A novel dry powder influenza vaccine and intranasal delivery technology: induction of systemic and mucosal immune responses in rats

Juan Huang a, Robert J. Garmise b, Timothy M. Crowder c, Kevin Mar a, C. Robin Hwang a, Anthony J. Hickey b,c, John A. Mikszta a, Vincent J. Sullivan a,

a BD Technologies, 21 Davis Drive, Research Triangle Park, NC 27709, USA
b School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA
c Department of Biomedical Engineering and Mathematics, School of Medicine, University of North Carolina, Chapel Hill, NC 27599, USA

Received 17 December 2003; received in revised form 28 May 2004; accepted 29 June 2004

Available online 12 August 2004

Abstract

Intranasal (IN) vaccination represents an attractive non-invasive alternative to needle-based injection and provides superior protection at mucosal surfaces. However, new formulations are needed to improve efficacy and reduce the refrigerated storage and distribution requirements associated with standard liquid vaccines. Here, we describe a powder formulation of whole inactivated influenza virus and a novel IN delivery platform. The powder-formulated vaccine elicited a significant serum antibody response in rats that was at least as strong as that provided by the liquid vaccine administered IN or via intramuscular (IM) injection. Significant nasal IgA responses were also observed solely after IN delivery. This study demonstrates for the first time the generation of potent nasal mucosal and systemic immune responses using an IN delivered influenza vaccine powder and suggests an alternative approach to vaccination against influenza and other infectious diseases.

© 2004 Elsevier Ltd. All rights reserved.

Keywords: Powder vaccine; Influenza virus; Intranasal immunization

1. Introduction

Influenza is a highly contagious disease that causes high morbidity and mortality worldwide each year. Like most infectious diseases, influenza infections occur when the virus invades the host through the thin and permeable mucosal surface. The establishment of mucosal immunity, the first line of defense against such pathogens, is of major importance in preventing infectious diseases. However, the majority of commercial vaccines used for prevention of infectious diseases are administered by parenteral injection. This route typically does not induce strong mucosal immunity [1,2]. Intranasal (IN) vaccination has been shown to elicit both mucosal and systemic immunity in animal studies and human clinical trials [1–4]. IN vaccination offers advantages of easy and non-invasive delivery, potentially reducing the need for administration by medically trained personnel. Most IN vaccines employ liquid formulations and require refrigeration for storage and distribution. These cold-chain requirements may be greatly reduced or eliminated through the use of dry powder formulations, which are potentially superior to liquid formulations in their sterility and stability, thereby facilitating mass vaccination [4–7].

The advantages of mucosal immune protection, along with the stability of powder formulations, have led to increasing interest in respiratory delivery of vaccines in dry powder form. Powder processing methods are now available for producing aerosolizable, powder forms of vaccines tailored specifically for delivery to either the nasal cavity or lungs [4–8].

In the present study, inactivated influenza virus, A/PR/8/34 H1N1, was processed to a dry powder formulation and administered to rats. The immune responses were compared to those elicited by a conventional liquid formulation...
administered IN or by intramuscular (IM) injection. It is generally accepted that the inactivated virus alone cannot induce an efficient mucosal immune response [9–11]. Chitosan, a biocompatible and biodegradable polymer derived from crustacean shells, has been used as a bioadhesive for IN delivery in order to boost the mucosal immune response [7,12]. In the present experiments, the mucoadhesive function of chitosan was employed in order to elicit mucosal immunity to dry powder influenza vaccine formulations delivered IN. These dry powder IN formulations generated potent nasal mucosal immunity, in addition to systemic immune responses comparable to those achieved through IM injection.

2. Methods

2.1. Influenza virus and preparation of powder formulation

Whole inactivated influenza virus of the H1N1 strain, A/PR/8/34 (Charles River SPAFAS, North Franklin, CT), was propagated in the allantoic cavity of SPF eggs, purified from sucrose gradient, inactivated by Beta-propio-lactone, and re-suspended in Hepes-Saline. This virus was used as a liquid preparation without addition of adjuvants, as provided by the vendor, or formulated as a powder. For powder preparation, virus was frozen in a dry ice—isopropanol slurry. Lyophilization (Virtis Model 10-324 Bench Freeze Dryer, Gardiner, NY) for 24 h was followed by moisture content determination, virus was frozen in a dry ice—isopropanol slurry. Vibrational milling was undertaken for 2 h with the desired range between 45 and 125 m. Powders were sized using a laser diffraction instrument (Malvern 2600 Series, Worcs, UK) and sieved using a sieve shaker (Gislon SS-S, Worthington, OH) in tap mode for 2 h with the desired range between 45 and 125 μm. Powder blends (2.8% w/w chitosan) were vibrated using the milling vial alone, i.e. without the ball, for 30 min to achieve uniformity.

2.2. Vaccine stability test

Liquid preparation of whole inactivated influenza virus samples were added to wells containing 50 μl of 0.5% chicken red blood cells (Charles River SPAFAS) and incubated at RT for 1 h. The endpoint HA titer was defined as the reciprocal of the highest influenza virus dilution showing complete hemagglutination of chicken red blood cells.

2.3. Animals and immunizations

Female Brown Norway rats (125–175 g, Charles River, Raleigh, NC) were kept under standard conditions in AAALAC approved facilities under IACUC approved protocol. Rats were fully anesthetized by intraperitoneal injection of ketamine, xylazine and ACEpromazine before immunization. Rats were immunized three times IM with the liquid formulation or IN with either the liquid or the powder formulation on days 0, 28, and 49. Each dose contained 100 μl of 0.5% chicken red blood cells (Charles River SPAFAS) and incubated at RT for 1 h. The endpoint HA titer was defined as the reciprocal of the highest influenza virus dilution showing complete hemagglutination of chicken red blood cells.
tein content as above) whole inactivated influenza virus at

2.5. Sample collection

Serum was obtained, under anesthesia, from the orbital sinus 3 weeks post priming and 2 weeks post boosting. Two weeks following the third immunization, rats were euthanized by CO2 inhalation and nasal washes were collected by

weeks following the third immunization, rats were euthanized by CO2 inhalation and nasal washes were collected by

anesthetized rat. The plunger of the syringe containing 3 ml of air was depressed, bursting the films at either end of the

capsule, and distributing the vaccine in the nasal cavity.

Particle size distribution of freeze-dried inactivated influenza virus. The data is graphed as relative frequency versus particle size. Relative frequency is the percent of the total particle count occurring within each particle size range.

2.5. Sample collection

Serum was obtained, under anesthesia, from the orbital sinus 3 weeks post priming and 2 weeks post boosting. Two weeks following the third immunization, rats were euthanized by CO2 inhalation and nasal washes were collected by cannulating the trachea and rapidly instilling 1 ml of PBS containing 20 g/ml aprotinin (Sigma). Fluid emerging from the nostrils was collected and stored at −20°C.

2.6. ELISA assays

Whole virus-specific antibody titers were determined by ELISA. Plates (96-well, Nalge Nunc Maxisorb, Rochester, NY) were coated with 100 µl of 1 µg/ml (based on total protein content as above) whole inactivated influenza virus at 4°C overnight, then blocked at 37°C for 1 h with PBS Tween 20 (PBST, Sigma) containing 5% non-fat dry milk. After washing, serial dilutions of sera or nasal wash (100 µl/well) were added and incubated at 37°C for 1 h. Plates were washed again, then incubated at 37°C for 45 min with horseradish peroxidase (HRP) conjugated secondary antibodies: goat anti-rat Ig (H+L) (Southern Biotechnology Associates, Inc, Birmingham, AL) or goat anti-rat IgA or IgE (Bethyl Laboratories, Montgomery, TX). Plates were developed by incubating for 30 min at room temperature with 3,3′,5,5′-tetramethyl Benzidine (TMB) substrate (Sigma). After stopping the reaction using 0.5 M H2SO4, plates were read at 450 nm (Tecan US Inc, RTP, NC). The endpoint titers were defined as the highest reciprocal dilution of sera or nasal wash yielding an OD450 value at least 3× background obtained using samples obtained prior to immunization. All standards, samples, and controls were analyzed in duplicate. Virus-specific IgG1, IgG2a and IgG2b were measured by ELISA as above, substituting reagents provided in IgG isotyping kits (Bethyl Laboratories). IgG1, IgG2a and IgG2b concentrations were determined using standard curves, as per the manufacturer’s instructions. The sensitivity of isotyping ELISA is in the range of 3–1000 ng/ml. Samples were diluted to fall within the standard curve range.

2.7. HA inhibition (HAI) assay

Test sera were inactivated at 56°C for 10 min to destroy complement and HAI inhibitors. Twenty-five milliliter of double serial diluted test sera were then added to wells containing 25 µl of four hemagglutinating units of influenza virus followed by adding 50 µl of 0.5% chicken red blood cells to each well. Plates were then incubated at room temperature for 1 h. The endpoint HAI titer was defined as the reciprocal of the highest serum dilution that completely inhibited hemagglutination of the chicken red blood cells.

3. Results

3.1. Delivery device and powder characterization

A specialized powder delivery device was developed for this study (Fig. 1a). This device consists of a powder-filled capsule that is contained within a plastic housing unit [13]. The capsule is loaded with powder vaccine and sealed with rupturable films at each end. The housing unit features a Luer-fitting attachment designed to connect to a standard syringe. Depressing the syringe plunger pushes air through the device, causing the rupturable capsule films to burst, thus creating a powder plume that exits the device through a diffuser. For the studies described herein, the exit diffuser of the device designed for human use [13] was reduced in diameter in order to accommodate the small nasal passages of rats.

Particle size for the powder formulations used in this study were measured by laser diffraction, and characterized in terms of both median size (calculated as median volume diameter) and distribution of particle size (calculated as span). Span is a measure of particle size distribution defined as $D(50) - D(10)/D(50)$, where $D(n)$ represents the volume diameter at a cumulative volume percent [14]. The median volume diameter of milled trehalose was 18.79 ± 1.71 µm with a span of 3.47 ± 0.93 µm (n = 3). The median volume diameter of milled chitosan was 141.57 ± 44.29 µm with
a span of 3.66 ± 0.52 µm (n = 3). The median diameter of milled trehalose: whole inactivated influenza virus was 37.06 ± 4.54 µm with a span of 4.47 ± 3.36 µm (n = 3) (Fig. 1b).

3.2. Vaccine powder stability study

A major advantage of powder vaccine formulations is the increased stability as compared to conventional liquid formulations. To assess stability, powder and liquid vaccine formulations were subject to various storage conditions for a period of up to 12 weeks. Results are shown in Fig. 2a (4°C and 25% RH), Fig. 2b (25°C and 25% RH) and Fig. 2c (40°C and 75% RH). At 4°C and 25% RH, powder vaccine retained 100% of pre-storage HA activity for up to 12 wks (not tested for storage over 12 weeks). However, the HA activity of the liquid formulation under these conditions dropped to 25% after week 8 (Fig. 2a). Similarly, at 25°C and 25% RH, the powder vaccine retained 100% of pre-storage HA activity, whereas the HA activity of the liquid dropped to 25% at week 4 and was further reduced to 12.5% of pre-storage levels at week 8 (Fig. 2b). The repeat experiment showed similar results. Only under the harshest conditions tested (40°C and 75% RH) did the powder formulation show a drop in stability over time; under these conditions HA activity was reduced to 25% pre-storage levels at week 2 and 12.5% at week 4. In a repeat experiment under this condition, the powder vaccine retained 100% of pre-storage HA activity at week 2 and dropped its HA activity to 25% at week 4 (Fig. 2c). Nonetheless, the powder formulation was still more stable than corresponding liquid, which lost 100% of activity by week 2. Altogether, the results demonstrate the improved vaccine storage stability provided by the powder formulation.

3.3. Serum total antibody response and hemagglutination inhibition (HAI) titers

To evaluate the immune responses elicited by powder influenza vaccine, various formulations were prepared and inoculated IN into rats. The immune responses following inoculation of the powder formulation with or without chitosan, as well as the responses to the liquid formulation administered IN or IM were assessed. IN delivery of liquid vaccine provided equivalent serum antibody titers as those obtained by IM injection (Fig. 3a). The serum antibody response provided by IN delivery of powder vaccine without chitosan was weaker than that provided by the liquid vaccine administered IM or IN. Notably, however, rats immunized with the powder/chitosan blend generated an overall 14–52-fold increase in serum antibody titers as compared to those immunized with the powder formulation lacking chitosan (P = 0.08 at day 21 by ELISA). Over the course of the study, the powder/chitosan group showed at least as strong serum antibody titers as those elicited by conventional IM injection. Similar trends were observed in a second independent experiment. HAI titers also displayed a similar pattern (Fig. 3b, P = 0.05 when comparing the powder with chitosan to the powder without chitosan).

3.4. Nasal IgA titers

Nasal lavage fluids were assessed for influenza-specific IgA responses in order to evaluate nasal mucosal immune response following IN delivery of dry powder vaccine. Positive nasal IgA responses were seen only after nasal administration, regardless of formulation (Fig. 3c). No detectable IgA
Fig. 3. Immunogenicity of whole inactivated influenza virus in the rat immunization model. Rats were given three immunizations with 100 μg whole inactivated influenza virus. Milled trehalose was used as a negative control. Data represent mean ± S.E.M. (a) Serum anti-influenza total antibody titers; \( n = 3 \) for IN liquid group, \( n = 4 \) for all other groups. (b) Serum HAI titers. Sera were collected two weeks after the third immunization. HAI titers of individual rats are indicated by closed symbols, \( n = 3 \) for IN liquid and IN powder/chitosan groups, \( n = 4 \) for all other groups. (c) Nasal IgA titers. Nasal lavage fluids were collected two weeks after the third immunization. IgA titers of individual rats are indicated by closed symbols, \( n = 3 \) for IN liquid group, \( n = 4 \) for all other groups.

Fig. 4. Serum IgG1/IgG2a/IgG2b profiles. Following the third immunization (d63), sera were analyzed for the presence of influenza-specific IgG1, IgG2a and IgG2b. Bars indicate mean ± S.E.M.

3.5. Serum antibody isotypes

Serum IgG1, IgG2a and IgG2b isotypes were measured as an indirect assessment of T-helper (Th) cell activity (Fig. 4). In rats, IgG1 and IgG2a antibodies are associated with Th2 activity, while IgG2b antibody is suggestive of a Th1 response [15]. All groups generated a mixed and balanced response consisting of IgG1, IgG2a and IgG2b. There was no major shift of the Th1/Th2 response across the different formulations. In addition, antigen-specific IgE responses were not detected in any of the groups (data not shown), suggesting negligible allergic effects. Taken together, the data show that the IN powder/chitosan blend elicited a mixed IgG subclass response, which may be important for protection against influenza and other pathogens.

4. Discussion

The high morbidity and mortality caused worldwide each year by influenza are well documented [16,17]. In the United States alone, over 50,000 deaths per year were estimated to be attributable to influenza in the 1990s [18]. More recently, the Centers for Disease Control has estimated deaths in the United States from influenza and its complications at 36,000 per year [19]. Widespread vaccination is the main defense against the epidemic spread of the disease [18]. Current efforts to control influenza are based on the use of annual IM-administered inactivated vaccines. Recently, a live attenuated, cold-adapted trivalent IN influenza vaccine, PhlaMus™, has been approved by the FDA. This vaccine provides mucosal immunity and was shown to be safe and well-tolerated.
In healthy pediatric and adult populations [20–22]. However, it is not indicated for certain populations, such as children under age five, the elderly and the immunocompromised [23]. The vaccine must be stored as a frozen liquid and thawed immediately before use in order to maintain potency. Thus, further improvements in influenza vaccination are needed.

The new powder formulation of whole, inactivated influenza virus described here is at least as potent as conventional liquid formulations and may ultimately provide a safe and effective alternative to currently available influenza vaccines, as well as a means to avoid the logistical burden and high cost of cold chain storage. The latter represents a major opportunity for improved vaccine dissemination and cost savings. The World Health Organization (WHO) estimates vaccine wastage in developing countries at roughly 50% [24–26], adding about $120 million to the cost of global vaccination efforts [27]. The major causes of wastage are cold chain failures and limited vaccine shelf life due to thermal instability. Cold chain equipment costs are typically estimated at 1 to 3% of the total cost of immunization [26], adding roughly $90 million in additional costs to global immunization programs. In addition to these economic considerations, cold chain failures can contribute to serious disease outbreaks [28]. Thus, the cold chain requirements associated with liquid vaccines are costly and impractical for developing countries. The introduction of room temperature stable, powder forms of vaccines can be expected to result in significant reductions in vaccine transportation and wastage costs.

Our stability results indicate that powder influenza vaccine formulations as described here are more stable than liquid formulations. The reduction in stability observed under 40 °C and 75% RH storage conditions may have been due to the absorbance of moisture since vials were stored open and fully exposed to the set humidity conditions in this study. The stability enhancement achieved with this powder formulation is likely attributable to the effect of trehalose in stabilizing the protein components of the vaccine. The trehalose:virus ratios described here (99:1 and 500:1, for animal and stability studies, respectively) are well above those typically required for stabilization of biologicals [29]. The excess trehalose here is used as a bulking agent, to simplify powder handling operations. Further stability improvements may be achievable by modifying the formulation with respect to parameters such as trehalose: virus ratio, lyophilization and milling conditions, and choice of mucosal adjuvant. The dry powder formulation and simple delivery platform described herein may provide significant advantages in comparison to current liquid IN and IM products, which require frozen storage or refrigeration.

While dry powder vaccines have the potential to overcome the stability shortcomings of liquid vaccines, to date there have been very few reported studies investigating IN delivery of dry powder vaccines. In contrast, vaccination with liquid formulations delivered via the IN route has drawn considerable attention recently. In addition to influenza, IN vaccination strategies have been investigated for a number of vaccines including HIV, hepatitis B, measles, anthrax, plague, diphtheria, pertussis, tetanus, bacterial meningitis, respiratory syncytial virus, rotavirus and others [30,31]. In many cases, increased local and systemic immune responses have been demonstrated. In most of these studies, only liquid formulations were evaluated. Anderson et al. showed that a powder form of rinderpest virus elicits protective immune responses in cattle [5]. In addition, Illum et al. reported increased serum IgG and nasal IgA titers following IN delivery of a pertussis/chitosan powder vaccine to guinea pigs [7]. In these studies, however, there was no comparison to standard parenteral routes. Furthermore, the powders used in these prior studies were not fully characterized and there was no description of a device suitable for reproducible IN powder delivery in humans.

LiCalis et al. have developed a pulmonary powder delivery device and characterized powder formulations of measles vaccine [6,8]. However, the vaccine was developed specifically for pulmonary rather than IN delivery. Our study is the first to demonstrate the generation of strong nasal mucosal and systemic immune responses using the combination of an IN delivery platform and powder formulation of influenza vaccine. This combination of delivery device and formulation is likely to be applicable to other vaccines for which mucosal immunity is desired. In preliminary studies, we have observed occasional antigen-specific IgA responses in vaginal fluid of rats following IN delivery of these vaccine powders (data not shown), consistent with the observation that application of immunogens to one mucosal inductive site can elicit antibodies in adjacent or physiologically-related effector sites [32]. The nasal IgA responses elicited by IN powder delivery in this study were more variable than those induced by the liquid vaccine. This variability may be due, at least in part, to the variability in particle size distribution and particle morphology observed for these powders, leading to variability in emitted dose. Additional work to further optimize the formulation is underway to address this issue.

Most IN vaccines are unable to elicit immune responses without an adjuvant. Enterotoxin-based adjuvants, such as cholera toxin or Escherichia coli heat-labile enterotoxin, are commonly used as mucosal adjuvants in animal studies. However, potential toxicity in humans is a major concern for such adjuvants. Other potential adjuvants such as nanoparticles, microspheres, synthetic lipopeptides, cytokines, and CpG-containing oligonucleotides have also been studied [33–37]. Chitosan has been proven to be nontoxic and used as an IN bioadhesive in preclinical and clinical studies to increase the bioavailability of drugs and boost mucosal immune response [7,38–40]. The mucosal adhesive properties of chitosan enhance the interaction with the nasal mucosal epithelia, thus extending residence time at the site of action [7,41]. In addition, chitosan transiently opens the tight junctions of the nasomucosal membrane [7,42]. These two features are likely to contribute to the adjuvant properties of chitosan observed in this study. It would be desirable to obtain a direct comparison of the performance of dry powder and liquid forms of whole...
inactivated influenza, both of which contain an equivalent mucosal adhesive. Due to very low solubility of the chitosan at or near neutral pH, it was deemed that the low pH needed for maintenance of a 2.8 wt.% chitosan solution would not be suitable for use with the whole inactivated influenza virus used in this study. Doing so would increase the likelihood of hydrolysis of hemagglutinin and other viral proteins at low pH. It may be possible to prepare lower molecular weight chitosan, or chitosan derivatives with enhanced solubility that are suitable for such use. However, it is not known whether the antigen presentation characteristics of the current study would be obtained with such a chitosan. Our results demonstrate that chitosan increases the immune response to an IN administered influenza vaccine in powder form.

In summary, this study suggests a novel approach to vaccination using a dry powder formulation and IN delivery platform specifically designed for single dose, non-invasive administration. The prospect of a low dose, stable, portable IN vaccine delivery system has the potential to expand the use of the IN vaccination route and improve the outcomes achieved in mass vaccination programs resulting in substantial health and economic benefits.

Acknowledgements

The authors would like to thank Noel Harvey and Jim O’Connell for technical advice and assistance in manuscript preparation, Matthew Mitchell for statistical support, and Pat McCutchen for layout and preparation of figures.

References


