International Journal of Gender, Science and Technology
..ISSN: 2040-0748
UGC Care Group I Journal
Vol-07 Issue-02 Aug 2019

How the Effectiveness of Aluminum Salt Adjuvants in a Model Lysozyme Vaccine Is Affected by Particle Size and Antigen Binding

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Abstract

The immunogenicity of vaccines made using aluminum salt adjuvants may be diminished if these particles aggregate during the freezing and drying processes, according to certain claims. We used lysozyme as a model antigen and evaluated this notion by looking at the immune response in a mouse model to several vaccine formulations—liquid, freeze-thawed, and lyophilized. Particle size distributions (PSDs) and degrees of antigen-adjuvant binding were shown to vary greatly due to the different processing procedures and excipient quantities. Vaccines adjuvanted with aluminum hydroxide or aluminum phosphate showed anti-lysozyme titers that were unaffected by the degree of antigen binding to the adjuvant and were independent of the PSD. Copyright 2008 by Wiley-Liss, Inc. and the American Pharmacists Association, Journal of Pharmaceutical Science, 97, 5252–5262, 2008. Plurality of particles, adjuvant, lysozyme, aluminum hydroxide, and aluminum phosphate

INTRODUCTION

In order to stimulate an adequate immune response, adjuvants are necessary for vaccines that include recombinant proteins.1, 2 The only adjuvants used in U.S.-approved vaccinations that are now available for purchase are aluminum hydroxide, aluminum phosphate, and aluminum salt adjuvants. In contrast to aluminum phosphate, which has a plate-like molecular structure, aluminum hydroxide, also known as boehmite (AlOOH),3 is composed of needle-like particles with sizes of 2 nm. main particles in the 50 nm range and their phology.5 When combined in a solution, the two adjuvants produce stable porous aggregates with a diameter of 1–10 mm.4,5 Several factors are likely responsible for the incompletely known mechanisms of action of aluminum salt adjuvants.6–9 The first theory put up was that these particle adjuvants would serve as a depot at the injection site, allowing for the gradual release of the antigen after delivery.nine, ten Newer theories suggest that it helps get the antigen to cells that can present it, however this process has also been called into doubt.5,6 It is believed to stimulate the immune system and release Th2 cytokines11,12, and to destabilize protein antigens on the adjuvant's surface, allowing them to be more easily broken down by proteolytic enzymes, which are necessary for antigen removal. It is common for vaccines made with aluminum-salt adjuvants to lose some of their effectiveness after being frozen or lyophilized to make them more storage stable.the number of The reason for the decrease in effectiveness is believed to be the adhuvant particles

clumping together when they are frozen.16 According to research by More-field et al.17, the smaller the aggregated adjuvant particles, the more interconnected they are. Furthermore, it was shown by Nygaard et al.18 that the immunological response to polystyrene particles in mice is mostly determined by the particle diameter, which in turn determines the surface area per mass of adjuvant and the number of particles per mass of adjuvant, rather than the quantity of adjuvant delivered. Both Zapata et al.19 and Maa et al.20 have noted the aggregation of gels composed of aluminum hydroxycarbonate and magnesium hydroxide upon freezing and thawing.16 Adding glass-forming excipients like trehalose or using fast freezing during lyophilization processes to minimize the time that adjuvant surfaces are exposed to freeze-concentrated liquid can minimize the agglomeration of aluminum salt adjuvant particles, which is related to surface charge alteration and crystallization of buffer salts during processing, as we demonstrated in a previous study.20

The PZC, the pH at which the particles' net surface charges are zero, is a defining feature of aluminum hydroxide and aluminum phosphate adjuvants. The PZC ranges from 9 to 11, 10, 21 for aluminum hydroxide and from about 22, 23 for aluminum phosphate. cells with dendrites. Because of pH changes, competing proteins, and other factors, it is probable that the binding strength of the adjuvant will be changed after injection into the patient, even if the antigen has been adsorbed to it in the produced product.25, 30–32

We postulated, based on these prior investigations, that process circumstances leading to adjuvant agglomeration would reduce the immunogenicity of a model vaccine formulation. Also, we thought the immune response would be boosted if the adjuvant surfaces were processed and formulated in a way that allowed for increased amounts of antigen binding. To control the antigen binding capacity and particle size distribution (PSD) of vaccine particles made of lysozyme, a model antigen, and either an aluminum hydroxide or aluminum phosphate adjuvant, we employed a number of process methods in this study, such as freeze-thawing, lyophilization, and spray-freeze drying. Next, a mouse model was used to assess vaccine formulations with different PSDs for their capacity to elicit an immunological response.

DATA AND PROCEDURE

Supply items

Ferro Pfanstiehl of Cleveland, OH, supplied the trehalose (very pure, minimal endotoxin). The St. Louis, MO-based Sigma Chemical Company was sourced for the acquisition of succinic acid. the 2.0% aluminum hydroxide adjuvant (AH) and the 2.0% aluminum phosphate adjuvant (AP) from AlhydrogelTM and Adju-phosTM,no more than five to seven. purchased products manufactured by Brenntag Biosectoroften aided by interactions between electrostatic charges

in the presence of the adjuvant and the protein,10 and hence, it is common practice to choose solution conditions so that the charges of the two substances are opposing to one another.10 To have greater control over the antigen-adjuvant interactions, buffer salts such phosphate,24-26 succinate,20, and citrate,24,27 may be adsorbed and exchanged on the surface of

the adjuvant, changing its surface charge. Some argue that antigen-adjuvant binding isn't necessary for vaccination effectiveness, even though the World Health Organization recommends that at least 80% of the antigen be adsorbed onto the adjuvant in formulation.15, 27, 29, 30 According to a new research, for an adjuvant to be effective in facilitating absorption by a target cell, the antigens must be contained inside its porous interstitial spaces rather than being securely bonded to its surface. the Clifton, NJ-based E.M. Sergeant Pulp & Chemical Co., Inc. Seikagaku Corporation of Japan supplied the lysozyme (LYS) in its solid crystal form. Glass lyophilization vials (68000316 and 68000344) with volumes of three and five milliliters, as well as stoppers (19500360), were procured from West Pharmaceu-tical Services. American Regent, Inc. (Shirley, NY) supplied the sterile injectable water used in all formulations.

The process of lyophilization

To freeze-dry the materials, we used a Lyophilizer from FTS Systems, the Lyostar. The following are the two cooling rates used to freeze the samples: (i) "tray-freezing," defined as putting samples in a lyophilizer, allowing them to equilibrate for one hour at 08C, and then chilling the shelves at 0.58C/min to —408C;

and (ii) spray-freezing liquid N2 by dropping about 20 mL of droplets into it. Lyophilization vials with a volume of 3 mL were used for tray-frozen samples and 5 mL for spray-frozen samples. The spray-frozen samples were swiftly moved to the lyophilizer, which was set up on shelves that had been precooled to 408C, after being frozen in liquid N2. To reduce radiative heating from the lyophilizer walls, the sample vials were placed at regular intervals and surrounded by a row of water-containing vials.

The samples were dried in two stages. The first stage, known as primary drying, involved heating the shelf to 208C and vacuuming it at 60 mTorr for 20 hours. The second stage, called secondary drying, was carried out at the same vacuum pressure and involved ramping the shelf temperatures from 20 to 08C at 0.28C/min, then to 308C at 0.58C/min, and finally held at 308C for 5 hours. In preparation for injection, the samples were vacuum-sealed and reconstituted with water. The samples that were frozen and then thawed were placed in air in a controlled environment at a temperature of 21.28C.

Distributed Sizes of Particles

The Beckman-Coulter LS230 was used to measure PSDs. There were three duplicates of each run for each formulation, and each run needed three 1-mL samples. The surface area weighted averages of three runs are reported as PSDs.

The Coomassie Plus Reagent (Pierce) was used to assess the amount of lysozyme bound to the adjuvant in the Coomassie Blue Total Protein Assay. To make a standard curve, we first made stock dilutions of a lysozyme solution and measured the protein content at 280 nm. Coomassie reagent was added to sample aliquots, which were then incubated at room temperature for 10 minutes. Absorbance values were measured at 595 nm using a FLUOstar OPTIMA microplate reader (BMG Labtech, Durham, NC). Mass balancing the total protein in the formulation with the measured protein in solution allowed us to determine the amount of protein bound to the adjuvant.

Care for Mice

The Institutional Animal Care and Use Committee at the University of Colorado Health Sciences Center gave its approval for all animal-related research under Protocol #75003806(03)1D. Lab Animal Care at the University of Colorado Health Sciences was the site of all surgeries. Five mice per cage were provided with acidified water and food.

Immunization and Collection of Serum from Mice

There are three of each gender. Each formulation of the aluminum hydroxide- and aluminum phosphate-adjuvanted lysozyme vaccines was tested for its immunogenicity in 5- to 7-week-old BALB/c mice from Jackson Laboratories in Bar Harbor, ME. The vaccines were freeze-dried (FD), spray freeze-dried (SFD), and freeze-thawed (FT). About sixteen hours before injection, the processed samples were frozen or reconstituted, and the liquid vaccines were made. Under the skin of the back, patients received 100 mL injections of a well mixed mixture containing 1 mg of lysozyme. Unprocessed buffer or lysozyme in buffer without adjuvant was administered into control animals. On the fourteenth day, a booster shot was given. Before each injection and again 14 days after the booster shot, anesthetized blood was drawn by retro-orbital hemorrhage. Before analysis, the serum was transferred to a clean centrifuge tube, centrifuged at 12500 rpm for 5 minutes, and then frozen at 808C. Elispot Assay for Enzyme-Linked Immunosorbent Assay

Elisa testing was used to find out how each vaccination affected the immune system. 50 mL of 50 mM sodium bicarbonate pH 9.6 was added to each well of 96 well plates (Nunc, Rochester, NY) containing 0.5 mg of lysozyme. The plates were then left to sit at 48C overnight. Dishes were

acidified by 7.4 pH phosphate-buffered saline (PBS) for washing. Nonspecific sites were blocked using 1% bovine serum albumin (BSA, Thermo Fisher Scientific, Waltham, MA) in PBS. The plates were let to dry and then kept at 48C until they were ready to be used.

Before being placed to the 96-well plate, serum samples were allowed to thaw at room temperature. They were then diluted in PBS with 1% BSA in a series of 20 to 1:3.5 106 dilutions. 50 mL of each sample was used for the experiment. Nightly incubation at 48C was performed on the samples. Following a PBS wash, 50 mL of IgG1, IgG2a, or IgE goat anti-mouse antibodies (Immunology Consultants Lab, Inc., Newberg, OR) were added to the plates at a 1:10000 dilution and left to incubate for 2 hours at room temperature while being spun at 400 rpm. After washing, the plates were incubated with 100 mL of Pierce's Ultra-TMB. A 100 mL solution of 1 N HCl was used to stop the reaction after it had been developed for 15 minutes. A ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA) was used to read the plates at an absorbance of 450 nm. Using a four-parameter fit performed in Softmax Pro software, anti-lysozyme titers were defined as the dilution factor that produced an absorbance value that was one standard deviation higher than the average of the negative control.

Quantitative Evaluation

The mean standard deviation is used to display the data. Every vaccination was

compared to two sets of data: one from the adjuvant and one from the negative control, which was a liquid vaccine of the same formulation. If a was less than 0.05 when using a T-test, the differences were deemed significant.

SUMMARIES AND ANALYZES

Average Particle Size of Aluminum Phosphate and Aluminum Hydroxide AdjuvantsThe immunogenicity of the adsorbed antigens is supposedly heavily dependent on the PSD of vaccination adjuvants, according to previous research.sums of 15, 16, and 18 Here, we developed a vaccine. by adjusting the processing (freeze/thaw and dried/reconstituted), cooling rate (tray-frozen or spray-frozen), and excipient concentration in the formulation, lysozyme-adsorbed onto aluminum phosphate or hydroxide particles can have a wide range of surface areas.20 Past research has extensively investigated the immunogenicity and antigen-adjuvant binding of lysozyme vaccines5,13,25-27,31,32,34,35.5,27

The lyophilized formulations in this investigation were all readily reconstitutable with water, and the adjuvant particles were evenly distributed after a little shaking. Vaccines that have been lyophilized or freeze-thawed tend to settle after a few minutes of sitting, especially in formulations that do not include trehalose. In contrast, all liquid vaccines take around 30 minutes to settle.

Figure 1 shows the surface-area weighted mean particle diameter for aluminum hydroxide particles (a) and aluminum phosphate particles (b) that were created using these approaches. With faster cooling rates and higher excipient levels, the two adjuvants follow trends similar to those presented in our previous study. This means that the surface area of the adjuvant particles is maximized and there is minimal adjuvant agglomeration. The mean diameter of the aluminum phosphate particles increases slightly after lyophilization and reconstitution.

Aluminum Phosphate and Aluminum Hydroxide Binding to Lysozyme in Processed Vaccine Formulations

One often used metric to measure vaccination effectiveness is the proportion of antigen bound to adjuvant. At least 80% antigen adsorption is recommended by the World Health Organization.28 At acidic pH's, lysozyme is positively charged because it has an isoelectric point of around 11. Additionally, at pH 4, both adjuvants will have a positive charge. However, this charge is lower than the PZC's of aluminum hydroxide (9-1110,21) and aluminum phosphate (5-7,22,23), thus adsorption is not likely to be advantageous. Having said that, our prior research shown that succinate in formulations reduced the zeta potential of aged and lyophilized aluminum hydroxide to almost zero, thus it stands to reason that succinate would modify the surface charge of the adjuvant.20 It has

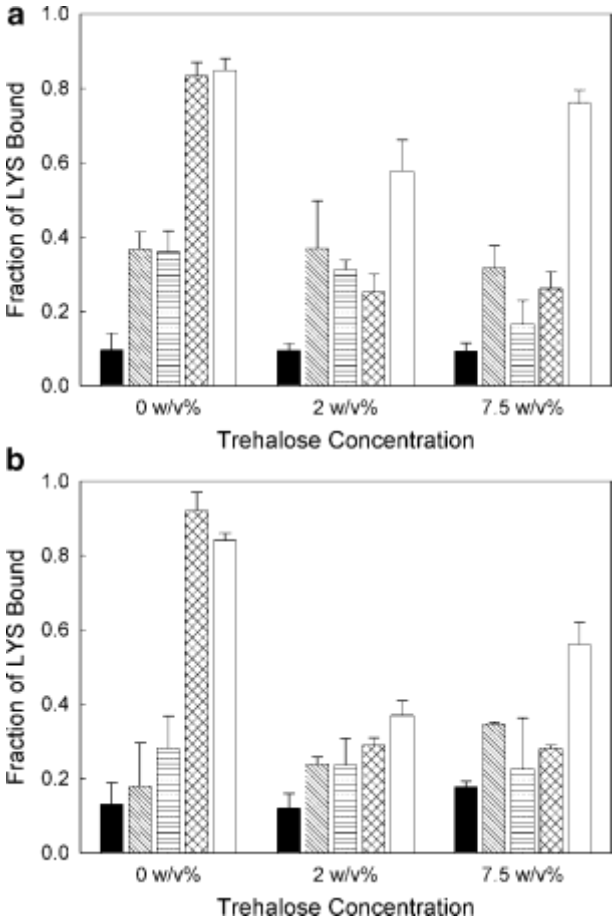
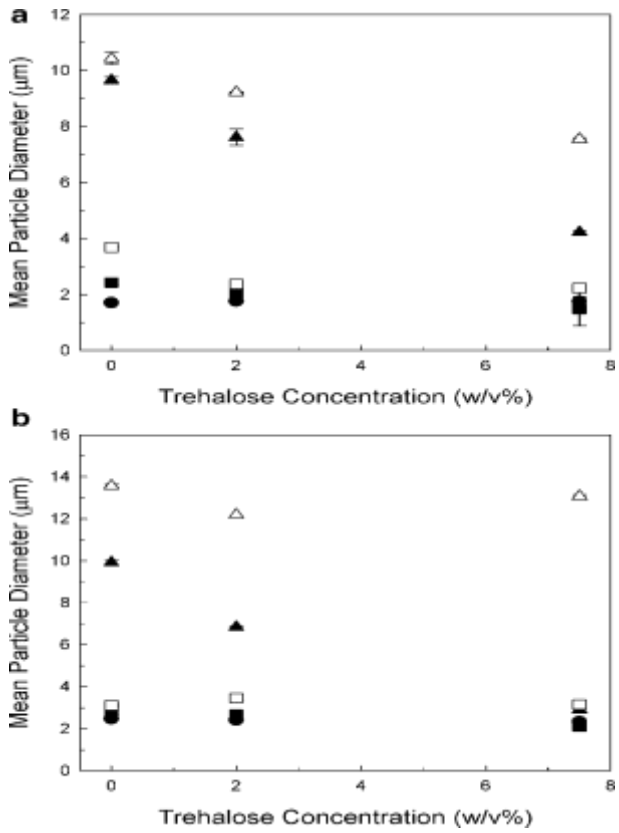


Figure 1 shows the average particle size of aluminum hydroxide and aluminum phosphate in different vaccine formulations with different concentrations of trehalose. The different forms of vaccine are shown as filled circles, filled triangles, open triangles, spray freeze-thaw, and open squares. There was a 0.2% adjuvant in 25 mM sodium succinate, pH 4.0, in all of the formulations.

altering the quantity of lysozyme that can bind to adjuvant may be possible by lowering of zeta potential.

Figure 2 shows the percentages of antigen bound to adjuvant for the following



lysozyme vaccine formulations: (a) aluminum hydroxide-adjuvanted and (b) aluminum phosphate-adjuvanted. The vaccines were freeze-thawed and then dried. The liquid vaccinations using aluminum hydroxide as an adjuvant had the least amount of lysozyme.

Figure 2. Protein fraction bound to adjuvant in vaccines made of aluminum hydroxide and aluminum phosphate, respectively. The following characteristics were used during processing: LIQ (solid black), FT (diagonally striped), SFT (horizontally striped), FD (checkered), and SFD (solid white). There was a pH of 4.0, 25 mM

sodium succinate, 10 mg/mL lysozyme, and 0.2% adjuvant used to prepare the vaccines.bound at a rate of 10-15%, with the SFD vaccinations achieving the greatest rate of 60-80%. Protein binding was highest in the FD and SFD formulations in 0 w/v% trehalose (80-90%) of the aluminum hydroxide-adjuvanted vaccines, and it was lowest in the liquid vaccines (10-15%). Several factors could contribute to the observed variations in binding, such as variations in excipient levels (which have been shown to influence binding in the past) and changes in surface area (due to particle agglomeration or changes in the adjuvant's surface charge, which impact electrostatic interactions) (36).

IN THE END

Even though it has been suggested in the past that the accumulation of aluminum salt adjuvants is what causesThis study examined a model lysozyme vaccine that had been formed with either aluminum hydroxide or aluminum phosphate. The samples were freeze-dried or freeze-thawed using two different freezing rates and different concentrations of trehalose. No PSD-dependency was found, suggesting that the immunogenicity of the vaccine was not negatively affected when these adjuvants were frozen or lyophilized. Even though we got a wide variety of antigen bound fractions from the adjuvant, we didn't see a clear association between anti-lysozyme titers and antigen bound fractions. When comparing the four dried formulations according to processing technique, three of them had a much stronger anti-lysozyme IgG1 response. However, when considering the vaccines in terms of their formulation, only a small number of them differed significantly from the positive controls. This suggests that, at the very least, lyophilized and spray-freeze dried vaccine preparations were equally effective as antigen eliciting liquid suspensions.

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