AAPS PharmSciTech 2007; 8 (4) Article 81 (http://www.aapspharmscitech.org).

Novel Dry Powder Preparations of Whole Inactivated Influenza Virus for Nasal Vaccination

Received: August 11, 2007; Final Revision Received: September 20, 2007; Accepted: September 24, 2007; Published: October 12, 2007

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ABSTRACT

The purpose of these studies was to enhance mucosal and systemic antibody production in response to increased local residence time of a whole inactivated influenza virus administered as a dry powder nasal vaccine formulation. Spray-freeze-drying (SFD) particles suitable for nasal delivery were characterized for physico-chemical properties and stability. Mucoadhesive compounds (MA) were characterized for their effects on nasal residence time of vaccine powders in rats compared with published in vitro data and elicited immune responses. SFD particles (D_{50} = 26.9μm) were spherical with a specific surface area of 1.25 m^2/g. Thermal analysis indicated SFD powders were amorphous and demonstrated improved stability with respect to liquid formulations under various storage conditions. In vitro physico-chemical studies and in vivo scintigraphic imaging experiments indicated sodium alginate (SA) and carboxymethylcellulose-high molecular weight (CMC-HMW) powder formulations most significantly increased residence time in Brown Norway rats. Intramuscular delivery provided equivalent serum antibody titers to intranasal (IN) powder without MA, in the presence of CMC-HMW, SA, and hydroxypropyl methylcellulose (HPMC-HMW) after initial dosing and all formulations except IN powder with chitosan after boosting. IN liquid provided equivalent serum antibody titers to all IN powders after the initial vaccination and significantly greater serum antibody titers than IN powder with chitosan after boosting. Trends were consistent between residence time studies and immune response; however, no statistically significant differences between powder and liquid formulations were observed. It was concluded that enhanced serum and mucosal antibody responses were elicited by a dry powder nasal vaccine, specifically, administered in the presence of sodium alginate.

KEYWORDS: intranasal, powder, influenza, vaccine, mucoadhesive, spray-freeze-drying.

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INTRODUCTION

There is growing interest in the development of stable powder vaccine formulations and delivery technologies to overcome refrigerated storage and distribution (ie, "cold chain") requirements associated with liquid-based vaccine stability and delivery. Dry powder formulations are potentially superior to liquid formulations in that they do not support microbial growth and are more stable, eliminating the necessity of the cold chain, thereby facilitating mass vaccination particularly in the developing world.[1-5] There is an urgent need not only to overcome cold chain requirements (for improved stability), but also to provide single-use, nonrefillable delivery technologies that require minimal training. Intranasal administration offers protection from influenza, since the virus uses the nasal route of entry to the host and intranasal (IN) vaccine elicits both a local and systemic immune response. This approach may ultimately provide a safe and effective alternative to the currently available influenza vaccines.

Earlier work was completed on a powder vaccine formulation suitable for nasal delivery that was obtained by lyophilization followed by milling and sieving under optimized conditions.[6,7] However, when delivered in vivo, the nasal immunoglobulin A (IgA) responses elicited by IN powder delivery were more variable than those induced by the liquid vaccine.[7] This variability may be explained, in part, by the differences in particle size distribution and morphology observed for these powders, leading to less reproducibility in emitted dose. Milling of lyophilized cakes with large pores produced from long channels formed by the crystallization and removal of water resulted in the production of needle-like particles, which were difficult to blend uniformly with other excipients. Alternative methods of manufacture, such as spray-freeze-drying (SFD) could potentially improve powder performance.

SFD, which combines the methods of spray-drying and freeze-drying, was developed recently.[8-13] This process is initiated by spraying liquid droplets, usually aqueous solutions, into liquid nitrogen, thus freezing them. The frozen particles are lyophilized at low temperature and pressure, eliminating the potential for heat damage and degradation of the solute. SFD frequently produces large porous particles with different aerodynamic properties than spray-dried particles.[11] Ideally, SFD particles are spherical and in a narrow particle size range. They rarely require further particle

size reduction (if required this is limited to deaggregation), minimizing the potential loss of activity, and rendering them suitable for blending with an added mucoadhesive compound (MA).

Residence time on the nasal mucosa is a determinant of effectiveness of vaccine delivery and response.[14] To afford protection, vaccines must present antigen to the target nasal-associated lymphoid tissue (NALT). Mucoadhesive compounds have been studied to prolong the residence time on a mucosal surface[14-21]; however, most of these systems use liquid formulations containing MA in solution. Powder systems require dissolution/hydration to occur for the system to be effective in impeding mucociliary clearance. Release of the active component from a powder to the nasal cavity must take into account several factors, including wettability, dissolution rate, and the interaction between adjacent MA particles and mucus.

The present work involved the manufacture and development of a dry-powder, inactivated, heat-stable influenza vaccine for nasal delivery that was intended to overcome the shortcomings of currently marketed influenza vaccines (cost of administration, need for refrigeration). Powders were manufactured by SFD and evaluated for their physico-chemical properties and storage stability. Since the antigen used in this study is a whole inactivated virus, the term stability is used to indicate retention of bioactivity. Mucoadhesive compounds were evaluated based on their potential ability to increase the residence time in the nasal cavity and, therefore, increase the immune response elicited by a nasal vaccine. It was proposed that maximal mucosal and systemic antibody production would be elicited by a dry powder nasal vaccine formulation containing whole inactivated influenza virus by increasing the local residence time.

Materials and Methods

Materials

Whole inactivated influenza virus (WIIV, H1N1 strain, A/PR/8/34, Charles River SPAFAS, North Franklin, CT; Lot#4-PRI000901, 2 mg/mL) was supplied as a liquid preparation. D-(+)-trehalose dihydrate (referred to as trehalose), hydroxypropyl methylcellulose (MW = 10 kD, HPMC LMW or MW = 90 kD, HPMC-HMW), carboxymethylcellulose sodium (MW = 90 kD, CMC LMW or MW = 700 kD, CMC-HMW), and sodium alginate (SA, 200–400 cps) were purchased (Sigma Chemical Co, St Louis, MO). Chitosan (Chit, MW= 161 kD, degree of deacetylation = 92%) was graciously donated (Vanson HaloSource, Bothell, WA).

SFD Powder Manufacture

Aqueous solutions containing 10% solids (trehalose alone for residence time studies or 1:1000 WIIV:trehalose for immune response studies) were passed through a sprayer (**7** mm, 2-fluid nozzle diameter, Büchi Mini Spray-Dryer, B-191, Flawil, Switzerland) at 10 mL/min with atomizing nitrogen gas (back pressure 1 atm) to deliver the suspension at 500 L/hr. Droplets collected in aluminum trays containing liquid nitrogen were lyophilized (manifold temperature –55°C, pressure 25 mtorr, 72 hours, Kinetics Flexi-Dry, Kinetics Thermal Systems, Stone Ridge, NY).

Physico-chemical Characterization

Particle Morphology and Size Determination

Powders on double-sided adhesive carbon tabs in representative areas of stubs were photographed (n = 3).[6] Each powder was suspended in 2% Span 80 in light mineral oil and subjected to laser diffraction particle size analysis (n = 3, Series 2600c, Malvern Instruments, Worcester, UK).[6]

Flow Property Assessment

Bulk/tapped densities and static angle of repose measurements were conducted for sieved fractions of trehalose, bulk and sieved MA, and SFD powders (n = 3). Density measurements used ~10 mL of powder poured into a graduated cylinder.[22] Carr's compressibility index (CCI) is calculated from the bulked and tapped density measurements. Static angle of repose measurement used ~10 mL of powder poured through a glass funnel onto a flat collection surface where the angle to the horizontal was measured.[23]

Surface Area Measurement

Single-point surface area analyses of sieved trehalose (1.5 to 1.75 g) and SFD (0.5 to 0.75 g) powders (Quantasorb Jr, Quantachrome Corp, Boynton Beach, FL) were performed. Weighed samples were degassed (25 mtorr, 60°C, 5 hours with nitrogen) followed by repetitive adsorption and desorption of the adsorbate (30% nitrogen in helium).

Thermal Analysis

Moisture content determination was conducted (Mettler LJ16 Moisture Analyzer, Mettler-Toledo, Columbus, OH). Each sample was loaded onto an aluminum tray coating the bottom with a thin layer of powder, then heated to 150°C (n = 3). Final versus initial powder weight was the water content in the sample expressed as a percentage of the total weight. Differential scanning calorimetry (Perkin Elmer DSC 6, Wellesley, MA) was performed. SFD powders were pretreated at 60°C for 5 hours to determine if the powder manufactured was amorphous. Analysis was performed between 50°C and 250°C. Ramp rates of 10°C per minute for bulk and 10°C and 40°C for SFD trehalose were employed.

Stability (Retention of Bioactivity)

WIIV was resuspended in HEPES-saline at a concentration of 2 μg/mL. The hemagglutination assay (HA) titer for the WIIV, as provided by the manufacturer, was 1:131,072 for 0.05 mL. Liquid preparation of WIIV from the vendor was diluted to 2 μg/25 μL with sterile saline and sealed in 1.7-mL centrifuge tubes. About 5 mg total 25-μm SFD powder for rat use containing 5 μg/5 mg trehalose was packed into each gelatin capsule (#3, Capsugel, Greenwood, SC) and sealed in a glass scintillation vial. Vials containing liquid or powder samples were stored at 37°C and 80% relative humidity (RH), 23°C and 40% RH, or 4°C and 50% RH. Samples were taken at intervals, for HA titers (compared with starting sample, 0) up to 12 weeks (n = 2). Powder was reconstituted with saline to 1 mg influenza virus/mL, based on hemagglutinin (H) content. Fifty microliters of double serial-diluted inactivated influenza virus samples were added to wells containing 50 μL of 0.5% chicken red blood cells (Charles River SPAFAS) and incubated at room temperature (RT) for 1 hour for the HA assay. The end point HA titer was the reciprocal of the largest influenza vaccine dilution showing complete hemagglutination of chicken red blood cells.

In Vivo Characterization

Animal Care

Female Brown Norway rats (125 to 175 g, Charles River, Raleigh, NC) were kept under standard conditions in Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities under Institutional Animal Care and Use Committee-approved protocol (06–265.0-A) in a controlled temperature (22°C ± 1°C) and light (12-hour light and dark cycles) room with free access to food and water. Prior to use rats were anesthetized by intraperitoneal injection of 40 mg/kg ketamine, 2 mg/kg xylazine, and 0.75 mg/kg acepromazine.

Residence Time Study

Liquid saline formulations or 1% MA in saline containing insoluble ^{99m}Tc-sulfur colloid (^{99m}Tc-SC) were delivered (25 μL) into the right nostril of an anesthetized rat. To label powder formulations, saline containing insoluble ^{99m}Tc-SC was centrifuged at 13 201*g* for 10 minutes. Supernatant was removed and the pellet was washed 3 times with ethanol. The pellet was allowed to dry before being blended with the rat SFD trehalose (with or without a MA) with a mortar and pestle. Five-milligram quantities were placed in gelatin capsules.

Powder formulations were delivered using compressed air (20 psig), by solenoid valve and 200 μL pipette tip (Eppendorf, Westbury, NY) containing the contents of 1 dosage capsule) to the right nostril of the anesthetized rat. The time points analyzed were 5, 15, and 120 minutes (n = 3) and 30, 60, and 180 minutes (n = 1). Rats were euthanized following cardiac puncture by CO_2 inhalation and nasal washes were collected by cannulating the trachea and rapidly instilling 4 aliquots of 1.5 mL of sterile saline. Fluid passed from the trachea through the nostrils was collected and analyzed in a radioisotope calibrator (Model CRC-4, Capintech, Inc, Pittsburgh, PA). Estimates of dose were adjusted for radioactive decay.

Residence time differences were assessed by regression analysis (SigmaPlot, Systat Software, Inc., San Jose, CA). A 2-compartment model was employed because there was clear evidence of rapid removal of radioactivity from the nasal cavity due to unhindered clearance followed by a gradual elimination attributable to material sequestered in some manner, perhaps adhered to mucus or epithelium. The particle size (650 ± 60 nm) of the ^{99m}Tc-SC was too large to be absorbed through the nasal epithelium; the only means of elimination was mucociliary clearance from the nasal cavity and ingestion. The equation for modeling clearance was**:**

$$y = ae^{(-bt)} + ce^{(-dt)} \quad (1)$$

where y is the radioactivity remaining and t is the time in minutes. The model was fitted to the average value of replicates and each individual point.

Vaccine Evaluation

Rats (n = 6/group) were immunized and boosted (intramuscularly [IM] 2 μg/100 μL liquid, IN 2 μg/25 μL liquid or 5 μg/5 mg powder on days 0 and 21). The actual dose delivered to the animal was 2 μg. SFD WIIV/trehalose was prepared with 3% wt/wt CMC-HMW, SA, chitosan, or HPMC-HMW.

Sample Collection

Blood samples obtained, under anesthesia; from the tail artery 3 weeks after vaccination were centrifuged (13 201*g* for 10 minutes); serum supernatant was removed and stored at −80°C. Two weeks following the boost, rats were euthanized by CO_2 inhalation, following cardiac puncture. Nasal washes were collected, by intubating the trachea and rapidly instilling 1 mL of sterile saline, and subsequently stored at −80°C.

Quantification of Antibody Response

Whole virus-specific antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). Plates (96-well, Nalgen 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 hour with phosphate-buffered saline (PBS) Tween 20 (PBST; Sigma) containing 5% nonfat dry milk. After washing, serial dilutions of sera or nasal wash (100 μL/well) were added and incubated at 37°C for 1 hour. Plates were washed again, then incubated at 37°C for 45 minutes with horseradish peroxidase conjugated secondary antibodies: goat anti-rat Ig (H^+L) (Southern Biotechnology Associates, Inc, Birmingham, AL) or goat anti-rat IgA (Bethyl Laboratories, Montgomery, TX). Plates were developed by incubating for 30 minutes at room temperature with 3,3′,5,5′-tetramethyl benzidine substrate (Sigma). After stopping the reaction using 0.5 M H_2SO_4, plates were read at 450 nm. The end point titers were defined as the highest reciprocal dilution of sera or nasal wash yielding an OD_{450} value at least 3× background obtained using samples obtained before immunization. All standards, samples, and controls were analyzed in duplicate.

Statistical Analyses

Analysis of variance was performed on the logarithmic transformation of vaccine titer values at $\alpha = 0.05$ to determine any differences between the dosing groups. For comparisons of 2 means, Scheffé's method was used at $\alpha = 0.05$.

Results and Discussion

Particle Morphology and Size Determination

Figure 1 shows scanning electron micrographs of spherical, porous SFD particles. SFD trehalose particles exhibited areas of surface coating. This coating may be explained by rapid solvent evaporation during the atomization process, transporting dispersed solids to the surface, and subsequent removal of solvent at the surface during lyophilization. However, this mechanism requires further study.

The particle size distribution shown in Figure 2 indicates modes at 4, 10, 20, and 40 μm, obtained by laser diffraction. The volume median diameter of the SFD trehalose powder was 24.9 μm with a span of 1.5 μm. The volume median diameter of the SFD powder was 26.9 μm with a span of 1.3 μm. The volume median diameter of the SFD powder containing WIIV was 24.9 μm, with a span of 1.8 μm. These powders do not exhibit statistically different median sizes.

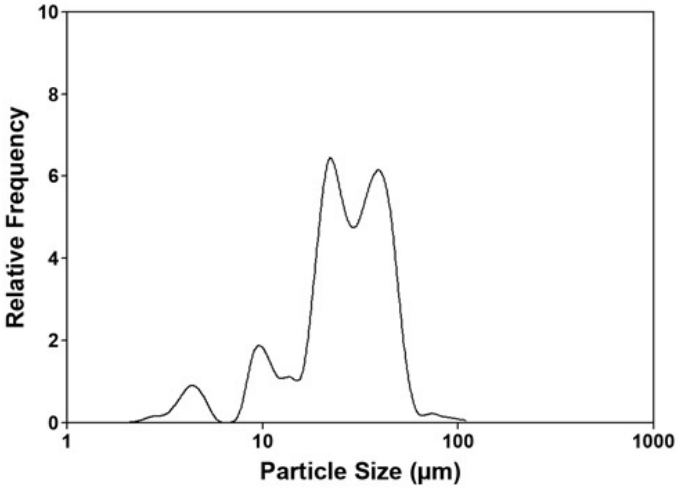

Figure 2. Particle size distribution of spray-freeze-dried (SFD) trehalose for use in rats.

Flow Property Assessment

The flow properties can potentially affect the performance of the final product. Free-flowing powders, in the size range of relevance to nasal delivery, are easily blended and dispersed as aerosols.

The bulk (~0.2 g/mL) and tapped (~0.2 g/mL) densities of the SFD powder were significantly lower than bulk trehalose (Table 1, ~0.5 and ~0.6 g/mL, respectively).

Large cohesive forces acting on a powder result in poor flow properties and render it easily compressible (ie, the higher the CCI). A free-flowing powder has a CCI less than ~20%

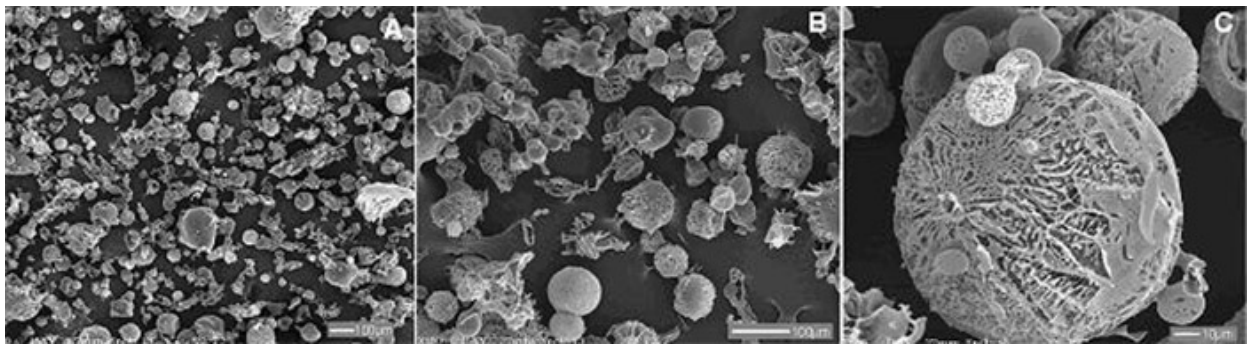

Figure 1. Scanning electron micrographs of spray-freeze-dried (SFD) trehalose for use at (A) ×60 magnification, (B) ×150 magnification, and (C) ×600 magnification.

Table 1. The Flow Properties, Specific Surface Area, and Moisture Content of Spray-Freeze-Dried (SFD) and Sieved Trehalose, Mean (SD), n = 3

Powder	Bulk Density (g/mL)	Tap Density (g/mL)	Carr's Compress. Index (CCI)	Static Angle of Repose (°)	Specific Surface Area (m^2/g)	Moisture Content (%)
SFD	0.17 (0.01)	0.26 (0.01)	34.8 (0.9)	36.1 (2.1)	1.25	5.39 (0.52)
Bulk sieved[1]	0.46 (0.01)	0.60 (0.01)	23.0 (1.3)	21.6 (1.5)	0.49	9.64 (0.06)

to 21%.[24] The sieved trehalose (~20% to 23%) had a lower CCI than the SFD trehalose (~35%). A large static angle of repose is indicative of large cohesive forces within the powder. As a guide, powders with static angles of repose less than 40° are considered to be free-flowing powders; with angles greater than 50°, the powders flow poorly or not at all.[25] Table 1 shows the angles of repose for the SFD and sieved powders studied. The static angle of repose of SFD (~36°) was larger than the sieved trehalose (~20° to 23°), but both would be regarded as free flowing. The sieved trehalose appeared to be free flowing when density and angle of repose data were considered. The SFD data was equivocal since CCI data seemed to contradict angle of repose data. However, it is clear that sieved trehalose appeared to flow more freely than SFD powder.

The ability of a powder to flow is one of the factors that affect the mixing of different materials and formation of a powder blend. Particle size, size distribution, shape, surface texture, surface energy, chemical composition, moisture content, and other factors influence flow.[26] This may be important for the blending of the virus and excipient with the mucoadhesive compound. Also, because nasal delivery requires a fluidization of the powder bed, it is conceivable that flow properties of powders could affect the emitted dose from the device and delivery of the vaccine.

Surface Area Measurement

The specific surface areas of the powders are shown in Table 1. The SFD powder (~1.2 m^2/g) exhibited significantly greater surface area than the sieved trehalose (~0.5 m^2/g). This is consistent with the literature.[9,11,27]

Thermal Analysis

The moisture contents of the trehalose powders are shown in Table 1. Moisture content was low in SFD powder because of the lyophilization process. Lyophilized powders generally have 3% to 5% moisture present. The sieved trehalose, as a dihydrate, has greater moisture content (~10%) than the SFD powders (~5%).

Differential scanning calorimetry scans of SFD powder and bulk trehalose are shown in Figure 3. The characteristic glass transition temperature (Tg) associated with the SFD amorphous solids required removal of the moisture from the powder. Consequently, powders were held at 60°C for 5 hours. SFD trehalose powder exhibits a Tg at 117°C, a crystallization peak at 174°C, and a sharp melting peak at 214°C, consistent with that in the literature.[28] The Tg of untreated SFD trehalose could not be resolved with this instrument, and further tests could be conducted with a modulated differential

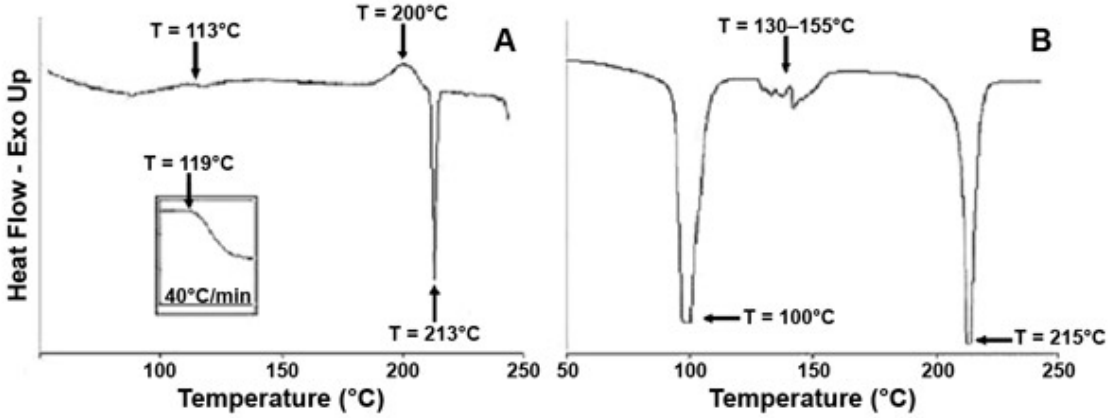

Figure 3. Differential scanning calorimetry of (A) spray-freeze-dried (SFD) and (B) bulk trehalose. After pretreated SFD samples were heated to 250°C at 10°C/min and also scanned at 40°C/min to better resolve the Tg at ~110°C. Bulk trehalose was heated from room temperature to 250°C at 10°C/min.

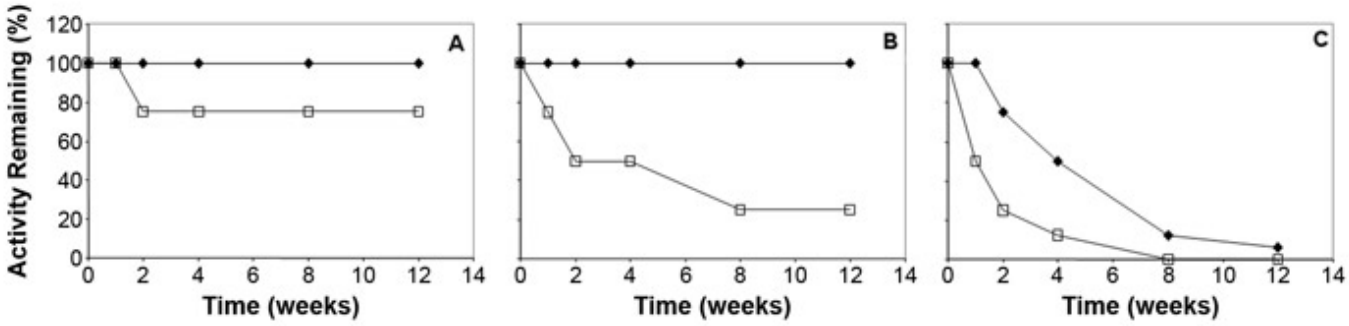

Figure 4. Vaccine stability of liquid (open square) or powder (closed diamond) vaccine stored under (A) 4°C, 50% RH, (B) 25°C, 40% RH, and (C) 37°C, 80% RH at different time points, compared with the week 0 sample. Each data point represents the mean of duplicate samples.

scanning calorimeter. Bulk trehalose dehydrate exhibited a sharp peak at 100°C, indicating removal of unbound surface moisture, at ~120°C, bound water of crystallization was removed. The melting peak can be seen at 214°C. Amorphous powder was produced by SFD and the relatively high Tg indicates that the trehalose should be stable under room temperature storage and handling conditions.

Stability (Retention of Bioactivity)

The storage conditions in these experiments were approximately those employed in regulated studies of refrigeration, room, and accelerated storage (5°C ± 3°C, 25°C ± 2°C, and 60% ± 5% RH, 40°C ± 2°C and 75% ± 5% RH.[29]) as dictated by existing laboratory equipment. At 4°C and 50% RH, powder vaccine retained 100% of HA activity for the duration of the 12 weeks (Figure 4). However, the HA activity of the liquid formulation under these conditions dropped to 75% after week 2. Similarly, at 23°C and 40% RH, the powder vaccine retained 100% of HA activity, whereas the HA activity of the liquid dropped to 50% at week 2 and was further reduced to 25% of prestorage levels at week 8. The powder formulation showed a drop in stability, to 75% at week 2, 50% at week 4, and 6.25% at week 12, over time under the accelerated storage conditions (37°C and 80% RH). The powder formulation was more stable than the corresponding liquid, which lost 100% of activity by week 8. While not ideal, the vaccine storage stability exhibited by the powder formulation was improved with respect to liquid.

The high Tg and low moisture content (< 3%) of the trehalose are 2 important factors in the increased stability of the powder formulation. To ensure the long-term stability of a biological macromolecules, particularly proteins and peptides, as a dried solid, the Tg of the amorphous phase must exceed the planned storage temperature.[30,31] Since water is a plasticizer of the amorphous phase, low residual moisture is needed to ensure stability.[32] The loss of activity seen under accelerated conditions may be explained by the residual moisture and large surface area of the SFD trehalose particles. Greater stability might be achieved by optimization of the freeze-drying process with a shelf lyophilizer to lower the moisture content of the powder. Additionally, greater stability could be achieved with control of surface area as an objective in a designed experiment. Increased surface area has been shown to lead to a decrease in stability of vaccine formulations.[33]

In vivo characterization

Residence Time Study

Correlation coefficients and parameters (a, b, c, and d) related to the nasal clearance following intranasal delivery of various formulations, determined by the regression analysis, are shown in Table 2. All of the curves exhibited good fit ($R^2 > 0.94$). Parameters a and b are representative of initial clearance of the radioactivity from the nasal cavity. They compose the term of a 2-compartment intravenous (IV) bolus equation that represents the distribution phase. With the exception of the liquid SA, the distribution constant (b) was larger for the powder formulations than the liquids. This is surprising because run-off and swallowing of the liquid formulations, unhindered clearance, might be expected to account for a large, immediate decrease in the radioactivity. However, viscosities of 1% HPMC-HMW and CMC-HMW were much greater than 1% SA, which may explain the increase in residence time.[34] Powders least effecting viscosity (trehalose alone and Chitosan with trehalose) cleared more rapidly than other formulations.

Parameters c and d are indicative of mucociliary clearance rate. They compose the term of a 2-compartment IV bolus equation that represents the elimination phase. The elimination constant (d) was very similar for all of the liquid formulations and powder No MA, CMC-HMW, and Chit, while the powder HPMC-HMW and SA were significantly lower. These results are expected because HPMC-HMW and SA exhibited the greatest viscosity, when fully dissolved, and swelling index, respectively.[34] Based on the in vitro results,

Table 2. Correlation Coefficient (r^2) and Parameters Determined by Regression Analysis of 2-Compartment Model Curves Determined by the % Radioactivity Remaining as a Function of Time

	Liquid Formulations				Powder Formulations				
	No MA	HPMC-HMW	CMC-HMW	SA	No MA	HPMC-HMW	CMC-HMW	SA	Chit
r^2	0.99	0.96	0.97	0.97	0.94	0.99	0.94	0.99	0.95
a	43.06	28.41	34.26	16.82	51.88	72.86	43.80	51.19	34.32
b	0.06	0.21	0.09	46.35	480000	0.23	0.56	0.25	13000
c	55.33	72.03	63.95	83.18	48.12	27.34	56.20	48.67	65.68
d	0.003	0.004	0.002	0.006	0.006	0.000	0.003	0.000	0.005
AUC (μCi*min)	8578	9290	10160	9018	5410	5056	7448	8972	7465

the powder CMC would be expected to have a lower elimination constant.

In vitro experiments were potential predictors of the effect of MA on the residence time of liquid and powder preparations in the nasal cavity.[34] Wettability of the powders was determined by placing a drop of buffer on a compact of the MA and measuring the contact angle. Dissolution rate of powders was determined by exposing them to buffer and measuring the viscosity over time with a capillary or cone and plate viscometer in unstirred and stirred systems. Swelling indices indicate the rate and amount of water uptake of the powders and were measured by placing compacts of the powders in buffer and measuring the weight gained by water uptake as a function of time.

The contact angle of the HPMC-HMW and HPMC-LMW (55° and 41°, respectively) were significantly greater than the other polymers, indicating a prolonged dissolution time ($P < .05$). For both the unstirred and stirred systems, the viscosities of the CMC polymers and SA were significantly greater than the HPMC polymers ($P < .05$). The swelling indices of CMC-HMW and SA (5.0 and 4.7, respectively) were significantly greater than the other MA ($P < .05$). Based on these in vitro properties, the rank order of MA on increasing residence time is predicted to be SA, CMC-HMW > Chitosan > HPMC-HMW.

The areas under the curve (AUC) are shown in Table 2. The AUC of the liquid formulations were greater than the powder formulations, with the exception of SA. However, the AUCs except trehalose alone and HPMC trehalose were similar (roughly 8000 to 10 000 μCi*min). The rank of the AUC of the liquid formulations (CMC-HMW > HPMC-HMW, SA > No MA) was consistent with the viscosities of the 1% solutions (HPMC-HMW > CMC-HMW > SA > No MA). However, it should be noted that larger viscosity does not directly correlate to increased residence time (No MA, with respect to, HPMC-HMW). The rank of the powder formulations (SA > CMC-HMW, Chit > HPMC-HMW, No MA) was consistent with the contact angle (Chit < CMC-HMW < SA < HPMC-HMW), viscosity (unstirred at 60 minutes, SA > CMC-HMW > HPMC-HMW > Chit, No MA), and swelling index (SI) at 60 minutes (CMC-HMW, SA > Chit > HPMC-HMW > No MA).

Formulation Evaluation

After the initial vaccination, IM delivery of liquid vaccine provided equivalent serum antibody titers to those obtained by IN powder without MA, CMC-HMW, SA, and HPMC-HMW, and significantly greater serum antibody titers than IN liquid and IN powder with chitosan (Figure 5a, $P < .05$).

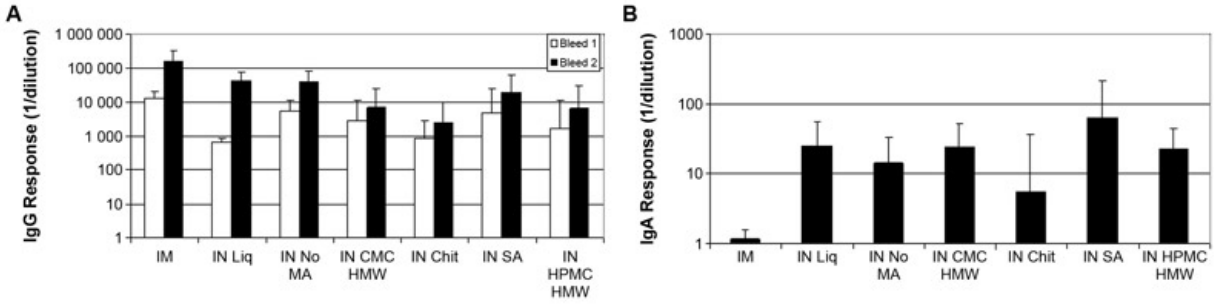

Figure 5. (A) Serum antibody titers (3 weeks after initial dosing [Bleed 1] and 2 weeks after boost [Bleed 2]) and (B) mucosal antibody titers elicited after vaccination of vaccine dosed either intramuscularly (IM) or intranasaly (IN) in saline or IN powder with or without 3% wt/wt mucoadhesive compounds (MA) (n = 6, positive standard deviation).

After boosting, IM delivery of liquid vaccine provided equivalent serum antibody titers to those obtained by IN liquid and IN powder with No MA, CMC-HMW, SA, and HPMC-HMW, and significantly greater serum antibody titers than IN powder with chitosan ($P < .05$). After the initial vaccination, IN liquid vaccine provided equivalent serum antibody titers to those obtained by IN powder without MA, CMC-HMW, SA, chitosan, and HPMC-HMW. After boosting, IN liquid provided significantly greater serum antibody titers than IN powder with chitosan and was equivalent to all other formulations ($P < .05$). All antibody responses elicited were significantly greater than naïve serum ($P < .05$).

Initial studies of the formulation (WIIV and trehalose were freeze-dried, milled, sieved to 45- to 125-μm particles and blended with chitosan) and nasal dosing of a dry powder influenza vaccine to rats has been described elsewhere.[6,7] Antibody responses were elicited by 100 μg powder vaccine that generated strong nasal mucosal and systemic immune responses. The chitosan mucoadhesive powder formulation generated significantly greater antibody titers compared with IM injection, IN liquid, or IN powder without mucoadhesive formulations at this dosing level after initial vaccination. Similar serum antibody titers were seen in chitosan mucoadhesive powder, IM injection, and IN liquid. Comparable nasal IgA titers were generated in the IN groups containing antigen, while no nasal IgA response was seen in animals dosed IM or with excipients alone intranasally.

In the present experiments, similar serum antibody levels to the previous study were achieved after dosing IN liquid 2 μg H after the initial vaccination and boosting and the IM after boosting to the previous experiment. However, a 10-fold higher response was generated by IM injection after initial vaccination than the previous experiment. The IN powder containing chitosan elicited a 10-fold higher response after the initial vaccination, but a comparable response after boosting. One possible explanation for this could be due to a potential adjuvant effect caused by impurities in the chitosan. In the present studies, highly purified (< 2% impurities) chitosan was blended with the WIIV-SFD particles. This chitosan did not require cryo-milling to produce particles in the desired size range. MA used in the present study was assayed to quantify for the presence of endotoxin. There was no more than 0.11 ng of endotoxin present in the MA in any dose of vaccine, an order of magnitude lower than normally administered as an adjuvant.[35] In both the previous and current studies, after initial vaccination and a boost, comparable serum antibody levels were seen when dosed IM, IN liquid, or IN powder with MA. Hemmaglutination inhibition (HAI) titers and IgG isotyping also displayed a similar pattern of response as ELISA (data not shown).

Substantial IgA titers were seen in all rats after intranasal immunization (Figure 5b). All of the nasal IgA titers elicited from nasal formulations were significantly greater than IM injection except for IN powder with chitosan ($P < .05$). There were no statistically significant differences seen between any of the IN formulations. The production of a mucosal immune response in the form of IgA production demonstrates that intranasal delivery of the WIIV will produce both a local and systemic immune response.

One powder, containing SA, was shown to have an increased residence time in the nasal cavity. No statistically significant differences were noted between formulations in eliciting both IgA and IgG responses. However, the trend was that SA elicited the highest IgA response of all treatments including solution. Even as it elicited one of the higher IgG responses, arguably the trehalose alone and liquid treatments elicited greater responses. While trends in the immune responses may be interpreted to support the original hypothesis, powder preparations containing WIIV were not statistically different in their effect.

CONCLUSIONS

Maximal mucosal and systemic antibody production would be elicited by an IN powder with SA, equivalent to IM injection. The experiments described address the potential to overcome cold chain requirements (needed for vaccine stability) and to provide single-use, nonrefillable delivery technologies that require minimal training. An inactivated, heat-stable, unit-dose vaccine was developed that elicited a systemic immune response in Brown Norway rats comparable to, and a local mucosal response superior to, IM injection. The latter observation indicates a benefit when used for influenza prevention, since the virus enters the host by this route. Further development of this approach may ultimately provide a safe and an effective alternative to the currently available influenza vaccines.

REFERENCES

1. Smith DJ, Bot S, Dellamary L, Bot A. Evaluation of novel aerosol formulations designed for mucosal vaccination against influenza virus. *Vaccine.* 2003;21:2805–2812.

2. Anderson J, Fishbourne E, Corteyn A, Donaldson AI. Protection of cattle against rinderpest by intranasal immunisation with a dry powder tissue culture vaccine. *Vaccine.* 2000;19:840–843.

3. LiCalsi C. Christensen T, Bennett JV, Phillips E, Witham C. Dry powder inhalation as a potential delivery method for vaccines. *Vaccine.* 1999;17:1796–1803.

4. LiCalsi C. Maniaci MJ, Christensen T, Phillips E, Ward GH, Witham C. A powder formulation of measles vaccine for aerosol delivery. *Vaccine.* 2001;19:2629–2636.

5. Illum L, Jabbal-Gill I, Hinchcliffe M, Fisher AN, Davis SS. Chitosan as a novel nasal delivery system for vaccines. *Adv Drug Deliv Rev.* 2001;51:81–96.

6. Garmise RJ, Mar K, Crowder TM, et al. Formulation of a dry powder influenza vaccine for nasal delivery. *AAPS PharmSciTech.* 2006;7: article 19.

7. Huang J, Garmise RJ, Crowder TM, et al. A novel dry powder influenza vaccine and intranasal delivery technology: induction of systemic and mucosal immune responses in rats. *Vaccine.* 2004;23:794–801.

8. Costantino HR, Firouzabadian L, Wu C, et al. Protein spray freeze drying. 2. Effect of formulation variables on particle size and stability. *J Pharm Sci.* 2002;91:388–395.

9. Costantino HR, Firouzabadian L, Hogeland K, et al. Protein spray-freeze drying. Effect of atomization conditions on particle size and stability. *Pharm Res.* 2000;17:1374–1383.

10. Gombotz WR, Healy MS, Brown LR, Auer HE, inventors. Alkermes Controlled Therapeutics, Inc., assignee. Process for producing small particles of biologically active molecules. US patent 6569458. May 27, 2003.

11. Maa YF, Nguyen PA, Sweeney T, Shire SJ, Hsu CC. Protein inhalation powders: spray drying vs spray freeze drying. *Pharm Res.* 1999;16:249–254.

12. Carrasquillo KG, Stanley AM, Aponte-Carro JC, et al. Non-aqueous encapsulation of excipient-stabilized spray-freeze dried BSA into poly (lactide-co-glycolide) microspheres results in release of native protein. *J Control Release.* 2001;76:199–208.

13. Carrasquillo KG, Carro JC, Alejandro A, Toro DD, Griebenow K. Reduction of structural perturbations in bovine serum albumin by non-aqueous microencapsulation. *J Pharm Pharmacol.* 2001;53:115–120.

14. Bacon A, Makin J, Sizer PJ, et al. Carbohydrate biopolymers enhance antibody responses to mucosally delivered vaccine antigens. *Infect Immun.* 2000;68:5764–5770.

15. Aspden TJ, Mason JD, Jones NS, Lowe J, Skaugrud O, Illum L. Chitosan as a nasal delivery system: the effect of chitosan solutions on in vitro and in vivo mucociliary transport rates in human turbinates and volunteers. *J Pharm Sci.* 1997;86:509–513.

16. Cheng Y-H, Dyer AM, Jabbal-Gill I, et al. Intranasal delivery of recombinant human growth hormone (somatropin) in sheep using chitosan-based powder formulations. *Eur J Pharm Sci.* 2005;26:9–15.

17. Illum L, Fisher AN, Jabbal-Gill I, Davis SS. Bioadhesive starch microspheres and absorption enhancing agents act synergistically to enhance the nasal absorption of polypeptides. *Int J Pharm.* 2001;222:109–119.

18. Soane RJ, Frier M, Perkins AC, Jones NS, Davis SS, Illum L. Evaluation of the clearance characteristics of bioadhesive systems in humans. *Int J Pharm.* 1999;178:55–65.

19. Soane RJ, Hinchcliffe M, Davis SS, Illum L. Clearance characteristics of chitosan based formulations in the sheep nasal cavity. *Int J Pharm.* 2001;217:183–191.

20. Ugwoke MI, Agu RU, Jorissen M, et al. Nasal toxicological investigations of Carbopol 971P formulation of apomorphine: effects on ciliary beat frequency of human nasal primary cell culture and in vivo on rabbit nasal mucosa. *Eur J Pharm Sci.* 2000;9:387–396.

21. Ugwoke MI, Agu RU, Vanbilloen H, et al. Scintigraphic evaluation in rabbits of nasal drug delivery systems based on carbopol 971p((R)) and carboxymethylcellulose. *J Control Release.* 2000;68:207–214.

22. Martin AN, Bustamante P. *Physical Pharmacy: Physical Chemical Principles in the Pharmaceutical Sciences*. Philadelphia, PA: Lea & Febiger; 1993.

23. Carstensen JT. *Pharmaceutical Principles of Solid Dosage Forms*. Lancaster, PA: Technomic Publishing Co.; 1993.

24. Carr RL, Jr. Evaluating flow properties of solids. *Chem Eng.* 1965;72:163–168.

25. Carr RL, Jr. Classifying flow properties of solids. *Chem Eng.* 1965;72:69–72.

26. Staniforth JN. Powder flow. In: Aulton ME, ed. *Pharmaceutics: The Science of Dosage Form Design.* 1st ed. New York: Churchill Livingstone; 2002:197–210.

27. Yu Z, III, Johnston KP, III, Williams RO, III. Spray freezing into liquid versus spray-freeze drying: influence of atomization on protein aggregation and biological activity. *Eur J Pharm Sci.* 2006;27:9–18.

28. Surana R, Pyne A, Suryanarayanan R. Effect of preparation method on physical properties of amorphous trehalose. *Pharm Res.* 2004;21: 1167–1176.

29. FDA. *US FDA Draft Guidance for Industry. Q1A(R2) Stability Testing of New Drug Substances and Products.* Bethesda, MD: USFDA; 2003.

30. Pikal MJ. Freeze-drying of proteins. In: Cleland JL, Langer R, eds. *Formulation and Delivery of Proteins and Peptides.* vol. 5567. Washington, DC: ACS Symposium Series; 1994:120–133.

31. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational design of stable lyophilized protein formulations: some practical advice. *Pharm Res.* 1997;14:969–975.

32. Carpenter JF, Chang BS. Lyophilization of protein pharmaceuticals. In: Avis KE, Wu VL, eds. *Biotechnology and Biopharmaceutical Manufacturing, Processing, and Preservation.* Buffalo Grove, IL: Interpharm Press; 1996:199–264.

33. Abdul-Fattah A, Truong-Le V, Yee L, et al. Drying-induced variations in physico-chemical properties of amorphous pharmaceuticals and their impact on stability II: stability of a vaccine. *Pharm Res.* 2007;24:715–727.

34. Garmise RJ, Hickey AJ. In vitro evaluation of mucoadhesive compounds for use in a dry powder nasal vaccine. Paper presented at: Respiratory Drug Delivery X; April 23-27, 2006; Boca Raton, FL.

35. Nelson M, Prior JL, Lever MS, Jones HE, Atkins TP, Titball RW. Evaluation of lipopolysaccharide and capsular polysaccharide as subunit vaccines against experimental melioidosis. *J Med Microbiol.* 2004;53:1177–1182.