Optimization of an Alum-Adsorbed Vaccine Powder Formulation for Epidermal Powder Immunization

Yuh-Fun Maa,[1,2,4] Cassandra Shu,[1,3] Mahmoud Ameri,[1,2] Cindy Zuleger,[1] Jenny Che,[1] Jorge E. Osorio,[1] Lendon G. Payne,[1] and Dexiang Chen[1]

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Purpose. To develop stable and effective aluminum salt (alum)-adsorbed vaccine powder formulations for epidermal powder immunization (EPI) via a spray freeze–drying (SFD) process.

Methods. Powder properties were determined using particle size analysis, tap density, and scanning electron microscopy. Alum coagulation was monitored via optical microscopy and particle sedimentation. Protein analysis was determined by the BCA protein assay, SDS-PAGE, and an enzyme immunoassay. *In vivo* immunogenicity and skin reactogenicity were performed on hairless guinea pigs and pigs, respectively.

Results. SFD of hepatitis B surface antigen (HBsAg) adsorbed to aluminum hydroxide or aluminum phosphate using an excipient combination of trehalose/mannitol/dextran produced vaccine powders of dense particles and satisfactory powder flowability and hygroscopicity. This formulation also offered excellent long-term stability to the powder and the antigen. The two most important factors influencing alum particle coagulation are the freezing rate and the concentration of aluminum in the liquid formulation for SFD. The SFD vaccines, when delivered to hairless guinea pigs by EPI or injected intramuscularly after reconstitution, were as immunogenic as the original liquid vaccine. A further study showed that EPI with SFD alum-adsorbed diphtheria–tetanus toxoid vaccine was well tolerated, whereas needle injection of the liquid formulation caused persistent granuloma.

Conclusions. Stabilization of alum-adsorbed vaccine by SFD has important implications in extending vaccination to areas lacking a cold chain for transportation and storage and may also accelerate the development of new immunization technologies such as EPI.

KEY WORDS: alum adjuvant; powder formulation; spray freeze–drying; epidermal immunization; HBsAg; vaccine.

INTRODUCTION

Aluminum salts (alum), aluminum hydroxide and aluminum phosphate, have become the most widely used vaccine adjuvants since their adjuvant effect was discovered in 1926 (1,2). Alum adjuvants may work by establishing an "antigen depot"; i.e., alum-adsorbed antigens persist in the tissue over a long period of time and provide a continuous stimulation of the immune system (2,3). Alum-containing vaccines are normally administered by intramuscular (IM) injection and have an excellent track record of safety in humans.

[1] PowderJect Vaccines, Inc., Madison, Wisconsin 53711.

[2] Current address: Alza Corporation, Mountain View, California 94043.

[3] Current address: Cell Genesys, Foster City, California 94404.

[4] To whom correspondence should be addressed. (e-mail: yuh-fun.maa@alza.com)

Skin is a sensitive immune organ and an attractive tissue for vaccination because of the presence of a large number of antigen-presenting cells, including epidermal Langerhans cells (LC) and dermal dendritic cells (4). Many clinical studies have found that intradermal (ID) injection may allow the use of a smaller vaccine dose than that used for IM injection (5–7). Alum-containing vaccines are currently not administered via the skin route, probably because of the risk of granuloma formation, as seen in some studies (8,9). We have recently reported that epidermal powder immunization (EPI), by delivering antigens to the LC-rich epidermis, is an effective way of raising immune responses in animals (10–13). We hypothesize that EPI with alum-adsorbed vaccines may avoid granuloma formation because the epidermis, the target tissue of EPI, regenerates in a 2- to 3-week cycle.

One of the challenges in testing the hypothesis is to prepare stable dry powder formulations because alum-adsorbed vaccines are notoriously unstable to dehydration when traditional methods are used (14–16). Limited success has been achieved by using a large amount of stabilizers (>99% of the total dry mass) or a low concentration of alum (<0.1% w/v) during dehydration (17,18). We previously reported that the freezing rate plays a critical role in stabilizing alum-adsorbed vaccines during dehydration (15), and spray freeze-drying (SFD), by quickly freezing small droplets in liquid nitrogen, could minimize the alum coagulation (15). SFD is a powder formulation process that has previously been used to generate fragile particles and porous dry powders (19–21). In this study, excipient compositions, alum concentration, and other variables in the SFD process are optimized to produce high-density powders suitable for EPI.

MATERIALS AND METHODS

Materials

Chemicals and excipients used in this study are summarized in Table I. All alum formulations were concentrated to the desired concentration by centrifugation (Allergra 6R centrifuge, Beckman Instrument, Palo Alto, CA) before use.

Methods

Powder Formulations

Alum-adsorbed hepatitis B surface antigen (HBsAg) and diphtheria–tetanus toxoid (dT) vaccines were prepared by SFD as previously described (15). Briefly, the liquid formulation was sprayed into liquid nitrogen using an ultrasonic atomizing nozzle (60-kHz frequency). The frozen particles in liquid nitrogen were transferred to a precooled (–55°C) shelf freeze dryer (Model #TDS2C2B5200, Dura-Stop, FTS System, Stone Ridge, NY). The freeze-drying conditions were set to –25°C for 18 h and 20°C for 10 h, with a shelf temperature ramping rate of 1°C/min and a chamber vacuum pressure of 100 mT throughout the cycle. As a control, the same liquid formulation was freeze-dried (FD) using the same lyophilization cycle without atomization.

Optical Microscopy

Visual analysis of the particles was performed using an optical microscope (Model DMR, Leica, Germany) with 10×

Table I. Chemicals/Excipients Used in the Study

Chemical	Lot	Source	Comment
Aluminum phosphate (Adjus-Phos, 2% $AlPO_4$)	8934	Accurate Chemical and Scientific (Westbury, NJ)	Manufactured by HCI Biosector (Frederikssund, Denmark)
Aluminum hydroxide [Alhydrogel, 3% $Al(OH)_3$]		Accurate Chemical and Scientific	Manufactured by Superflos Biosector (Vedbaek, Denmark)
Aluminum phosphate-adjuvanted DT		CSL Limited (Parkville, Australia)	Bulk containing 5% w/v aluminum phosphate adsorbed with both dT and tT at 563 Lf/mL
Aluminum hydroxide-adjuvanted hepatitis-B surface antigen (HBsAg)		Rhein Amaericana S.A. (Buenos Aires, Argentina)	20 μg HBsAg adsorbed to 0.5 mg of aluminum or 1.5-mg of aluminum hydroxide
HBsAg	9423	Rhein Amaericana S.A.	Provided at the concentration of 2.44 mg/mL
Dextran (MW 10,000 Da)	18H0568	Sigma (St. Louis, MO)	Reagent grade
Sodium lauryl sulfate	17H0459	Sigma	Sodium dodecyl sulfate (SDS); MW = 288 Dalton
Mannitol	127H0960	Sigma	Reagent grade
Trehalose dihydrate	28H3797	Sigma	Reagent grade

eyepiece lens and 10× objective lens. The system was equipped with a Polaroid camera system for image output.

Scanning Electron Microscopy

The external morphology of coated particles was examined using an Amray 1810T scanning electron microscope (Amray, Bedford, MA) (15). Powder samples were first sputter coated with gold using a Hummer JR Technics unit (Pergamon Corporation**,** King of Prussia, PA) before microscopy.

Particle Size Analysis

The mean geometric/aerodynamic diameter of the particles in the volume distribution was determined using a time-of-flight particle size analyzer (Aerosizer, API, Minneapolis, MN) (15). The mean volumetric size was calculated by the software using the density of 1.0 g/mL, and the particle population between 10% (D_{10}) and 90% (D_{90}) was reported for particle size distribution. Each analysis required approximately 3 to 5 mg of the powder sample.

Powder Tap Density

Each powder sample, approximately 100 to 300 mg, was weighed in a 2-mL glass vial (12 × 35 mm, E & K Scientific Products, Cat. # E025010). The vial was gently tapped against the lab bench 20 times. By visual inspection, water of an equivalent volume to that of the powder was placed into an empty vial of the same type. The tap density of the powder sample could be calculated by dividing the powder sample weight by the water sample weight (assuming water density = 1 g/mL).

Alum Gel Coagulation Analysis

The degree of alum gel coagulation was determined by optical microscopy and gel sedimentation. The powder sample was first reconstituted in water to a concentration of 100 mg/mL without agitation. The gel solution was pipetted onto a glass slide and examined under an optical microscope (Model DMR, Leica, Germany). The same gel solution was then loaded into a 15-mL polystyrene conical tube (Falcon, Becton Dickinson, Franklin Lakes, NJ), and the sedimentation rate of the alum gel was monitored.

Protein Assay

The protein assay was performed using the BCA kit from Pierce (Rockford, IL) by following the manufacturer's protocol.

HBsAg Adsorption to Alum Salts

Adsorption of HBsAg to aluminum hydroxide or aluminum phosphate was prepared by slowly adding antigen to the alum suspension diluted in saline (pH 5.5) while stirring. The mixture was gently stirred overnight at room temperature (RT). The pH was then raised to 7.2, and the mixture was centrifuged at 4,500 rpm for 20 min at RT. The supernatant was decanted, and the pellet was resuspended in 0.9% normal saline solution. The amount of adsorbed protein was determined by measuring the remaining protein in the supernatant by bicinchoninic acid (BCA) protein assay. In all experiments, adsorption efficiency was >99% for both aluminum phosphate and aluminum hydroxide.

SDS-PAGE

Coomassie colloidal-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Nu-PAGE gel from Novex (San Diego, CA) (4–12% MES, running buffer, sample buffer, and/or dithiothreitol reducing agent) as previously described (15).

Stability and in Vitro Antigenicity of HBsAg

The stability of hepatitis B surface antigen (HBsAg) powder formulations was investigated after storage at 25°C and 40°C for up to 26 weeks. Each powder sample (5 to 10 mg) was weighed into a HPLC vial, sealed, and incubated in a stability chamber. The potency/antigenicity of HBsAg was determined by a quantitative enzyme immune assay using AUSZYME® Monoclonal kit (Abbott Laboratories, Abbott Park, IL).

Immunization and Serum Collection

Hairless guinea pigs (six animals per group, 8–10 weeks old, mixed sexes, Charles River, Wilmington, MA) were used to assess the immunogenicity of powder formulations because their skin structure and thickness are similar to those of hu-

mans. Immunizations were performed on days 0, 28, and 49. For EPI, 1 mg of powder containing 1 μg of HBsAg adsorbed to 25 μg elemental aluminum was delivered to the left inguinal skin of the animals with a powder delivery device at 40-bar helium pressure as previously described (10). Control animals were immunized with 0.20 mL of liquid vaccine in saline by intramuscular (IM) injection using a 26-gauge needle. Animals received boost immunization (same dose and route) on days 28 and 49. Blood was collected via the carotid blood vessel before each vaccination and 14 days after the final immunization.

Local Reactogenicity Test

Pigs were anesthetized with a 1:1 mixture of Rompun and Telazol. The abdomen of each pig was tattooed on either side of the shot site. Each site received 2 mg powder formulation by EPI. Control sites received intradermal (ID) injection of reconstituted powders or unprocessed liquid vaccine. After immunization, each injection site was inspected weekly by palpation.

Skins of the immunization sites were excised on day 42, fixed with 10% formalin, and embedded with paraffin. Sections of 6 μm thickness were cut, stained with hematoxylin and eosin (H&E), and visualized under a light microscope (Nikon, Melville, NY).

ELISA

The antibody response to HBsAg was determined using a previously described ELISA method (10). The endpoint titers of the sera were determined by four-parameter analysis using the Softmax Pro 4.1 program (Molecular Devices, Sunnyvale, CA) and defined as the reciprocal of the highest serum dilution with an OD reading above the background by 0.1. A reference serum with a predetermined titer was used on every plate to calibrate the titers and adjust assay-to-assay and plate-to-plate variation.

RESULTS

Powder Properties: Effect of Excipient Composition

Appropriate particle size (20–70 μm), high density, and good powder flowability are the most important powder properties for EPI. Of these properties, only particle size is less sensitive to excipient compositions but is determined by atomization conditions. Ultrasonic atomization is a low-shear process and controls droplet size by the frequency of the ultrasound. The 60-kHz frequency nozzle produces particles primarily within the range of 20 to 70 μm. More specifically, the typical particle size distribution as determined by the time-of-flight method is of D_{10} = 25 μm, D_{50} = 40 μm, and D_{90} = 60 μm.

A pilot experiment was conducted to select excipients for SFD. Initial experiments tested the combination of trehalose and mannitol. Increasing the ratio of trehalose to mannitol resulted in increased density of SFD particles because of increased particle shrinkage. Powders had the highest tap density, 0.5 to 0.7 g/mL, as the content of trehalose was increased to 50 to 80% while the total solid content was kept at 30% or higher (≤70% of water). It is noteworthy that the highest solid content achievable is determined by water solubility of the individual excipient. In this case, mannitol has the lowest water solubility of approximately 15% at room temperature. Outside the range of 50 to 80% trehalose, the tap density decreased to lower than 0.5 g/mL. However, powder flowability deteriorated with increasing trehalose content. These powders became highly hygroscopic on exposure to ambient humidity, 40 to 60% relative humidity. In some cases, powders began to liquefy with obvious surface melting detectable under optical microscopy. When the polymer dextran was added to the trehalose/mannitol mixture, the SFD powders were less hygroscopic, and tap density remained high, >0.5 g/mL. Tap density of SFD powders increased linearly with the total solid content of the spraying solution. After evaluating various combinations, trehalose:mannitol:dextran = 30%/30%/40% (T/M/D = 3/3/4 by weight ratio) at 35% of the total solid content was found to be optimal. SEM images for the powder formulation containing aluminum hydroxide (36 μg alum/mg powder, Fig. 1A) or aluminum phosphate (50 μg alum/mg powder, Fig. 1B) show obvious particle shrinkage. This powder had a tap density of approximately 0.6 g/mL, and it showed no sign of particle agglomeration or fusion with moisture content of ~2% after SFD. These powder formulations were free-flowing on processing under the ambient condition of 40 to 60% relative humidity.

Alum Stability: Minimizing Alum Coagulation

Alum coagulation was evaluated under optical microscopy on the reconstituted powders prepared by SFD based on the formulation of T/M/D = 3/3/4 and compared with the coagulation of the freeze-dried (FD) counterparts. No coagulation was observed for the reconstituted SFD aluminum hydroxide formulation (36 μg alum/mg powder, Fig. 1C) and aluminum phosphate powder (50 μg alum/mg powder, Fig. 1D) as shown by the sandy morphology. In contrast, FD counterparts (Fig. 1E, aluminum hydroxide; Fig. 1F, aluminum phosphate) were highly coagulated. The sedimentation rates were also determined on these reconstituted solutions. Alum particles settled from the SFD solutions in the vial, i.e., the top solution became clear, in approximately 5 h, whereas the FD suspensions settled within 1 min.

The effect of excipients on the alum coagulation was studied using SFD and FD powders that were prepared with different excipient combinations (trehalose:mannitol with or without dextran). Alum coagulation was consistently seen with FD, but not SFD, powder formulations. Similarly, varying the ratio of trehalose:mannitol:dextran had little effect on the alum coagulation. Therefore, excipient composition, which had a major impact on the particle density and hygroscopicity, had little effect on the alum coagulation. This is in agreement with our previous observation that alum coagulation is more dependent on the drying rate and droplet formation by atomization than the excipient composition (15).

Alum Stability: Critical Concentration of Aluminum Salt

A series of experiments were conducted to determine the effect of alum concentration in the liquid formulation on alum coagulation during SFD. SFD powders were prepared using liquid formulations of different total solid content, containing aluminum phosphate or aluminum hydroxide at different concentrations. All of these formulations were prepared using

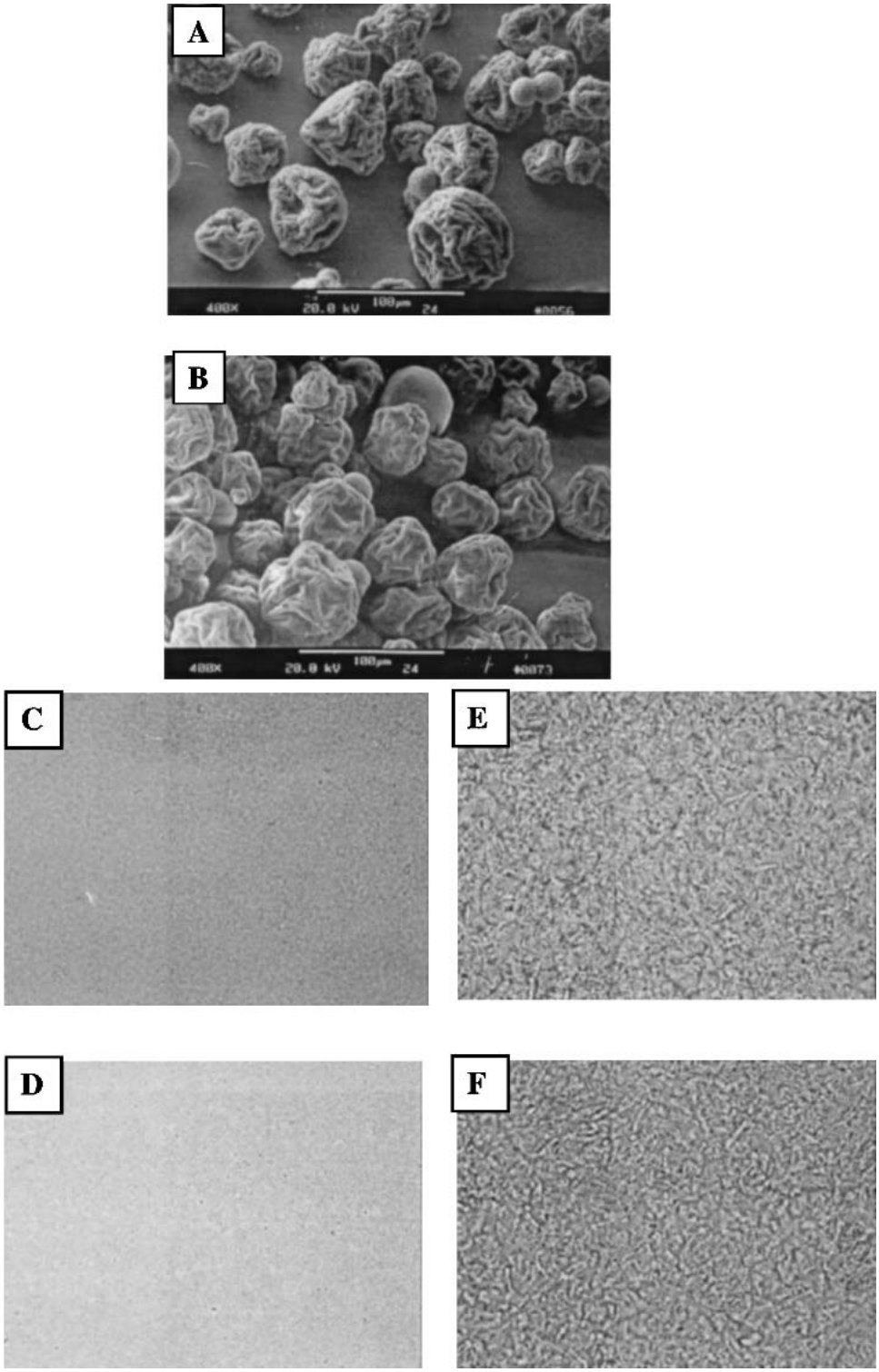

Fig. 1. Scanning electron and optical micrographs of powder formulations. Scanning electron micrographs of SFD powder formulation containing aluminum hydroxide (A) or aluminum phosphate (B). Optical micrographs for reconstituted SFD aluminum hydroxide (C) and SFD aluminum phosphate (D), FD aluminum phosphate (E), FD aluminum phosphate (F). All formulations were based on T/M/D = 3/3/4 at 35% w/w of total solid content. (Magnification: 1 cm represents 250 μm).

the same excipient composition (T/M/D = 3/3/4). Some representative formulations are summarized in Table II. The concentration of alum salt in the liquid formulation before SFD appears to be the most important factor. In the range of 26 to 34 mg/mL of alum salt in the liquid formulation, there is an obvious transition of alum gel into a coagulated form in the reconstituted SFD powders when examined by optical microscopy. Formulation A (26.5 mg/mL) is coagulation free (Fig. 2A), whereas Formulation B (30.3 mg/mL) is slightly coagulated (Fig. 2B). As the concentration was further in-

Table II. Effect of Aluminum Concentration on the Stability of SFD Formulations

Sample	Aluminum (mg/mL liquid formulation before SFD)	Aluminum (μg/mg SFD powder)	Total solid content before SFD (% w/w)	Coagulation
A[a]	26.5	76.0	35	no
B[a]	30.3	76.0	40	partially
C[a]	34.0	114.0	30	yes
D[a]	28.4	114.0	25	no
E[a]	20.1	50.3	40	no
F[b]	11.5	28.7	40	no

[a] Aluminum phosphate was formulated with trehalose/mannitol/dextran = 30%/30%/40%.
[b] Aluminum hydroxide was formulated at the same composition as the aluminum phosphate formulation.

creased to 34.0 mg/mL (Formulation C), alum coagulation became serious (Fig. 2C).

Alum coagulation does not directly correlate with the quantity of alum salt in the powder. Formulations C and D have the same alum content (114 μg aluminum/mg powder), but Formulation D is coagulation-free (Fig. 2D). The difference in the coagulation results from the difference in alum concentration in the liquid suspension for SFD, which was 28.4 mg/mL for formulation D and 34 mg/mL for formulation C. The critical alum salt concentration for the SFD process was 30 mg/mL in the liquid gel. Alum coagulation shows no correlation with the total solids content in the liquid suspension used for SFD (Table II). However, a higher total solids content resulted in more dense SFD particles. SFD of formulations with a total solids content of 35% or higher has produced suitable powder for EPI.

Stability Evaluation

The powder's physical stability and the antigen's biochemical/chemical stability were investigated using the preferred formulation of T/M/D = 3/3/4. Powder samples were stored at 40°C up to 6 months and then analyzed by particle size analysis and optical microscopy. There were no obvious changes before or after incubation in terms of particle size or powder agglomeration/fusion (pictures not shown but identical to Fig. 1A,B). The same formulation composition was used to prepare a powder formulation containing 20 μg of HBsAg per 1 mg powder. Powder samples were then incubated at 25°C up to 6 months or 40°C up to 2 months. As determined by the AUSZYME assay, the potency/antigenicity of active HBsAg remained stable over the duration of incubation at both temperatures (Fig. 3) considering the variation of the assay. The chemical stability of HBsAg was determined using SDS-PAGE. No additional bands, compared to the untreated HBsAg, were observed in either the nonreducing or reducing conditions (gels not shown but identical to those in Ref. 15).

Immunogenicity Studies

The immunogenicity of SFD HBsAg–aluminum phosphate and HBsAg–aluminum hydroxide was compared in hairless guinea pigs. The formulations containing aluminum phosphate and aluminum hydroxide (E and F in Table II, respectively), which are widely used in commercial vaccine products, were prepared at a HBsAg:aluminum ratio of 1:25 as in the commercial vaccine. These two formulations were administered to guinea pigs by either EPI with dry powders or IM injection after reconstitution with water. All groups showed a significant increase in the antibody response after the first and the boost vaccinations (6 and 9 weeks after prime, respectively) were administered (Fig. 4). The antibody titers in the animals vaccinated by IM injection with the reconstituted powders were equivalent to that induced by the original liquid vaccine ($p > 0.05$), suggesting that SFD preserved the adjuvant activity of alum. Animals vaccinated by EPI developed significant antibody responses, and the titers are comparable to that elicited by IM injection of the unprocessed liquid vaccine ($p > 0.05$). The two alum salts appear to be equally immunogenic when delivered as dry powders in the skin.

Local Reactogenicity

The local reactogenicity to EPI with SFD alum-adjuvant dT vaccine at the immunization sites was examined and compared with that of ID injection. The domestic white pig was chosen as the animal model for this test because its epidermis is structurally similar to that of the human. Both EPI and ID injection with alum-adsorbed dT caused erythema responses. The size of the erythema area is greater and more intense for EPI, but erythema completely resolved within 48 h. The site of EPI was yellowish for an additional 2 to 3 days but then returned to its normal color. The site of EPI became visually indistinguishable from normal skin 7 days posttreatment. EPI with aluminum hydroxide or aluminum phosphate did not result in granuloma by visual examination (Table III). In contrast, most of the ID injection sites had a solid lump that could be detected by palpation starting from day 7 and until the end of the study (Table III). At the end of the study (42 days posttreatment), the size of the lumps ranged around 0.3 to 0.5 mm in diameter and 0.2 to 0.3 mm in thickness. Histologic examination of the EPI sites 42 days after treatment showed a skin structure (Fig. 5B) and cellular composition (Fig. 5C) that resembled normal skin (Fig. 5A). The lumps from the ID injection sites were typical granulomas composed of inflammatory cells with a connective tissue capsule (Fig. 5D). The inflammatory cells surrounded by a capsule of connective tissue completely replaced the normal tissue. A closer examination showed that macrophages were the main type of infiltrating cells (Fig.5E). Both aluminum hydroxide and aluminum phosphate induced granuloma when introduced by ID injection. EPI clearly offers a unique safety advantage over ID injection.

DISCUSSION

A powder formulation is the product of a drug/excipient composition being subjected to a particle-formation process. Therefore, particle formation plays a pivotal role in powder formulation as the technology platform. Selection criteria of a powder-formation process normally include simplicity, efficiency, scalability, and compatibility with various drug molecules/excipients. More importantly, the selected process needs to produce particles of desired properties for specific applications. Among many powder-formation methodologies

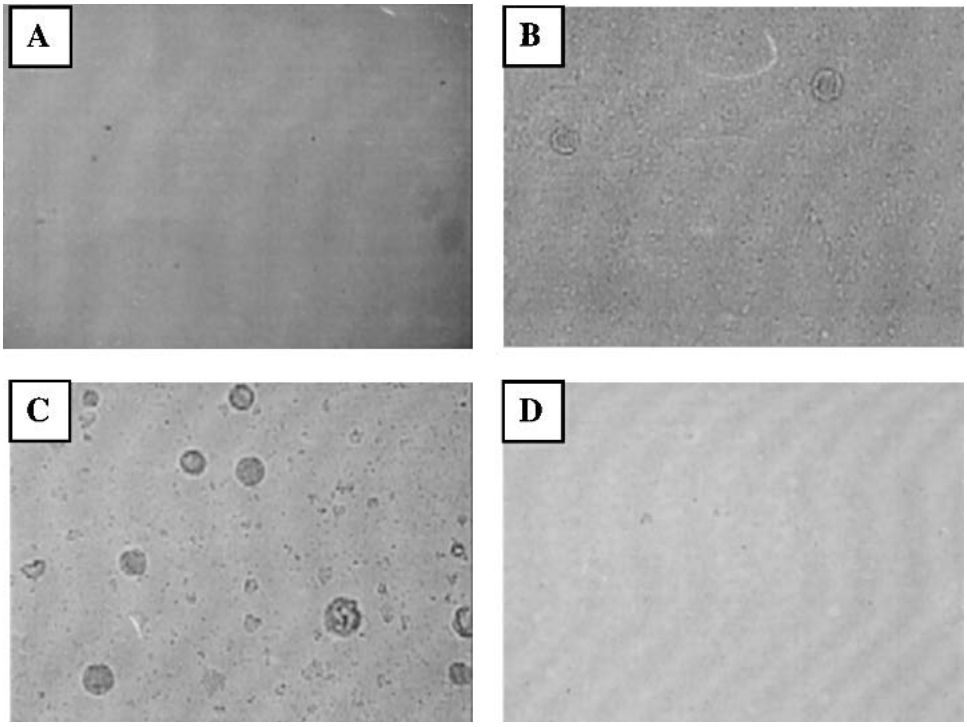

Fig. 2. Optical micrographs of reconstituted SFD powder. Formulation information is summarized in Table II, where aluminum phosphate was formulated with T/M/D = 3/3/4 containing polysorbate 80 at 0.5% of the total solid. Reconstituted Formulation A (A); reconstituted Formulation B (B); reconstituted Formulation C (C); and reconstituted Formulation D (D) (Magnification: 1-cm representing 250 μm).

reviewed recently (22), SFD is a relatively new technology but is the only effective approach for producing alum-adsorbed vaccine powder formulations because of the extreme fast freezing phenomenon. Fast freezing is the most important parameter in minimizing alum particle coagulation (15). However, SFD is not known to produce dense particles, which is important for effective skin penetration by EPI.

Optimization of Particle Properties: Particle Shrinkage and Excipient Effect

FD normally produces a dry cake of the same volume as the original solution. It is intuitive that the higher the solid content of the spraying solution, the denser the particle because it has more mass per unit volume. However, increasing the solids content alone is not sufficient to achieve the desired density without particle shrinkage because the solids content is limited by the solubility of each excipient present in the solution. Further, even if the 50% solids content (i.e., equal weight of solid and water) were achievable, the density of particles without shrinkage might be only half of that of the

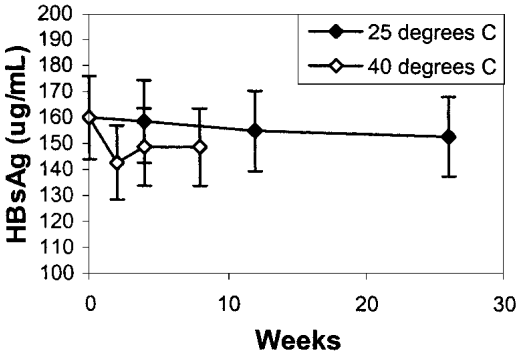

Fig. 3. Biochemical stability of HBsAg in the SFD dry powder formulation. The SFD HBsAg powders were prepared using T/M/D = 3/3/4 at 35% w/w of total solid content. The powder was incubated at either 25°C or 40°C. Samples were taken at different time points, and the HBsAg was determined by the AUSZYME assay.

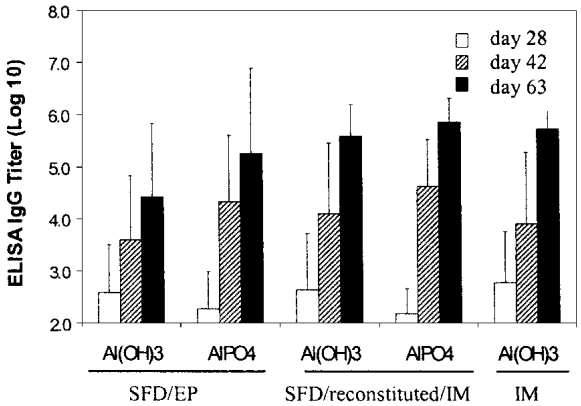

Fig. 4. Immunogenicity of HBsAg adjuvanted with either aluminum hydroxide or aluminum phosphate in guinea pigs. Groups of animals were vaccinated with a SFD formulations containing 1 μg HBsAg/25 μg aluminum as $Al(OH)_3$ or $AlPO_4$ by EPI or by IM after reconstitution. Control animals were vaccinated IM with the liquid commercial Hepatitis B vaccine containing 1 μg HBsAg/25 μg aluminum as $Al(OH)_3$. Animals received boost immunization (same dose and route) on days 28 and 49. Sera samples were collected before each immunization and 14 days after the last immunization and tested for total IgG titers using ELISA.

Table III. Granuloma Formation in Pigs following the Administration of Alum-Adjuvant dT[a]

Formulation	Alum	Route	Granuloma sites out of a total ten sites					
			D7	D14	D21	D28	D35	D42
Liquid A	$Al(OH)_3$	ID	10	8	8	7	7	7
Liquid B	$AlPO_4$	ID	10	10	10	10	8	8
Powder A	$Al(OH)_3$	EPI	0	0	0	0	0	0
Powder B	$AlPO_4$	EPI	0	0	0	0	0	0

[a] Each site was treated with 500 μg of alum-adsorbed dT by ID or EPI on day 0. Granuloma formation was initially determined by weekly palpation for 6 weeks and then confirmed by histology on day 42.

fully shrunken particle. Therefore, producing dense particles by SFD appears to be a daunting task because particle shrinkage is required when the voids left behind by sublimation of ice crystals collapse during drying. Such shrinkage should occur only inside the particle at a micro level. If shrinkage occurs at a macro level, particles might fuse together or completely collapse, losing the powder characteristics.

Freeze-drying conditions and excipient composition may be the two most important factors affecting particle shrinkage during FD. In an earlier study (23), annealing trehalose-based formulations at high temperatures around 0°C before primary drying induced a "shrunken" particle surface morphology. The authors attributed this phenomenon to the collapse of the freeze-concentrate structure during the primary and/or secondary drying, particularly for formulations containing excipients that would soften the mixture and promote flow of the solid phase. It was found that the addition of mannitol and/or dextran is particularly effective in plasticizing the mixture and promoting particle shrinkage. However, the potential risk of excessive collapse or ice melting during annealing prompted us to avoid using this strategy, so we evaluated excipient composition as the primary approach for particle property optimization. Tap density was used to estimate particle density because there are no accurate and reliable methods available at this time. Tap density could correlate with particle density if particle size/distribution and particle shape/morphology are similar. In this study, all formulations were produced under the same atomizing conditions, and the resulting particles are spherical.

The composition of the excipients was found in this study to have a strong influence on particle shrinkage and thereby on particle density, and it also affected other properties such as powder flowability/hygroscopicity. The basic requirement for excipient selection is that these excipients must be parenterally approved compounds and are commonly used in freeze-drying protein formulations. Amorphous sugars such as trehalose and sucrose are commonly used as protein stabilizers, and they are in general known to be soft and hygroscopic in the solid state. These characteristics would be particularly true for a SFD particle of a porous nature as a result of high specific surface area, which would render the solid product unstable in terms of poor powder flowability on adsorbing moisture. Adding bulking agents such as mannitol and dextran improves powder flowability as the hygroscopicity is reduced. In addition, mannitol and dextran improve other particle attributes such as particle strength/integrity required to sustain the impact on contacting the skin for EPI.

Stability Evaluation

Good powder flowability and low hygroscopicity are critical to the long-term stability of the powder. Sticky particles and powders that are prone to adsorb moisture tend to grow into large agglomerates and lose the original particle properties during processing and storage. The physical stability of the T/M/D = 3/3/4-based powder was demonstrated by storing the powder at 40°C up to 6 months. After storage, particle morphology and size were unchanged, and the powder remained free flowing.

HBsAg instead of alum-adsorbed HBsAg was evaluated for biochemical/chemical stability in this study because HBsAg adsorbed to alum surface might be less influenced by the formulation process compared to the unbound, free HBsAg. Although the combination of trehalose, mannitol, and dextran was used primarily from the powder property perspective, these excipients have been extensively used as protein stabilizers for freeze-drying. The combination of disaccharide and dextran was reported to offer excellent stability to actin (24). Amorphous trehalose or sucrose is capable of maintaining a high level of native protein conformation during freeze-drying (25). The preservation of antigen conformation, especially the epitopes or antigenic determinants, is imperative for stimulating protective immune responses. The addition of dextran increased the formulation's glass transition temperature without compromising the stability of alum and alum-adsorbed vaccines, as shown in Fig. 2. Mannitol served primarily as a bulking agent for improving particle integrity and powder flowability. Despite mannitol's tendency to crystallize on freeze-drying, it may only partially crystallize in this formulation where the mannitol content was <30%. The remaining amorphous mannitol would serve as a stabilizer similar to amorphous trehalose (26).

Alum Stability

We previously proposed a "minimal volume" hypothesis to address alum coagulation (15). We found that alum particle coagulation is inevitable when alum particles came into close approximation with one another, i.e., in the concentrated state. However, antigen molecules adsorbed to alum could still be released on reconstitution and remain immunogenic as long as the scale of alum coagulation was minimized. We determined that the freezing rate is the most critical parameter in minimizing alum particles from large-scale coagulation during freeze-drying. Faster freezing causes a greater rate of nucleation, a lower rate of ice crystal growth, and the formation of smaller ice crystals. The tiny ice crystals divide the alum-containing freeze concentrate into much smaller compartments as compared to the compartments formed by large ice crystals generated by slower freezing rates. Based on this concept, SFD was found to achieve the greatest freeze rate possible by atomizing alum-containing liquid formulation into liquid nitrogen (–196°C). A simple mathematical model based on a steady-state heat-transfer condition estimated that droplets of this tiny volume (4.7×10^{-9} cm^3 for a droplet of 30-μm in diameter) would freeze in a few milliseconds in liquid N_2, which is about 3 orders of magnitude faster than the

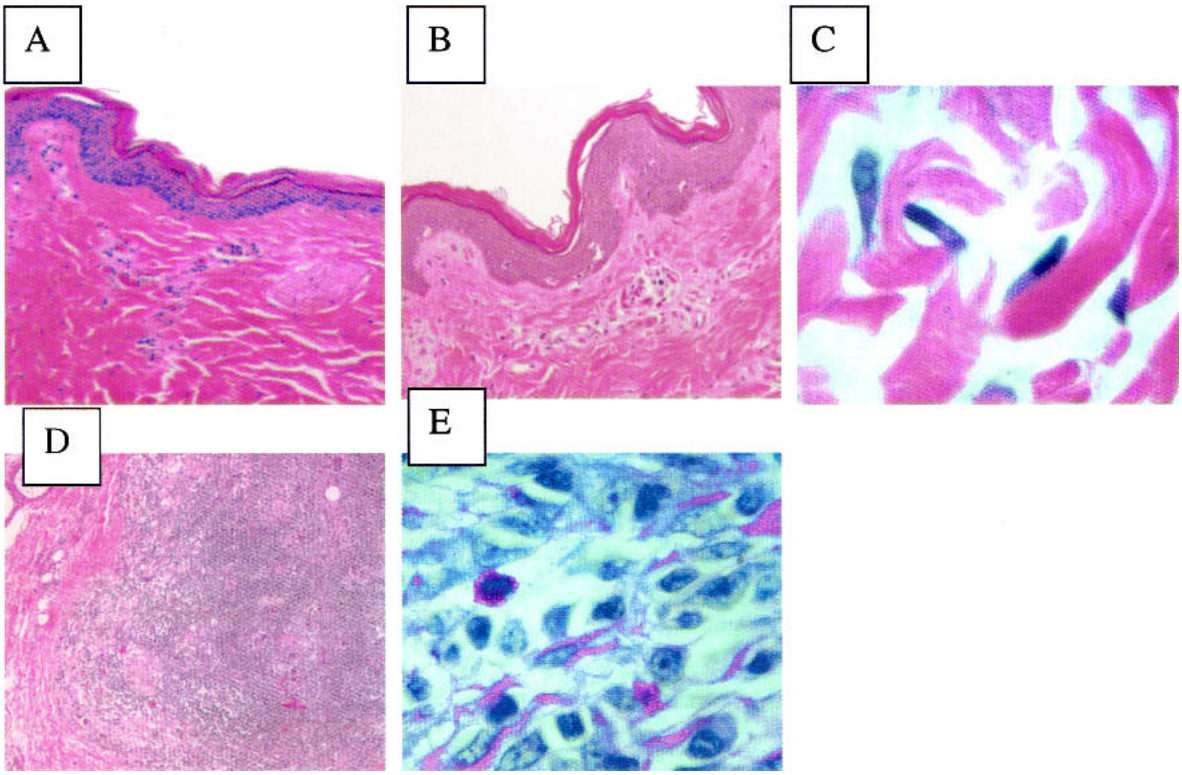

Fig. 5. Histologic changes of the immunization sites. Data are digital light microscopic images of the H&E-stained section from a normal skin (A, 10× magnification.); site of EPI (B, 10×; C, 75×); and site of ID injection (D, 10×; E, 75×). Skin samples were collected 56 days posttreatment.

freezing time for 1-mL liquid submerged in liquid nitrogen (22).

Alum-adsorbed vaccines of low alum concentrations (<0.1 w/v%) have been successfully freeze dried using a normal freezing protocol with preserved immunogenicity (17,18). We hypothesize that alum particles might not form large-scale, dense coagulates under such low concentrations so that the antigen may still be released from the loosely coagulated alum matrix. These formulations might be impractical considering that most commercial alum-adjuvant vaccines are injected at 2 to 3% w/v. It was demonstrated previously that these formulations fail to stabilize alum salt from coagulation when freeze-dried at alum salt concentrations higher than 1% (15). Therefore, alum salt concentrations play a critical role in alum coagulation during dehydration. We confirmed that alum coagulation still occurred even under the extreme fast freezing by SFD when the alum concentration in the liquid formulation before atomization exceeded 30 mg/mL. A possible explanation is that the high-concentration alum gel forms a dense matrix and affects the pattern of ice formation, where ice crystals are less likely to push the freeze concentrate into small compartments compared to the case where the alum gel concentration is lower.

In Vivo Animal Model: Reactogenicity and Immunogenicity

In this study, hairless guinea pigs were used to assess the immunogenicity of SFD alum-HBsAg administered by EPI. The hairless guinea pig is a useful experimental model for skin studies because the skin is structurally similar to that of humans and is immunologically competent (27,28). The pig was used to assess the local reactogenicity to alum-adjuvanted vaccine administered by EPI or ID injection because the pig has a large skin surface area that allows multiple administrations of a vaccine in a defined anatomic area for comparative studies. Equally important is that pig skin is morphologically, biochemically, and histochemically similar to the human skin, although the pig skin immune system is not well characterized (29). Thus, information learned from the hairless guinea pig and pig models may have important implications for human vaccination in terms of local reactogenicty and immunogenicity of the SFD alum-adjuvant vaccine. The results of this study indicate that SFD alum-adjuvant vaccines when administered by EPI are both well tolerated and immunogenic. In contrast, the liquid vaccine having the same compositions when injected ID resulted in granuloma formation. The safety advantage of EPI is likely because the target tissue, stratum corneum and epidermis, is regenerated in a 2- to 3-week cycle, and residual alum is sloughed-off at the same time. Alum delivered to deeper tissues such as the dermis was retained for a long time and evoked a chronic inflammatory response that leads to granuloma formation.

CONCLUSIONS

We developed an SFD process to produce alum-adsorbed vaccine powder formulations for EPI and identified a preferred excipient composition of T/M/D = 3/3/4. This formulation process resulted in particles of optimal particle density and flowability/hygroscopicity. These powders possessed good long-term physical stability, and the SFD antigen was chemically and biochemically stable. More importantly,

alum coagulation was minimized, and the antigen was immunogenic in an animal model. The safety of alum-adjuvant vaccines, when administered by EPI, was demonstrated in this study by preventing granuloma formation. Overall, the powder formulation is stable, safe, and efficacious.

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