Immunogenicity in mice of anthrax recombinant protective antigen in the presence of aluminum adjuvants

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Abstract

The only US-licensed anthrax vaccine for human use, as well as several experimental vaccines containing solely purified recombinant protective antigen (rPA), are formulated using aluminum hydroxide (Al(OH)3) as an adjuvant. It has been suggested that effective adjuvanticity of aluminum salts for protein antigens depends, at least partially, on the degree of adsorption of the antigen to the adjuvant. On the other hand, the ease of antigen desorption from the adjuvant in a quantitative fashion may facilitate the assessment of vaccine characteristics in the laboratory. In this regard, aluminum phosphate (AlPO4), although deemed a “weaker” adjuvant than Al(OH)3, appears superior to the latter. To investigate the possibility of formulating rPA vaccines with AlPO4, as well as the significance of the adsorption of this antigen to the aluminum salt for adjuvanticity, we studied the effect of AlPO4 combined with rPA was examined. Antibodies against rPA were measured using an ELISA. Results indicated that AlPO4 is able to significantly increase the antibody response to rPA, irrespective of its degree of adsorption to the adjuvant. Based on these results, in a second experiment mice were immunized twice, with different formulations of rPA containing either AlPO4 or Al(OH)3, and rPA-antibodies were measured using ELISA and an in vitro toxin neutralization assay. Comparable immune responses to rPA were obtained with both aluminum salts. Additionally, results with AlPO4 as adjuvant confirmed that, in this mouse model, binding of the protein to the adjuvant is not essential for adjuvanticity, whereas the amount of adjuvant has an influence on the antibody response induced.

Keywords: Anthrax vaccine; Aluminum-containing adjuvant; Immunogenicity; Mice

1. Introduction

The degree of public preparedness to protect and treat humans from infections caused by Bacillus anthracis has come under scrutiny in light of recent events in the United States. An important component of a preparedness program is the availability of an effective and well-tolerated vaccine [1]. For more than 30 years only one anthrax vaccine has been available for limited human use in the country. This vaccine consists of the culture filtrate of a non-encapsulated, non-proteolytic, avirulent strain of B. anthracis adsorbed on aluminum hydroxide (AVA, anthrax vaccine adsorbed) [2]. Although AVA is safe and effective [3,4], its extensive use is hampered by the six-dose immunization schedule, and the limited scale-up capacity of the manufacturing process. These limitations have prompted the development of improved anthrax vaccines. AVA contains protective antigen (PA) as a major component [5], and research in animal models has indicated that antibodies to PA can protect against an aerosol
challenge of *B. anthracis* [6,7]. Therefore, research efforts on alternative anthrax vaccines have focused on the investigation of vaccine formulations based on recombinant PA (rPA) only. In some preparations currently undergoing pre-clinical evaluation rPA is adsorbed to an aluminum-based adjuvant [8].

Aluminum salts are currently the only adjuvants included in vaccines licensed by the FDA [9]. For some antigens, Al(OH)₃ has been found to be a more potent adjuvant than AlPO₄ [10]. Al(OH)₃ is the adjuvant used for AVA; however, no studies have confirmed the superiority of Al(OH)₃ for vaccines based on rPA. Generally, Al(OH)₃ has a higher adsorption capacity than AlPO₄, and some antigens have shown better adsorption to Al(OH)₃ at a roughly neutral pH [10]. Adsorption can be improved by optimizing antigen–adjuvant interactions, for example, on the basis of a difference in charge. The isoelectric point of the antigen and the zero-charge point of the aluminum-containing adjuvant predetermine the charges of these components in a formulation mixture [11]. It is believed that effective adjuvanticity is a function of the degree of adsorption of the antigen on the aluminum-containing adjuvant, and this in turn is the basis of the depot theory [10]. Al(OH)₃ might have a more potent adjuvant effect because at roughly neutral pH (6.5 ± 0.5) the ionization state of PA (negative) and the surface charge of the aluminum salt (positive), yield an optimized adsorption as a result of electrostatic attraction. However, recent studies on the in vivo absorption of aluminum-containing vaccine adjuvants from the injection site have revealed the rapid appearance of aluminum in the blood, calling some aspects of the depot theory into question [12]. Additionally, to our knowledge the role of adsorption in the stimulation of the immune response to rPA by aluminum-containing adjuvants has not yet been investigated.

In this study we compared the effect of AlPO₄ and Al(OH)₃ on the immunogenicity of rPA in mice, examining the effect of adsorption of rPA to AlPO₄ on its adjuvant activity. AlPO₄ was chosen primarily because of its low zero-charge point, which we expected to minimize adsorption of rPA, due to its closeness to the protein’s isoelectric point (pI). Furthermore, the adsorbed protein can be removed from AlPO₄ by dissolution, a mild and quantitative way of releasing adsorbed antigen for further analysis [13].

### 2. Materials and methods

#### 2.1. Antigen

The rPA (purified from *B. anthracis* [14]) used in these studies was kindly donated by Dr. S. Leppla of the NIAID.

#### 2.2. Adjuvants

Two commercially available aluminum-containing adjuvants were used, Alhydrogel® 1.3% (Superfos Biosector a/s, Denmark) and Adju-Phos® (HCI Biosector, Denmark). Alhydrogel®, an aluminum hydroxide gel, is a crystalline aluminum oxohydroxide (AlOOH), also known as boehmite. Adju-Phos®, an Aluminum phosphate gel, is an amorphous aluminum hydroxyphosphate [15].

#### 2.3. Characterization of formulations used for immunization

The formulations were prepared in two steps. In the first step the aluminum-containing adjuvant was thoroughly mixed with either phosphate-buffer (1% Na₂HPO₄, pH 7.5, AlPO₄), or aqueous sodium chloride (0.8% NaCl, pH ∼ 6.5, AlPO₄ or Al(OH)₃) at room temperature (RT, around 22 °C). Subsequently rPA was added and the formulation was thoroughly mixed again (using a Vortex mixer). The rPA formulations were kept at RT for at least 1 h, but not more than 2 h before use or dilution. After the incubation period the formulation was again thoroughly mixed before diluting it. All two-fold serial dilutions were made in the same solution used for the preparation of the corresponding formulation (either Na₂HPO₄-buffer or aqueous NaCl). For details see Tables 2 and 3. For the estimation of the degree of adsorption, aliquots of the preparations before use or dilution were centrifuged at 6000 rpm for 10 min to pellet the aluminum gel.
Table 3
Formulations used in 14 groups of 10 animals each for the immunization study to compare the effects of Al(OH)₃ and AlPO₄

<table>
<thead>
<tr>
<th>Group</th>
<th>rPA (µg/dose)</th>
<th>Adjuvant</th>
<th>Al (mg/dose)</th>
<th>Buffer</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 1a</td>
<td>15, 7.5</td>
<td>AlPO₄</td>
<td>0.09, 0.044</td>
<td>NaP</td>
<td>rPA not adsorbed</td>
</tr>
<tr>
<td>2, 2a</td>
<td>15, 7.5</td>
<td>AlPO₄</td>
<td>0.09, 0.044</td>
<td>NaCl</td>
<td>≥80% of rPA adsorbed</td>
</tr>
<tr>
<td>6, 6a</td>
<td>15, 7.5</td>
<td>AlPO₄</td>
<td>0.5, 0.25</td>
<td>NaCl</td>
<td>≥80% of rPA adsorbed</td>
</tr>
<tr>
<td>3a</td>
<td>–</td>
<td>AlPO₄</td>
<td>0.5</td>
<td>NaCl</td>
<td>Adjuvant alone</td>
</tr>
<tr>
<td>3b</td>
<td>–</td>
<td>Al(OH)₃</td>
<td>0.5</td>
<td>NaCl</td>
<td>–</td>
</tr>
<tr>
<td>4, 4a</td>
<td>15, 7.5</td>
<td>–</td>
<td>–</td>
<td>NaCl</td>
<td>PA alone</td>
</tr>
<tr>
<td>5, 5a</td>
<td>15, 7.5</td>
<td>Al(OH)₃</td>
<td>0.09, 0.044</td>
<td>NaCl</td>
<td>~100% of rPA adsorbed</td>
</tr>
<tr>
<td>7, 7a</td>
<td>15, 7.5</td>
<td>Al(OH)₃</td>
<td>0.5, 0.25</td>
<td>NaCl</td>
<td>~100% of rPA adsorbed</td>
</tr>
</tbody>
</table>

Two-fold dilutions were prepared from formulations 1, 2, 4, 5, 6, and 7 each, and administered to six groups of animals, identified as 1a, 2a, 4a, 5a, 6a, and 7a.

Two weeks after the last immunization, blood was collected from their tail veins and serum separated and frozen (−20°C). If a booster dose was needed, it was administered immediately after the first bleeding, and blood was collected again 28 days later.

2.5. Anti-PA-ELISA

The anti-PA-ELISA used was developed at LMQDC/CBER, and validated according to the parameters suggested in the ICH guidelines (Pombo M, et al., Validation of an anti-PA ELISA for the potency testing of anthrax vaccine in mice, in press). In brief, rPA-coated micro-titer plates (1 µg/mL in 0.05 M Carbonate solution, pH 9.6) were incubated at RT for 2 h with test and reference sera (diluted in the wells using PBS, containing 0.05% Tween 20 and 2% FBS). After incubation, plates were washed three times with 0.8% NaCl, containing 0.05% Tween 20, and affinity purified, alkaline phosphatase-labeled Goat anti-Mouse Ig conjugate (H+L; Kierkegaard and Perry, Gaithersburg, MD), diluted in PBS containing 0.05% Tween 20 and 2% FBS, was added. After 2 h incubation period and three washes, substrate (pNPP, Sigma Chemicals, St. Louis, MO) was added, and the plates were incubated for another 30 min. Absorbance was measured at 450 and 505 nm and units were calculated relative to an interim in-house reference serum (800 EU/ml), using a reference-line algorithm [16] (Unitcal, PhPlate AB, Stockholm, Sweden). All numerical values (ELISA units) provided by the software were used in the calculations. The antibody level was considered zero if no value could be calculated.

2.6. TNA

An in-house modified toxin neutralization assay for mouse sera, based on USAMRIID’s [17], was used. In this test, serum-mediated neutralization of anthrax lethal toxin is detected as suppression of cell death. J774A.1 cells were plated in 96-well micro-titer plates and incubated (37°C, 5% CO₂) overnight. Serum samples and standards were prediluted with assay medium and incubated with lethal toxin (PA + LF, lethal factor) for 1 h at 37°C. Subsequently the cells were incubated with these mixtures for 4 h at 37°C. Cell viability was estimated using a vital dye (MTT [18]). Three controls (assay control, sample control, and a medium titer control) were run in each experiment to assure comparability. Absorbance was read at two wavelengths (A 570 and A 690 nm). Units were calculated relative to the same interim in-house reference serum as for the anti-PA-ELISA (800 U/ml) using a four-parameter logistics program (KC4 v3.0, Bio-Tek Instruments, Inc., Winooski, VT). All values (TNA units) provided by the software were used in the calculations. Neutralizing antibody levels were considered zero if OD values corresponding to a 1:25 dilution of the test serum were below the lower asymptote of the standard curve.

2.7. Statistical analysis

All statistical tests (one-way ANOVA and all pairs Tukey-Kramer analysis at an alpha value of 0.05) were performed using JMP version 5 (SAS Institute, Cary, NC).

3. Results

3.1. Immunization study with AlPO₄ as adjuvant

This study was designed to assess whether AlPO₄ mixed with rPA was able to significantly increase immunogenicity of rPA, as well as whether full adsorption of rPA onto AlPO₄ was necessary to obtain suitable adjuvanticity. We chose AlPO₄ because its use makes possible a mild and quan-
titative removal of adsorbed rPA for further studies, and also because its physico-chemical characteristics enabled us to assess the effect of reduced rPA adsorption on immunogenicity. Binding of rPA to AlPO₄ was expected to be less efficient than to Al(OH)₃ due to the low zero-charge point (4.5) of AlPO₄ [19]. Additionally, increasing amounts of phosphate ions in the formulation buffer are able to decrease the adsorption rate of proteins to an aluminum adjuvant under certain circumstances [11]. Adsorption of rPA to AlPO₄ was substantially reduced by using 0.09 mg Al and 1% Na₂HPO₄ (NaP) as formulation buffer; repeated protein determinations in supernatants of AlPO₄-rPA formulations in aqueous NaCl indicated that, on average, less than 20% of the rPA remained in solution (Table 1). We immunized mice (10 animals per treatment group) with a single dose of one of the following formulations: 1) AlPO₄ at 0.09 mg Al per 0.5 mL dose (in 0.8% NaCl), 2) 15 µg of rPA per 0.5 mL dose (in 0.8% NaCl), or 3) 15 µg of rPA plus AlPO₄ at 0.09 mg Al per 0.5 mL dose, suspended in either 0.8% NaCl (adsorbed rPA), or 1% NaP (non-adsorbed rPA). The groups injected with either AlPO₄ or rPA alone were used as controls. Additionally, two other groups were immunized with two-fold serial dilutions (made in the corresponding saline solutions) of the original formulations. Details of the four different formulations used in this experiment are shown in Table 2. Antibodies against rPA were measured using an anti-PA ELISA. Results for the undiluted formulations are shown in Fig. 1. Our findings indicate that the addition of AlPO₄ to rPA significantly increases the antibody response to this protein, irrespective of its adsorption. Furthermore, the ELISA results revealed a clear dose-dependent antibody response to the protein combined with but not adsorbed to the adjuvant. Reducing the amount of antigen per dose to one half and one fourth of the original amount of 15 µg resulted in a sharp decrease in the anti-PA antibody level induced (Fig. 2). However, for the adsorbed protein, a significant difference between the anti-PA antibody levels induced by 15, 7.5, and 3.75 µg of rPA could not be detected. This seems to indicate that adsorption enhances immunogenicity of lower doses of antigen, relative to the mere presence of adjuvant.

3.2. Immunization study with AlPO₄ or Al(OH)₃ as adjuvant

In a second immunization experiment, we compared the adjuvant effect of the two aluminum salts, AlPO₄ and Al(OH)₃. Again, the results of the estimated adsorption of rPA to the adjuvants, for the chosen formulations, are shown in Table 1. Since we had done the first experiment with AlPO₄ at 0.09 mg Al, we decided to use the same amount of aluminum for the comparison of the two aluminum adjuvants. However, in preliminary immunization experiments with rPA in mice (data not shown), 15 µg of rPA adsorbed to Al(OH)₃ containing 0.5 mg Al in a 0.5 mL dose induced a suitable antibody response. To test if lowering the amount of aluminum affects the anti-PA antibody level induced, we decided to use both adjuvants also at 0.5 mg Al. For both adjuvants the percentage of adsorption seemed to be independent of the aluminum amount used, at the protein/adjuvant ratios tested. Specifically, the lower amount of either adjuvant (0.09 mg Al)
adsorbed the same percentage of rPA as the higher amount (0.5 mg Al) of the corresponding adjuvant. The groups for the second immunization experiment were set up as shown in Table 3. In addition to using two different amounts of either AlPO₄ or Al(OH)₃, all the animals received a second immunization 28 days after the first one, and were bled 28 days after the second immunization. Two different doses of antigen (15 and 7.5 μg) were given in this experiment. Antibodies against rPA were measured using ELISA, as well as an in vitro toxin neutralization assay (TNA).

Our ELISA findings from the first experiment indicating that addition of AlPO₄ to rPA significantly increases the antibody response to this protein, irrespective of its adsorption, were supported by the TNA results after the first injection. In the second experiment, formulations containing 15 μg of rPA and AlPO₄ at 0.09 mg Al per dose, either adsorbed or not, induced a significantly higher level of toxin-neutralizing antibodies than rPA alone (Fig. 3).

After the second injection, all the adjuvant-containing rPA preparations (adsorbed and non-adsorbed), with the exception of the one containing AlPO₄ at 0.5 mg Al, induced significantly higher levels of anti-PA antibodies, relative to rPA alone (by ELISA, Fig. 4). Neutralizing antibodies measured by TNA after the second injection revealed a slightly different picture. As seen in Fig. 5, only the formulation with rPA adsorbed to the low amount of AlPO₄ appeared to induce a significantly higher level of neutralizing antibodies, when compared to rPA alone. When tested by ELISA, there was no substantial difference between the antibody levels induced by rPA adsorbed to Al(OH)₃ at 0.09 and 0.5 mg Al. However, the amount of AlPO₄ appeared to have an influence on the anti-PA antibody level induced. Both the ELISA (Fig. 4) and the TNA results (Fig. 5) suggest that amounts of AlPO₄ containing a fraction of 0.5 mg Al per dose may be more effective in stimulating rPA immunogenicity in the mouse. In addition, immunization with formulations that contained rPA adsorbed to AlPO₄ at 0.25 mg Al or less per dose, tended to result in higher levels of toxin-neutralizing anti-PA antibodies than all of the rPA-Al(OH)₃ formulations tested in the model.

4. Discussion

The malicious distribution of anthrax spores through the US mail system [20], in the wake of the events of September 2001, have incited research to improve the currently available anthrax vaccines. The majority of studies have focused on the antigenic composition of alternatives or the testing of
their efficacy in animal models [21–24]. Although a safe and effective vaccine (AVA) that contains Al(OH)₃ as an adjuvant is available in the US [3], detailed studies on the effect of vaccine formulation on immunogenicity appear to be lacking. Thus, we decided to study in a mouse model the possibility of using AlPO₄ as an adjuvant in anthrax vaccines containing rPA, as well as the role played by antigen adsorption on the adjuvant effect of this salt.

Our results indicate that a considerable amount of rPA is associated with the aluminum phosphate adjuvant at pH 6.0–6.5, which is close to the protein’s calculated isoelectric point (pI = 5.6; [25]). It has been suggested that protein adsorption tends to be maximal at the pI, because protein–protein interactions are minimal [11]. The point of zero-charge of AlPO₄ is closer to the calculated pI of rPA than the point of zero-charge of Al(OH)₃. Consequently, the ionic attraction between AlPO₄ and rPA at roughly neutral pH is very low. Therefore, electrostatic attraction due to ionization is presumably not the only interaction that keeps the protein bound to the adjuvant. Alternative adsorption mechanisms, such as hydrophobic bonding and hydrogen bonding, are also possible [11]. Recent findings [8] showing that the release of adsorbed rPA from Al(OH)₃ by interruption of electrostatic attraction becomes less efficient over time support this latter contention. Phosphate-free buffer might also have a positive effect on adsorption, in comparison to PBS, as phosphate ions are able to reduce the adsorptive characteristics of an aluminum-containing adjuvant [11]. Additionally, the actual aluminum adjuvant lot used might have an impact on the degree of adsorption. Callahan et al. showed that two lots of a commercially available aluminum-containing adjuvant displayed slightly different adsorptive characteristics for one and the same protein in a comparative study [11].

The supernatant of the AlPO₄–rPA preparation (0.09 mg of Al/dose) in 1% NaP buffer, pH 7.5, contained nearly 100% of the antigen used for the mixture, suggesting that the association of rPA with the adjuvant was negligible. Comparison of this formulation with one containing mostly adsorbed rPA revealed that both formulations induced comparable anti-PA antibody levels in mice. These findings indicate that, unlike diphtheria toxoid [10], the effectiveness of adjuvanticity in the mouse model used does not depend on complete adsorption of rPA onto the adjuvant. However, at lower concentrations of AlPO₄, adsorption appeared to improve immunogenicity, relative to the same amount of unadsorbed rPA in the presence of an equivalent amount of AlPO₄. Although the mechanisms by which aluminum adjuvants enhance the immune response are not well understood, some possibilities have been proposed [26]. The lack of detectable adsorption of rPA to AlPO₄ suggests that effects on antigen delivery and presentation may not adequately explain the adjuvant effect in our model. However, the aluminum salt still has the potential to enhance the immune response by direct or indirect stimulation or attraction of antigen presenting cells, by activation of complement or by induction of immunomodulatory cytokines.

When tested by ELISA, no substantial difference between the antibody levels induced by rPA adsorbed to either salt, at both aluminum amounts tested (0.09 mg and 0.5 mg) could be detected, indicating that AlPO₄ is a potential alternative to Al(OH)₃ as an adjuvant for rPA. However, TNA revealed a slightly different picture. Although our initial experiments showed that efficiency of adsorption to both adjuvants was not affected by the reduction of aluminum from 0.5 to 0.09 mg Al per dose, the low aluminum amount (0.09 mg Al per dose, approximately 1/10 of the amount allowed by the US CFR for use in humans without special authorization [27]) displayed different adjuvanticity depending on the type of adjuvant. Neutralizing antibody levels induced by Al(OH)₃ formulations exhibited a decreasing trend regarding the reduction of aluminum in the sample. In the case of AlPO₄, TNA results revealed that formulations of adsorbed rPA containing less than 0.5 mg Al per dose might be more effective in stimulating an anti-PA response in the mouse, not only by inducing higher antibody levels, but also by lowering variability between animals. These findings suggest that, for the AlPO₄–rPA formulation, adjuvanticity, at a constant antigen amount, not only becomes optimal at a certain adjuvant amount, above which improvement does not occur, but also that, in the immunogenicity model used, a higher amount of AlPO₄ interferes with the antibody response. In this regard, one of the most striking observations was that the AlPO₄ preparation using 0.09 mg of Al per dose and 15 μg of rPA tended to induce higher titers of neutralizing antibodies than any of the Al(OH)₃ formulations included in the same experiment. Hennessen has discussed similar findings using other antigens [28]. There have been suggestions that aluminum, as part of an adjuvant, has the potential to contribute to adverse reactions associated with vaccines [29]. Reduction of the aluminum concentration in a vaccine might therefore represent an advantage regarding vaccine acceptability.

 Vaccines containing aluminum-based adjuvants have a long history of reasonable safety and efficacy [9]. Consequently, using one of such adjuvants in a formulation analogous to one already licensed seems the obvious choice for an rPA-based vaccine, in the face of urgency. In addressing the development of an improved anthrax vaccine, rPA combined with a variety of aluminum-containing and experimental adjuvants has been tested for its protective efficacy, or its ability to induce antibodies in different species [30]. However, our results suggest that even moderate changes, which need to be empirically tested for each antigen under consideration, might result in substantial gains in product quality. The use of AlPO₄ as an alternative adjuvant for anthrax vaccines has been limited mostly by the fact that adsorption of PA has been perceived as poor. If complete adsorption were not essential for an appropriate immune response to PA, consideration could be given to the use of AlPO₄ instead of Al(OH)₃. A recent study on Haemophilus influenzae type b-CRM197 conjugate vaccines showed that the change of adjuvant from Al(OH)₃ to AlPO₄ resulted in comparable immune responses in healthy infants and did not impact the safety profile [31].
Moreover, AlPO₄ represents an advantage over Al(OH)₃ with regard to product characteristics, such as the ease of antigen desorption in a quantitative fashion that might simplify vaccine quality assessment (e.g. in vitro potency testing).

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[27] 249–76, Chapter 8.


