Influence of Particle Size and Antigen Binding on Effectiveness of Aluminum Salt Adjuvants in a Model Lysozyme Vaccine

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ABSTRACT: It has been suggested that agglomeration of aluminum salt adjuvant particles during freezing and drying can cause loss of immunogenicity of vaccines formulated with such adjuvants. In this study, we tested this hypothesis and examined the immune response in a murine model to various liquid, freeze-thawed, and lyophilized vaccine formulations, using lysozyme as a model antigen. The various processing techniques and excipient levels resulted in a wide range of particle size distributions (PSDs) and antigen–adjuvant binding levels. Anti-lysozyme titers were independent of the PSD for vaccines adjuvanted with either aluminum hydroxide or aluminum phosphate and also were unaffected by the level of antigen binding to the adjuvant. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:5252–5262, 2008

Keywords: lysozyme; aluminum hydroxide; aluminum phosphate; adjuvant; particle size distribution

INTRODUCTION

Vaccines containing recombinant proteins require an adjuvant in order to elicit a sufficient immune response.1,2 Currently, the only adjuvants appearing in approved vaccines marketed in the U.S. are the aluminum salt adjuvants, aluminum hydroxide, and aluminum phosphate. Aluminum hydroxide, chemically aluminum oxyhydroxide, or boehmite (AlOOH),3 consists of needle-like particles with diameters of 2 nm,4 while aluminum phosphate, chemically aluminum hydroxyphosphate (Al(OH)₃(PO₄)₂),3 has a plate-like morphology with primary particles in the range of 50 nm.5 In solution, the both adjuvants form stable porous aggregates 1–10 μm in diameter.4,5

The mechanisms of action of aluminum salt adjuvants are not completely understood, but are likely due to several different phenomena.6–9 The original mechanism proposed was that these particulate adjuvants act as a depot at the site of injection, wherein the antigen is slowly released after administration.9,10 However this mechanism has been questioned, and more recently proposed mechanisms are that it aids in delivery of the antigen to antigen-presenting cells,5,6 that it serves as an immunostimulator and elicits Th2 cytokines11,12 and that it destabilizes protein antigens on the surface of the adjuvant making them more susceptible to proteolytic degradation required for antigen presentation.13,14
When vaccines formulated with aluminum-salt adjuvants are processed by freezing or lyophilization to improve their storage stability properties, a loss of potency is often reported. This loss of potency has been attributed to agglomeration of the adjuvant particles during freezing. Morefield et al. showed that the degree of internalization of adjuvant particles is inversely related to the particle size of the adjuvant aggregates. Also, Nygaard et al. showed that the particle diameter (and thus surface area per mass of adjuvant and number of particles per mass of adjuvant) rather than the mass of adjuvant administered, is the dominant determinant of the immunological response to polystyrene particles in mice. Zapata et al. first reported the agglomeration of aluminum hydroxycarbonate and magnesium hydroxide gels after freezing and thawing, an effect that has also been observed by Maa et al. In a previous study, we showed that the agglomeration of aluminum salt adjuvant particles is related to surface charge alteration and crystallization of buffer salts during processing and can be minimized through the addition of glass-forming excipients such as trehalose and/or by using fast freezing during lyophilization processes to minimize the time that adjuvant surfaces are exposed to freeze-concentrated liquid.

Aluminum hydroxide and aluminum phosphate adjuvants exhibit a characteristic point of zero charge (PZC), the pH at which the net surface charge on the particles is zero. For aluminum hydroxide, the PZC is in the range of 9–11, and for aluminum phosphate the PZC is approximately 5–7. Protein binding to adjuvant is often facilitated by electrostatic interactions between the protein and the adjuvant, and thus the solution conditions are usually chosen so that the adjuvant and protein are oppositely charged in solution. The surface charge on the adjuvant can be modified by the adsorption and surface exchange of buffer salts, such as phosphate, succinate, and citrate, allowing for more control of antigen–adjuvant interactions. Although the World Health Organization recommends that at least 80% of the antigen should be adsorbed onto adjuvant in formulation, there is some debate as to the necessity of antigen–adjuvant binding for efficacy of vaccines.

A recent study proposes that the antigens need to be trapped in the porous interstitial spaces of the adjuvant, but not necessarily bound tightly to the adjuvant surface in order to facilitate uptake by dendritic cells. Even if all of the antigen is adsorbed to adjuvant in a formulated product, it is likely that this binding will be altered after injection into the patient due to the presence of other buffer salts, a pH shift, and/or competition with other proteins.

Based on these previous studies, we hypothesized that immunogenicity of a model vaccine formulation would decrease as a result of processing conditions that result in agglomeration of the adjuvant. Furthermore, we hypothesized that processing and formulation conditions that provided higher levels of antigen binding to the adjuvant surfaces would also increase immune response. In this study, we used various processing techniques including freeze-thawing, lyophilization, and spray-freeze drying to manipulate the particle size distribution (PSD) and antigen binding capacity of vaccine particles composed of lysozyme, a model antigen, and aluminum hydroxide or aluminum phosphate adjuvant. Vaccine formulations with varying PSDs were then testing for their ability to provoke an immune response in a murine model.

MATERIALS AND METHODS

Materials

Trehalose (high purity, low endotoxin) was obtained from Ferro Pfanstiehl (Cleveland, OH). Succinic acid was purchased from Sigma Chemical Company (St. Louis, MO). Alhydrogel\textsuperscript{TM} 2.0% aluminum hydroxide adjuvant (AH) and Adjuphos\textsuperscript{TM} 2.0% aluminum phosphate adjuvant (AP), made by Brenntag Biosector, were purchased from E.M. Sergeant Pulp & Chemical Co, Inc. (Clifton, NJ). Lysozyme (LYS) was obtained from Seikagaku Corporation (Japan) as a crystallized solid. Three- and 5-mL, 13-mm glass lyophilization vials (Product Numbers 68000316 and 68000344) and stoppers (Product Number 19500360) were obtained from West Pharmaceutical Services. All formulations were made using sterile water for injection from American Regent, Inc. (Shirley, NY).

Sample Preparation

Endotoxins were removed from the reconstituted lysozyme using Detoxi-Gel AffinityPak Prepacked Columns from Pierce (Rockford, IL), then the solution was dialyzed in 25 mM sodium succinate,
pH 4 using Slide-A-Lyzer Dialysis Cassettes, 7000 MW Cutoff from Pierce. After filtration through a 0.2 μm filter for sterilization, protein concentration was determined by UV absorbance. For preparing samples for injection, all aqueous solutions were passed through a 0.2 μm filter prior to formulation with the exception of the adjuvants, which are obtained sterile. Formulations were prepared consisting of 25 mM sodium succinate (pH 4.0), 10 μg/mL lysozyme, 0.2 w/v% of either aluminum hydroxide (AH) or aluminum phosphate (AP) adjuvant, and 0, 2, or 7.5 w/v% trehalose.

Lyophilization
An FTS Systems Lyostar lyophilizer was used for the freeze-drying of samples. Samples were frozen at two cooling rates as follows: (i) freezing by placing the samples in lyophilizer, equilibrating 1 h at a shelf temperature of 0°C, then cooling the shelves at 0.5°C/min to −40°C (“tray-freezing”); and (ii) spray-freezing by dropping by ~20 μL droplets into liquid N2. Tray-frozen samples were processed in 3-mL lyophilization vials, while the spray-frozen samples were processed in 5-mL lyophilization vials. Following freezing in liquid N2, spray-frozen samples were quickly transferred to the lyophilizer placed on shelves precooled to −40°C. Sample vials were spaced in the lyophilizer so that they were each separated from one another and were encircled with a row of vials containing water to minimize radiative heating from lyophilizer walls.

Primary drying of the samples was achieved by setting the shelf temperature to −20°C and applying vacuum at 60 mTorr for 20 h, and was followed by secondary drying (also at 60 mTorr), in which shelf temperatures were ramped from −20 to 0°C at 0.2°C/min, then to 30°C at 0.5°C/min and finally held at 30°C for 5 h. Samples were sealed under vacuum and reconstituted with water for injection prior to use. Freeze-thaw samples were thawed at laboratory temperature (21 ± 2°C) in air after freezing.

Particle Size Distributions
PSDs were measured using a Beckman-Coulter LS230. Three 1-mL samples were required for each run, and three replicates of each run were completed per formulation. Reported PSDs are surface-area weighted averages of three runs.

Coomassie Blue Total Protein Assay
Lysozyme bound to adjuvant was measured using the Coomassie Plus Reagent (Pierce). A standard curve was prepared by preparing stock dilutions of a stock lysozyme solution from which the protein concentration was determined by absorbance at 280 nm. Sample aliquots were mixed with the Coomassie reagent, incubated for 10 min at room temperature, and absorbance values were read on a FLUOstar OPTIMA microplate reader (BMG Labtech, Durham, NC) at 595 nm. Protein bound to adjuvant was derived from a mass balance from the measured protein in solution and the total protein in the formulation.

Mouse Care
All work including animals was approved by the University of Colorado Health Sciences Center Institutional Animal Care and Use Committee under Protocol # 75003806(03)1D. All procedures were done at the University of Colorado Health Sciences Center for Laboratory Animal Care. Mice were housed 5 to a cage with food and water (acidified) available ad libitum.

Mouse Immunization and Serum Collection
Male and female (three each) 5- to 7-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used to assess the immunogenicity of each of the liquid, freeze-thawed (FT), spray freeze-thawed (SFT), freeze-dried (FD), and spray freeze-dried (SFD) formulations of aluminum hydroxide- and aluminum phosphate-adjuvanted lysozyme vaccines. Liquid vaccines were prepared and processed samples were either thawed or reconstituted approximately 16 h prior to injection. Injections of 100 μL of the well-mixed formulation containing 1 μg of lysozyme were administered subcutaneously along the back. Control mice were injected with unprocessed buffer or with unprocessed lysozyme in buffer without adjuvant. A booster immunization was administered on day 14. Blood was collected via retro-orbital bleeding under anesthesia prior to each injection and 14 days following the booster immunization. Serum was separated by centrifugation at 12500 rpm for 5 min, transferred to a clean centrifuge tube, and frozen at −80°C until analysis.
Enzyme-Linked ImmunoSorbent Assay (ELISA)

The antibody response to each vaccine was determined by ELISA. Ninety-six-well plates (Nunc, Rochester, NY) were coated with 0.5 μg of lysozyme per well (50 μL) in 50 mM sodium bicarbonate pH 9.6 overnight at 4°C. Plates were washed with phosphate-buffered saline (PBS), pH 7.4. One percent bovine serum albumin (BSA, Thermo Fisher Scientific, Waltham, MA) in PBS was used to block nonspecific sites. Plates were allowed to dry and were stored at 4°C until use. Serum samples were thawed at room temperature and serially diluted in 1% BSA in PBS from 1:20 to 1:3.5 x 10^6 dilutions, and 50 μL of each sample was added to the 96-well plate. Samples were incubated overnight at 4°C. After washing with PBS, plates were incubated with 50 μL horse-radish peroxidase-conjugated goat-anti-mouse antibodies for IgG1, IgG2a, or IgE (Immunology Consultants Lab, Inc., Newberg, OR) at 1:10000 dilution for 2 h at room temperature with rotation (400 rpm). Plates then were washed and incubated with 100 μL of Ultra-TMB (Pierce). After 15 min of development, the reaction was quenched with 100 μL of 1 N HCl. Plates were read at an absorbance of 450 nm using a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). Anti-lysozyme titers were calculated as the dilution factor that gave an absorbance reading equal to one standard deviation greater than the average of the negative control as determined using a four-parameter fit analyzed using Softmax Pro software.

Statistical Analysis

Data are presented as mean ± standard deviation. Comparisons between sets of data were made between each vaccine and the negative control of the same formulation and each vaccine from the positive control (liquid vaccine) of the same formulation and adjuvant. Differences were considered significant if α < 0.05 using a T-test.

RESULTS AND DISCUSSION

Mean Particle Diameter of Processed Aluminum Hydroxide and Aluminum Phosphate Adjuvants

Previous literature suggests that the PSD of vaccine adjuvants is a critical factor in determining the immunogenicity of the adsorbed antigens. In this study, we prepared vaccine formulations composed of lysozyme adsorbed onto aluminum phosphate or aluminum hydroxide particles with a wide range of surface areas by altering the processing (freeze/thaw and dried/reconstituted), cooling rate (tray-frozen or spray-frozen), and excipient (trehalose) concentration in the formulation. Lysozyme is frequently used as a test antigen, with previous studies thoroughly examining antigen–adjuvant binding and the immunogenicity of these vaccines.

In this study, all of the lyophilized formulations were easily reconstituted with water, and the adjuvant particles were well dispersed upon gentle shaking. However, noticeable settling of lyophilized and freeze/thawed vaccines were observed within a few minutes of sitting, especially in formulations without trehalose (vs. about 30 min for all liquid vaccines).

The surface-area weighted mean particle diameter obtained by these methods is shown in Figure 1 for aluminum hydroxide particles (a) and aluminum phosphate particles (b). The mean diameter increases slightly for the aluminum phosphate particles following lyophilization and reconstitution, but the two adjuvants follow trends similar to those presented in our previous study, with faster cooling rates and higher excipient levels minimizing adjuvant agglomeration and maximizing the resulting surface area of the adjuvant particles.

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

The fraction of antigen bound to adjuvant is oftentimes cited as an important parameter for vaccine efficacy. The World Health Organization recommends that at least 80% of the antigen be adsorbed. Lysozyme has an isoelectric point of around 11, and thus is positively charged at acidic pH's. Both adjuvants will also be positively charged at pH 4, which is below the PZC’s of aluminum hydroxide and aluminum phosphate (9–11 and 5–7 respectively), and thus adsorption would not be expected to be favorable. However, it would be expected that the surface charge on the adjuvant would be altered in the presence of succinate in the formulation, as was shown in our previous study in which the zeta potential of aged and lyophilized aluminum hydroxide was lowered to nearly zero. This
reduction of zeta potential may alter the amount of lysozyme that can bind to adjuvant.

The fractions of antigen bound to adjuvant for the liquid, freeze-thawed and dried lysozyme vaccines are presented in Figure 2 for aluminum hydroxide-adjuvanted (a) and aluminum phosphate-adjuvanted (b) vaccines. In the aluminum hydroxide-adjuvanted vaccines, the liquid vaccines had the lowest percentage of lysozyme bound (10–15%) while the SFD vaccines had the highest percentage (60–80%). In the aluminum hydroxide-adjuvanted vaccines, the formulations with the greatest amount of protein bound were the FD and SFD formulations in 0 w/v% trehalose (80–90%) while the liquid vaccines also had the lowest percentage of protein bound (10–15%). The differences in binding could be a result of numerous factors including the differences in excipient levels, which has previously been showed to have an effect on binding, as well as changes in surface area with agglomeration of the particles or changes in surface charge of the adjuvant, affecting electrostatic interactions.
Anti-Lysozyme IgG1 Titers Following Injection with Processed Vaccines

Samples were injected into mice to assess the immunogenic response. Figure 3a and b shows the anti-lysozyme IgG1 titers following two injections of the vaccine for vaccines containing AH or AP. Lysozyme without adjuvant (in buffer alone) is shown on both graphs for comparison. Statistics for each data set are shown in Table 1 with α values for each sample compared to the negative control (buffer only) and the positive control (lysozyme with adjuvant) comparing similar formulations. The vaccine containing lysozyme without adjuvant did not produce a significant IgG1 response compared to the negative control as expected since an adjuvant is required for a significant immune response.1,2 For vaccines containing adjuvants, a majority of the formulations elicited a response significantly greater than that of the negative control (α < 0.05) with varying titers; yet, there were also some samples that did not produce an anti-lysozyme titer greater than the negative control. There were no mice (0/33) injected with lyophilized aluminum hydroxide that did not have a significant response and only one of the 36 mice injected with lyophilized aluminum phosphate (FD in 0% trehalose) did not have a significant response. This is compared with 4/18 mice injected with one of the liquid AH vaccines and 3/14 mice injected with the liquid aluminum phosphate vaccine that did not show an immune response greater than that of the negative controls, indicating that the lyophilized vaccines may be more immunogenic than their liquid suspension counterparts. Due to the large variance in the observed titers, however, only a few of the processed samples were significantly different (α < 0.05) in titer levels from unprocessed liquid adjuvanted vaccines of the same formulation. FD and SFD vaccines with aluminum hydroxide in 0 w/v% trehalose produced significantly greater anti-lysozyme IgG1 titers than those resulting from administration of the liquid controls (α < 0.02 for both). For vaccines with aluminum phosphate, the FT vaccine in 7.5 w/v% trehalose had a significantly lower response (α < 0.05) and the SFD vaccine in 2 w/v% trehalose had a significantly higher response (α < 0.01) than the corresponding liquid adjuvanted vaccine. For all formulations and process conditions, detectable levels of anti-lysozyme IgG2 or IgE antibodies were not observed.

To examine more closely the effect of processing conditions on immune response, titers were averaged across all trehalose concentrations with statistics based on processing condition alone (Fig. 4). When formulation is not considered, all vaccines produced a response significantly greater than that of the negative controls with the exception of injecting lysozyme without adjuvant. FD samples containing either aluminum phosphate or aluminum hydroxide, and SFD samples containing aluminum phosphate produced significantly higher anti-lysozyme IgG1 titers than those resulting from administration of the liquid

Figure 3. IgG1 titers from serum from mice immunized with various lysozyme vaccines containing (a) aluminum hydroxide and (b) aluminum phosphate adjuvant. Trehalose concentrations are (circles) 0 w/v% (squares) 2 w/v% and (triangles) 7.5 w/v%. Bar graphs indicate mean of the data. Annotation: Lys, lysozyme; AH, aluminum hydroxide adjuvant; AP, aluminum phosphate adjuvant; FT, freeze thaw; SFT, spray freeze thaw; FD, freeze-dried; SFD, spray freeze-dried.
adjuvanted vaccine \((a < 0.05)\). This is contrary to what was expected based on the PSD of the adjuvant after reconstitution, because the lyophilized samples containing adjuvant have the largest mean particle diameters. Figure 5a and b compares the anti-lysozyme IgG1 titers to the mean particle diameter, showing no particle size dependency on the aluminum hydroxide \((r^2 = 0.010)\) or aluminum phosphate \((0.0051)\) adjuvanted vaccines, respectively.

The weak dependency of titer on PSD of adjuvants may seem contradictory to previous studies. Nygaard et al. examined the immune response to ovalbumin adsorbed onto polystyrene particles, but they examined the production of anti-ovalbumin IgE and IgG2 antibodies. IgG2 is produced through a Th1 response, which is cell-mediated whereas IgG1 and IgE production are obtained through a Th2 response. Therefore, it might be expected that the size dependency on immunogenicity would be different for the two classes of responses. In our study, only negligible amounts of anti-lysozyme IgG2 or IgE antibodies were observed. Further-

### Table 1. Mean Log(Anti-Lysozyme IgG1 Titers) and Alpha Values Obtained From T-Test for Statistical Significance from Negative Control and Positive Control for Formulations with the Same Trehalose Concentration

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Mean Log(IgG1 Titer)</th>
<th>(\alpha) from Neg. Cont.</th>
<th>(\alpha) from Pos Cont.</th>
<th>Vaccine</th>
<th>Mean Log(IgG1 Titer)</th>
<th>(\alpha) from Neg. Cont.</th>
<th>(\alpha) from Pos Cont.</th>
</tr>
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<tr>
<td>Lys-0</td>
<td>0.29</td>
<td>0.682</td>
<td>N/A</td>
<td>Lys/AP-0</td>
<td>1.94</td>
<td>0.111</td>
<td>N/A</td>
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<tr>
<td>Lys/AH-0</td>
<td>0.91</td>
<td>0.541</td>
<td>N/A</td>
<td>Lys/AP-FT-0</td>
<td>0.43</td>
<td>0.932</td>
<td>0.117</td>
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<tr>
<td>Lys/AH-FT-0</td>
<td>1.69</td>
<td>0.130</td>
<td>0.397</td>
<td>Lys/AP-SFT-0</td>
<td>1.70</td>
<td>0.181</td>
<td>0.825</td>
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<tr>
<td>Lys/AH-FD-0</td>
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<td>0.014</td>
<td>Lys/AP-FD-0</td>
<td>3.27</td>
<td>0.029</td>
<td>0.290</td>
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<tr>
<td>Lys/AH-SFT-0</td>
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<td>Lys/SFT-0</td>
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<td>0.101</td>
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</table>

Annotation: Lys, lysozyme; AH, aluminum hydroxide adjuvant; AP, aluminum phosphate adjuvant; FT, freeze thaw; SFT, spray freeze thaw; FD, freeze-dried; SFD, spray freeze-dried.

**Figure 4.** Mean IgG1 titers of each processing technique to aluminum hydroxide (solid bars) and aluminum phosphate (striped bars) when averaged over all three concentrations of excipients. Stars indicated significantly different from the negative control \((\alpha = 0.05)\) while plus symbols indicate significantly different from positive control \((\alpha = 0.05)\). Annotation: Lys, lysozyme; AH, aluminum hydroxide adjuvant; AP, aluminum phosphate adjuvant; FT, freeze thaw; SFT, spray freeze thaw; FD, freeze-dried; SFD, spray freeze-dried.
more, the particle diameters that Nygaard et al. found to be the most immunogenic, those with diameters of 0.0588 and 0.202 μm, are well below the diameter of aluminum adjuvant particles (>1 μm) present in unprocessed stock solutions of adjuvants. Therefore, a comparison between these two studies may not be relevant. Maa et al., using an aluminum hydroxide-adsorbed hepatitis B vaccine as a model, investigated the dependency of immunogenicity in a murine model on the PSD of a dried formulation. In that study, however, the particle size measurements were based on dried powder formed by compressing, grinding, and sieving, and thus the PSDs obtained are not able to be directly compared to this study, in which we measured the PSD in liquid suspensions following thawing or reconstitution.

It has been reported that aluminum salt adjuvants have an optimal size range for adjuvant action of less than 10 μm, mainly because it is thought that antigen uptake by macrophages is an important determinant of adjuvant effectiveness. The mean particle diameters produced in this study were all 14 μm or smaller, and thus not considerably larger than this arbitrary cut-off value. It could be argued that the aggregate populations obtained in this study were not large enough to hinder the immune response, but with the lack of PSD data in existing studies that report diminished potency, it is impossible to draw that conclusion. It is possible that other properties of the vaccine could be important, such as protein conformation or the charge of the adjuvant, which could give rise to changes in immunogenicity or potency; however, these were not investigated in this study. Unfolding of the protein on the surface of the adjuvant caused by lyophilization could be a factor contributing to the increase in immunogenicity observed, however, this was not examined due to the complexity of measuring the antigen structure when bound to adjuvant.

Figure 6 examines the relationship between the fraction of protein bound to adjuvant and the resulting anti-lysozyme IgG1 titers. The various combinations of formulations and processing conditions had a dramatic effect on the fraction of protein bound to adjuvant, with binding fractions ranging from as little as about 10% to over 90%. Surprisingly, however, the observed anti-lysozyme IgG1 titers were insensitive to the fraction of antigen bound to either adjuvant (r² = 0.098 and 0.043 for antigen bound to aluminum hydroxide and aluminum phosphate, respectively). However, this response echoes more recent studies that also conclude that antigen–adjuvant binding may not be a necessity for immunogenicity. It has been suggested by others that the antigen needs to only be in the vicinity of the adjuvant in order to facilitate uptake by macrophages by being enclosed in porous spaces of the adjuvant. It could also suggest that the potentiation of the immune response by aluminum adjuvants may also be due to indirect activation...
of antigen-presenting cells and associated inflammation at the site of injection.\textsuperscript{12}

\textbf{CONCLUSIONS}

Although previous studies suggest that agglomeration of aluminum salt adjuvants is the cause of the loss of immunogenicity when vaccines formulated with such adjuvants are frozen or lyophilized, no PSD-dependency was observed in this study on a model lysozyme vaccine formulated with either aluminum hydroxide or aluminum phosphate for samples freeze/thawed or freeze-dried utilizing two freezing rates and varying trehalose concentrations. A large range of the fraction of antigen bound to adjuvant was also obtained, but no direct correlation between anti-lysozyme titer and fraction of antigen bound was observed. Three of the four dried formulations produced a significantly greater anti-lysozyme IgG1 response when grouped by processing technique, but only a few of the vaccines were significantly different from the positive controls when formulation is taken into consideration, indicating at the very least that both lyophilized and spray-freeze dried vaccine preparations were equally effective as liquid suspensions at eliciting an IgG1 response to the antigen.

\textbf{ACKNOWLEDGMENTS}

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