness of inactivated influenza vaccines [9]. As a result, intramuscularly delivered inactivated vaccines are less efficacious in patient populations at-risk of developing severe influenza infection, such as children, the elderly, and individuals with chronic debilitating diseases [10, 11]. In contrast, intranasally delivered live-attenuated influenza vaccines more closely resemble the natural route of infection, and generate both mucosal and systemic antibodies and a CD8⁺ T cell response [1, 6, 12-14]. The broadened immune response elicited by live-attenuated vaccines corresponds to enhanced protection in children when compared to intramuscularly administered inactivated vaccines [15, 16].

Mucosally applied influenza vaccines composed of inactivated viral components and delivered with an adjuvant are attractive vaccine candidates because they can elicit strong mucosal and systemic protective immune responses and could be safely administered to immunocompromised patient populations. Mucosal vaccines can efficiently induce secretory IgA at mucosal surfaces, thereby preventing or limiting infection at the site of influenza virus entry [17]. Adoptive transfer of secretory IgA, but not other antibody isotypes, prevents virus-induced pathology in the upper respiratory tract [18]. Influenza-specific IgA antibodies directed against the HA protein also exhibit a higher degree of reactivity and protection against heterotypic viruses than influenza-specific IgG [19, 20]. Thus, the induction of mucosal IgA should be viewed as a critical component of adjuvanted influenza vaccines given the year-to-year antigenic drift of seasonal influenza viruses.

The effective induction of protective immunity following mucosal immunization will likely require co-administration of an adjuvant. Nanoemulsions are oil-in-water emulsions produced by mixing a water immiscible liquid phase into an aqueous phase by high stress mechanical extrusion. NEs were initially developed as agents with broad-spectrum antimicrobial activity and were later identified as a promising class of mucosal adjuvant [21-27]. While previously developed mucosal adjuvants such as cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) have been hampered by toxicity and clinical side-effects, the nanoemulsions we have developed have not been found to elicit inflammatory responses and have shown an acceptable safety profile in a number of species, including humans [28-31].

In this study, we used a mouse model to evaluate the mucosal NE adjuvant, W₈₀5EC, in an intranasally applied inactivated vaccine against the 2009 pandemic H1N1 A/Wisconsin/WSN/34939/09 influenza virus. The W₈₀5EC NE contains Generally Recognized as Safe (GRAS) materials that are also included on the FDA list of inactive ingredients in approved pharmaceutical products, but does not contain toxins or biological immune activators. The results presented here define the W₈₀5EC NE as a promising adjuvant for intranasally-applied, inactivated influenza vaccines and suggest the W₈₀5EC NE will provide a new platform for the development of vaccines to protect against mucosal pathogens.

Materials & Methods

Mice

Mice were acquired from Charles River Laboratories International, Inc. (USA) and the Jackson Laboratory (USA). All experiments involving mice were performed in accordance with guidelines set by the Institutional Animal Care and Use Committee at the University of Wisconsin–Madison.

Growth and purification of viruses

The pandemic (H1N1) 2009 influenza virus A/Wisconsin/WSLH 34939/09 (herein referred to as A/Wisc/09) was isolated from a patient in Wisconsin during the 2009 influenza pandemic [32]. The virus was amplified in Madin-Darby canine kidney (MDCK) cells. Mouse-adapted influenza A/Puerto Rico/8/34 (H1N1) (PR8) was purchased from ATCC (Rockville, MD) and used in experiments to screen potential nanoemulsion formulations for adjuvant activity. PR8 was grown in 10-day old embryonated chicken eggs. Methods for virus purification and the generation of mouse-passaged A/Wisc/09 are described in the Supplemental Materials.

Adjuvants and vaccine preparation

Nanoemulsions were prepared and provided by NanoBio Corporation (Ann Arbor, MI). The chemical and physical characteristics of the different NE formulations discussed in this study are presented in Supplementary Table 1.

Immunizations and challenge

Eight week old female CD1 mice were intranasally immunized twice at an interval of four weeks with vaccines containing 5×10^5 or 5×10^6 plaque-forming units (PFU) of β -propiolactone (BPL)-inactivated A/Wisc/09 virus with or without the 20% W₈₀5EC NE in a total volume of 10 μ l. 5×10^5 and 5×10^6 PFU inactivated virus corresponds to 0.09 μ g and 0.9 μ g of total viral protein, respectively, as estimated by the BCA Protein Assay Kit (Thermo Scientific) and is equivalent to 0.026 μ g or 0.26 μ g HA determined by SDS-PAGE analysis and quantification of viral protein bands. For intranasal inoculation, mice were anaesthetized with Isoflurane using an anesthesia machine (Summit Anesthesia Solutions, USA) attached to an oxygen supply. Equal quantities of vaccine virus with or without alum were delivered intramuscularly in a volume of 50 μ l. Cohorts of mice immunized with PBS or the 20% W₈₀5EC NE without any vaccine virus served as vehicle-only controls. For experiments evaluating survival after a lethal virus dose challenge following vaccination, mice were anaesthetized and inoculated intranasally with 100 LD₅₀ (3.3×10^6 PFU) of the mouse-adapted A/Wisc/09 virus prepared in 50 μ l PBS.

Hemagglutination Inhibition (HAI) Assay

The HAI assay was performed according to the WHO Global Influenza Surveillance Network Manual for the laboratory diagnosis and virological surveillance of influenza.

Viral load measurements

Lungs and nasal turbinates were disrupted in 1 ml of Minimal Essential Media (MEM) containing 0.3% BSA using a tissue homogenizer (Yasui Kikai, Japan). Viral loads were determined by plaque assay on MDCK cells and expressed as PFU/gm.

Statistics

Two-tailed nonparametric Mann-Whitney tests were used for analysis of virus-specific antibody titers, HAI antibody titers, and viral loads. A repeated measures ANOVA was used to compare weight loss curves. Differences with P values of 0.05 or less were considered significant.

Results

Screening of NE formulations with best adjuvant activity

We tested a panel of NEs with varied ratios of cationic and nonionic surfactants for their ability to enhance influenza-specific immune responses (Supplementary Table 1). These variations alter the mucoadhesive and penetration capabilities of the emulsions, and also enhance the solubilization of antigen in the oil phase. The adjuvant effect of each NE formulation was tested using a well-established influenza vaccination procedure in outbred CD1 mice, in which mice were immunized twice at 4 week intervals with an intranasal application of vaccine composed of 5×10^5 PFU inactivated A/PuertoRico/8/34 influenza virus delivered in the 20% NEs or PBS only as control [33]. This prime-boost vaccination schedule was selected on the basis of previous experiments that compared single versus multiple doses of intranasal vaccines containing the W₈₀5EC nanoemulsion and has been used to evaluate novel nanoemulsion formulations for their potential as intranasal vaccine adjuvants [26, 33].

All NE formulations tested resulted in higher influenza-specific serum IgG titers when compared to a PBS only vaccine (Figure 1A). The X-1091-AX1e-001-07 formulation, consisting of a NE with a 1:6 ratio of cationic-to-non-ionic surfactants, elicited significantly higher influenza-specific IgG serum antibody titers than any other formulation tested (Figure 1A). We next tested influenza-specific IgA levels in the bronchoalveolar lavage fluid of vaccinated mice 4 weeks following the second intranasal application of vaccine. Mice immunized with each of the NE formulations tested had detectable influenza-specific IgA in the bronchoalveolar lavage (BAL) fluid, with the greatest influenza-specific IgA levels being elicited by the X-1091-AX1e-001-07 NE formulation (Figure 1B). The X-1091-AX1e-001-07 NE formulation also elicited the highest levels of a panel of cytokines from restimulated splenocytes harvested from vaccinated mice (Supplementary Table 2), reflecting enhanced influenza-specific cellular responses following immunization. Collectively, these results demonstrate that of the NE formulations tested, the X-1091-AX1e-001-07 formulation had the greatest adjuvant effect on influenza-specific immune responses elicited following intranasal vaccination. This NE formulation was selected for further evaluation and was designated as W₈₀5EC.

W₈₀5EC NE augments influenza-specific serum antibody responses during intranasal vaccination

We sought to evaluate the quality of antibody responses elicited following intranasal vaccination using the W₈₀5EC NE. Mice were immunized and boosted according to the timeline depicted in Supplementary Figure 1. The vaccine containing the equivalent of 5×10^6 PFU of inactivated A/Wisc/09 virus prepared in the W₈₀5EC NE elicited higher serum HAI antibody titers compared to vaccines prepared in PBS only (Figure 2). At a vaccine dose containing 5×10^5 PFU inactivated virus, the W₈₀5EC NE induced detectable HAI antibody responses in some mice, whereas no detectable HAI antibody was detected in mice receiving the same dose of inactivated virus in PBS. As expected, control mice that received the W₈₀5EC NE without inactivated virus did not reveal detectable serum HAI antibody titers (Figure 2). These data show that intranasal vaccines containing inactivated

influenza virus and prepared in the W₈₀5EC NE formulation elicited greater average HAI serum antibody responses in mice compared to equivalent vaccines prepared in PBS.

We measured the levels of total influenza-specific IgG and IgA in the serum of mice following intranasal immunization with vaccines prepared in the W₈₀5EC NE or PBS. Mice immunized intramuscularly with vaccines prepared with or without Alum were included for comparison. At vaccine doses of 5×10^6 PFU and 5×10^5 PFU, intranasally delivered vaccines formulated with the W₈₀5EC NE induced higher average virus-specific IgG titers than those formulated with PBS (Figure 3, left panel). The influenza-specific IgG titers elicited by intramuscularly delivered vaccines were comparable to those of the intranasally delivered vaccines that contained the W₈₀5EC NE at the 5×10^6 PFU dose, but were higher than intranasally delivered vaccines that contained the W₈₀5EC NE at the 5×10^5 PFU dose (Figure 3, left panel).

At the 5×10^5 PFU vaccine dose, vaccines containing the W₈₀5EC NE induced significantly higher levels of serum IgA than vaccines containing PBS, although no effect of the W₈₀5EC NE was apparent at the 5×10^6 PFU vaccine dose (Figure 3, right panel). On average, intranasal vaccination with the W₈₀5EC NE elicited higher serum influenza-specific IgA than intramuscular vaccination with or without Alum adjuvant (Figure 3, right panel). These data indicate that the vaccines containing the W₈₀5EC NE augmented influenza-specific IgG and IgA levels in the serum relative to vaccines delivered in PBS.

W₈₀5EC NE improves influenza-specific mucosal antibody responses during intranasal vaccination

Next, we evaluated mucosal antibody responses following immunization by measuring influenza-specific IgG and IgA titers in BAL fluid and nasal wash 4 weeks after the booster vaccination. Influenza-specific IgG titers in the BAL fluid were higher in mice immunized intranasally with vaccines prepared in the W₈₀5EC NE compared to vaccines prepared in PBS, although this effect was statistically significant only at the 5×10^6 PFU vaccine dose (Figure 4A, left panel). Among intranasally immunized mice, those receiving vaccines prepared in the W₈₀5EC NE displayed modestly higher average influenza-specific IgA titers at both the 5×10^6 and 5×10^5 PFU dose, indicating an adjuvant effect of the W₈₀5EC NE on mucosal antibody responses (Figure 4A, right panel).

Vaccines containing the W₈₀5EC NE elicited significantly higher IgA titers in the nasal wash at the 5×10^6 PFU dose compared to vaccines prepared in PBS (Figure 4B, right panel). At the 5×10^5 PFU dose, vaccines containing the W₈₀5EC NE elicited a higher average influenza-specific IgA response in the nasal wash, but the difference was not statistically significant. The influenza-specific IgA responses in the nasal wash were generally higher among mice vaccinated intranasally compared to those vaccinated intramuscularly (Figure 4B, right panel). The W₈₀5EC NE appeared to have no significant benefit on the generation of influenza-specific IgG in the nasal wash at either antigen dose (Figure 4B, left panel). In summary, these data demonstrate a marked adjuvant effect of the W₈₀5EC NE on the generation of influenza-specific IgA antibody responses at respiratory mucosal surfaces following intranasal vaccination, and suggest that needleless vaccines containing W₈₀5EC NE may more efficiently drive protective immune responses.

Reduced viral burden and morbidity following challenge of mice vaccinated with W₈₀5EC NE-adjuvanted vaccines

Cohorts of mice were challenged with 100 LD₅₀ of live virus following intranasal immunization with vaccines prepared with or without the W₈₀5EC NE according to the timeline in Supplementary Figure 1. By day 3 post-challenge, all three mice immunized with

a vaccine containing 5×10^6 PFU inactivated virus and prepared in the W₈₀5EC NE had no detectable virus in their lungs, whereas all three mice vaccinated with an equivalently dosed vaccine prepared in PBS had viral loads exceeding 10^5 PFU/g tissue (Figure 5A, left panel). No differences in lung viral burden were noted at the lower antigen dose of 5×10^5 PFU when comparing mice immunized with vaccines containing W₈₀5EC NE to mice that received vaccines prepared in PBS on day 3 post-challenge (Figure 5A, left panel). By day 6 post-challenge, the mice immunized with the 5×10^5 PFU dose vaccine prepared with the W₈₀5EC NE had no detectable virus in the lung, whereas the three mice receiving the equivalent vaccine prepared in PBS still had detectable lung virus (Figure 5A, right panel). Similarly, no virus was detected in nasal turbinates from mice immunized with the 5×10^6 PFU dose prepared in the W₈₀5EC NE on day 3 post-challenge, whereas virus was still recovered from mice that were immunized with the equivalently dosed vaccine prepared in PBS (Figure 5B, left panel). No notable differences in nasal turbinate viral loads on day 3 or day 6 post-challenge were noted between cohorts of mice immunized with the 5×10^5 PFU dose regardless of whether the virus was prepared in W₈₀5EC NE or PBS. As expected, cohorts of mice that received a vehicle-only vaccine containing the W₈₀5EC NE had high levels of virus in the lungs and nasal turbinates at both day 3 and day 6 post-challenge (Figure 5). These data demonstrate that intranasal immunization with vaccines prepared in the W₈₀5EC NE result in accelerated clearance of virus from respiratory organs following a high-dose challenge compared to equivalent vaccines prepared in PBS.

Mice immunized with a vaccine prepared in the W₈₀5EC NE at the 5×10^6 PFU dose experienced significantly less body weight loss than mice that received the equivalently dosed vaccine prepared in PBS when challenged with 100 LD₅₀ of A/Wisc/09 H1N1 influenza virus (Figure 6A, left panel). All mice survived the high-dose challenge when vaccinated with the 5×10^6 PFU dose vaccines regardless of whether they were prepared in the W₈₀5EC NE or PBS (Figure 6A, right panel). At the 5×10^5 PFU vaccine dose, addition of NE did not significantly alter the rate or degree of weight loss (Figure 6B, left panel). No significant differences in survival between the cohorts immunized with vaccines prepared in the W₈₀5EC NE or PBS were noted at the 5×10^5 PFU vaccine dose (Figure 6B, right panel). At both doses of viral antigen, the intramuscularly delivered Alum-adjuvanted vaccine protected mice from challenge and was comparable to the protection afforded by vaccination through the intranasal route (Figure 6). As expected, control mice that received NE alone as a vehicle-only control lost weight until the animals succumbed to infection or reached a predetermined clinical endpoint and were considered moribund and euthanized (Figure 6). These data indicate that, with appropriate antigen dosing, intranasal vaccines containing the W₈₀5EC NE can reduce morbidity following high-dose influenza virus challenge.

Discussion

Influenza vaccines that elicit a strong, robust and long-lived protective immune response at respiratory mucosal surfaces would provide an immediate first-line of defense at the portal of influenza virus entry. While intramuscularly delivered inactivated vaccines have proved effective, they do not generate high-titer mucosal immune responses and elicit suboptimal immune responses in the elderly and people with chronic debilitating conditions. Live-attenuated influenza vaccines elicit influenza-specific mucosal antibodies and a strong cellular immune component, but are not recommended for use in patient populations that are most at risk of severe influenza virus infection. There is a continued need to develop more effective and safe influenza vaccines.

We have addressed the limitations of current influenza vaccine strategies by developing an intranasally delivered vaccine consisting of inactivated virus and a novel NE adjuvant. Our results indicate that a W₈₀5EC NE-adjuvanted inactivated influenza vaccine delivered

intranasally elicits a strong mucosal influenza-specific IgA response, offering an important advantage over immune responses driven by parenteral vaccination. The generation of high-titer influenza-specific IgA responses at the respiratory mucosa is an important component of the protective immune responses elicited by influenza vaccination, as IgA-deficient mice suffer increased lethality to influenza virus challenge following intranasal immunization with an H1N1 subunit vaccine despite having similar systemic and mucosal influenza-specific IgG responses [35]. The polymeric immunoglobulin receptor (pIgR) is required for normal active transport of dimeric IgA across the epithelial layer into mucosal secretions. Cross protection against antigenically drifted influenza viruses was severely abrogated in pIgR-deficient mice indicating that dimeric influenza-specific IgA at mucosal surfaces is a critical component immune protection against antigenically drifted strains of influenza virus [36]. As influenza viruses exhibit antigenic variability from year-to-year, the next generation of influenza vaccines should aim to drive a strong mucosal IgA component to provide increased immune protection against antigenically drifted variants during inter-pandemic influenza seasons. The intranasally-delivered W₈₀5EC NE-adjuvanted inactivated vaccine developed herein is a step towards this goal.

We observed that serum IgG levels following intramuscular vaccination were higher than those generated following intranasal vaccination with the W₈₀5EC nanoemulsion. While the intranasal route may require higher antigen doses to generate levels of influenza-specific serum IgG comparable to those elicited following intramuscular vaccine delivery, intranasal vaccination with the W₈₀5EC nanoemulsion elicits higher levels of mucosal influenza-specific IgA than equivalently dosed intramuscular vaccines. A number of factors may contribute to the differences in antibody responses between intranasally- and intramuscularly-delivered vaccines. For example, differences in effective antigen dose (i.e. the amount of antigen available for uptake) might be less during intranasal delivery due to mucosal surface physiology. Further, the contribution of specific immune cell subsets (i.e. dendritic cell subsets) involved shaping the immune response may vary according to the anatomical site used for immunization.

Intramuscular vaccination elicited higher IgG responses at mucosal sites including BAL fluid and nasal wash when compared to the intranasally delivered vaccine. Serum IgG is thought to reach mucosal surfaces primarily by passive diffusion, so it is perhaps not surprising that the higher levels of influenza-specific IgG in the serum are reflected in higher levels of influenza-specific IgG at mucosal surfaces. Consistent with this concept, a linear relationship has been reported between influenza-specific serum IgG levels and IgG at mucosal surfaces [37], and our finding is in agreement with previous reports [38, 39].

It can be noted that the HAI titers between the two vaccine dosing groups are greater than the difference in binding IgG titers. Different aspects of the influenza-specific antibody response are measured in the HAI as compared to the ELISA. HAI antibody titers reflect only the subset of influenza-binding antibodies that can inhibit virus-mediated agglutination of turkey red blood cells. This subset of antibodies is mainly directed against epitopes on the hemagglutinin protein. In contrast, all influenza virus proteins present in the virion are used as target in the ELISA to measure influenza-specific IgG antibody titers.

A major hurdle in mucosal vaccinology has been the development of safe and effective mucosal adjuvants. In this work, we document a strong adjuvant effect of a novel NE, W₈₀5EC, during intranasal immunization with an inactivated influenza vaccine. The W₈₀5EC NE does not contain specific pro-inflammatory components and has displayed an acceptable safety profile in humans and a number of animal models [30, 31] and therefore should continue to be actively pursued as a novel mucosal adjuvant. An important next step will be to evaluate the adjuvant activity of W₈₀5EC NE in the context of influenza vaccines

that are associated with reduced inherent immunogenicity, including split virion vaccines, subunit vaccines, and vaccines consisting of recombinant influenza HA protein.

Conclusions

We have identified the W₈₀5EC NE as a strong mucosal adjuvant that enhances influenza-specific systemic and mucosal antibody responses when included in intranasally delivered vaccines composed of inactivated viral components. Inactivated mucosal influenza vaccines containing the W₈₀5EC NE represent a promising vaccination platform that addresses the major limitations of currently licensed influenza vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

The effect of nanoemulsions on intranasal influenza vaccination was examined in mice

The W₈₀5EC nanoemulsion is identified as a candidate mucosal vaccine adjuvant

W₈₀5EC nanoemulsion improved antibody responses following intranasal vaccination

Vaccines with the W₈₀5EC nanoemulsion reduced morbidity after influenza challenge

Figure 1. Screening NE formulations for mucosal adjuvant activity

A. Mice were immunized and boosted with intranasal vaccines prepared in the indicated nanoemulsion formulations or PBS only as a control. The titer of influenza-specific serum IgG in vaccinated mice four weeks after the booster immunization was determined by indirect ELISA. B. Mice were immunized and boosted with intranasal vaccines prepared in the indicated nanoemulsion formulations or PBS as a control. The relative amounts of influenza-specific IgA in bronchoalveolar lavage fluid collected from vaccinated mice four weeks after the booster immunization was determined by indirect ELISA. Bronchoalveolar lavage fluids were tested using a 1:2 dilution. Error bars represent the standard deviation; 3-5 mice per group. Statistically significant differences ($P < 0.05$) are indicated.

Figure 2. Intranasal vaccines prepared in the W805EC NE increase hemagglutination inhibiting antibody levels

Mice were intranasally administered vaccines containing the indicated dose of β -propiolactone-inactivated H1N1 influenza virus in the W805EC NE or phosphate buffered saline (PBS) according to the time line in Supplementary Figure 1. Sera, collected 56 days following the first immunization, were serially diluted and tested for the ability to inhibit influenza virus-mediated agglutination of turkey red blood cells. Data points represent antibody titers in individual mice. Horizontal bars indicate the geometric mean. HAI: hemagglutination inhibiting.

Figure 3. Intranasal vaccines prepared in the W805EC NE increase influenza-specific serum IgG and IgA levels

Mice were administered vaccines containing the indicated dose of β -propiolactone-inactivated pandemic (H1N1) 2009 influenza virus according to the time line in Supplementary Figure 1. Intranasally delivered vaccines were prepared in the W805EC NE or phosphate buffered saline (PBS), and intramuscularly delivered vaccines were prepared in Alum or PBS. Sera were collected 56 days after the first immunization and influenza-specific IgG (left panel) and IgA (right panel) titers were determined by indirect ELISA. Data points represent antibody titers in individual mice. Horizontal bars indicate the geometric mean. Statistically significant differences ($P \leq 0.05$) are indicated.

Figure 4. Intranasal vaccines prepared in the W805EC NE increase mucosal influenzaspecific IgG and IgA levels

Mice were administered vaccines containing the indicated dose of β -propiolactone-inactivated H1N1 influenza virus according to the time line in Supplementary Figure 1. Intranasally delivered vaccines were prepared in the W805EC NE or phosphate buffered saline (PBS), and intramuscularly delivered vaccines were prepared in Alum or PBS. Bronchoalveolar lavage fluid (A) and nasal wash (B) were collected 56 days after the first immunization and influenza-specific IgG (left panels) and IgA (right panels) titers were determined by indirect ELISA. Data points represent antibody titers in individual mice. Horizontal bars indicate the geometric mean. Statistically significant differences ($P < 0.05$) are indicated.

Figure 5. Intranasal vaccines prepared in the W805EC NE decrease viral loads in respiratory organs following high-dose viral challenge

Mice were intranasally administered vaccines containing the indicated dose of β -propiolactone-inactivated H1N1 influenza virus in the W805EC NE or phosphate buffered saline (PBS) according to the time line in Supplementary Figure 1. Four weeks after the second administration of vaccine, mice were challenged with 100LD50 of mouse-adapted A/Wisc/09 virus. Lungs (A) and nasal turbinates (B) were collected at day 3 (left panels) and day 6 (right panels) following challenge and virus titer was determined using a plaque assay. Data points represent viral loads per gram of tissue (PFU/g) in individual mice. Horizontal bars indicate the mean. Statistically significant differences ($P < 0.05$) are indicated.

Figure 6. Intranasal vaccines prepared in the W805EC NE reduce morbidity following viral challenge

Mice were intranasally or intramuscularly administered with vaccines containing 5×10^6 PFU (A) or 5×10^5 PFU (B) β -propiolactone-inactivated pandemic (H1N1) 2009 A/Wisc/09 virus according to the time line in Supplementary Figure 1. Vaccines were prepared in the W805EC NE or phosphate buffered saline (PBS) for intranasal delivery, or in Alum for intramuscular delivery. Cohorts of mice received the W805EC NE with no viral antigen (0 PFU groups) as a control. Four weeks after the second administration of vaccine, mice were challenged with 100LD50 of mouse adapted A/Wisc/09 virus and survival and weight loss was monitored daily for two weeks. Error bars represent standard deviation. A repeated measures ANOVA was used to calculate P values for overall weight loss curves of mice receiving vaccine that included NE and those receiving vaccine without NE. Statistically significant differences ($P < 0.05$) are indicated.