Synthesis and characterization of *Pseudomonas aeruginosa* alginate–tetanus toxoid conjugate

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Chronic infection with *Pseudomonas aeruginosa* is the main proven perpetrator of lung function decline and ultimate mortality in cystic fibrosis (CF) patients. Mucoid strains of this bacterium elaborate mucoid exopolysaccharide, also referred to as alginate. Alginate-based immunization of naïve animals elicits opsonic antibodies and leads to clearance of mucoid *P. aeruginosa* from the lungs. Alginate was isolated from mucoid *P. aeruginosa* strain 8821M by repeated ethanol precipitation, dialysis, proteinase and nuclease digestion, and chromatography. To improve immunogenicity, the purified antigen was coupled to tetanus toxoid (TT) with adipic acid dihydrazide (ADH) as a spacer and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) as a linker. The reaction mixture was passed through a Sepharose CL-4B column. The resulting conjugate was composed of TT and large-size alginate polymer at a ratio of about 3 : 1; it was non-toxic and non-pyrogenic, and elicited high titres of alginate-specific IgG. Antisera raised against the conjugate had high opsonic activity against the vaccine strain. The alginate conjugate was also able to protect mice against a lethal dose of mucoid *P. aeruginosa*. These data indicate that an alginate-based vaccine has significant potential to protect against chronic infection with mucoid *P. aeruginosa* in the CF host.

**INTRODUCTION**

The most common pathogen responsible for the morbidity and mortality seen in cystic fibrosis (CF) patients is *Pseudomonas aeruginosa* (Mathee *et al.*, 1999; Koch & Heiby, 1993). One of the clinically most important features of infection by *P. aeruginosa* is the tendency of this bacterium to change to a mucoid phenotype, as a result of the production of a polysaccharide known as alginate or mucoid exopolysaccharide (MEP) (Doggett, 1966). In pathogenesis, this exopolysaccharide has potential roles as a mechanism for bacterial adherence, as a barrier to phagocytosis and as a mechanism to neutralize oxygen radicals (Govan & Deretic, 1996). Alginate also affects leukocyte functions, such as the oxidative burst and opsonization, and plays an immunomodulatory role via induction of proinflammatory cytokines and suppression of lymphocyte transformation (Pedersen, 1992).

Immunization with alginate antigen gives rise to antibodies that have opsonic activity and lead to clearance of mucoid *P. aeruginosa* from the respiratory tract in mice and rats (Pier *et al.*, 1983, 1990, 1994).

One of the most effective modern technologies applied to active vaccination has been the conjugation of surface carbohydrate capsular antigens to carrier proteins to increase their immunogenicity, particularly in young children (Lakshman & Finn, 2002; Makela, 2003; Pelton *et al.*, 2003). This converts polysaccharide from a T-cell-independent to a T-cell-dependent antigen, and elicits a higher and boostable immune response in animals (Sood *et al.*, 1996). The applicability of this technology to the alginate of *P. aeruginosa* has been investigated (Pier, 2005; Cryz *et al.*, 1991; Theilacker *et al.*, 2003). Cryz *et al.* (1991) used detoxified exotoxin A as a carrier protein, whereas Theilacker *et al.* (2003) used keyhole-limpet haemocyanin (KLH).

In the present study, we describe the synthesis and characterization of alginate–tetanus toxoid (TT) conjugate using the native non-depolymerized polymer of alginate.

**Abbreviations:** ADH, adipic acid dihydrazide; CF, cystic fibrosis; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; i.p., intraperitoneally; KLH, keyhole-limpet haemocyanin; MEP, mucoid exopolysaccharide; TT, tetanus toxoid.
METHODS

Purification of alginate. Alginate was purified from mucoid P. aeruginosa strain 8821M donated by Dr Parviz Owlia (Faculty of Medicine, Shahed University, Tehran, Iran), as previously described (Kashef et al., 2005). Briefly, 4 l of modified Mian’s medium was inoculated with the test strain and incubated at 37 °C for 72 h. Cultures were stirred with a magnetic bar for 3 to 5 h, and bacterial cells were removed by centrifugation for 1 h at 17 000 g at 4 °C. Crude alginate was precipitated from the supernatant by the addition of cold absolute ethanol to a final concentration of 80 % (v/v). The precipitate was collected by centrifugation at 3000 g for 30 min, washed twice in 80 % (v/v) ethanol and once in 96 % (v/v) ethanol, dialysed against distilled water for 48 h, and lyophilized. Freeze-dried crude alginate was redissolved at 2 mg ml⁻¹ in PBS, pH 7–2, supplemented with 5 mM MgCl₂ and 1 mM CaCl₂, and DNase I was pooled and concentrated. The final material was passed through a 0.1 m pore-size filter and stored at 4 °C.

Chemical analysis of alginate. The purified antigen was analysed for uronic acid content by the carbazole-borate assay (Knutson & Jeanes, 1986) with sodium alginate as the standard, for protein by the Bradford assay (Bradford, 1976) with BSA as the standard, for nucleic acid by the Limulus amoebocyte lysate assay with Escherichia coli endotoxin as the standard.

Protein. TT was obtained from Razi Vaccine and Serum Research Institute of Iran. TT preparations were concentrated by ultrafiltration with a molecular-size cutoff of 100 000 Da. Chromatography was performed with a Sephacryl S-200 (Pharmacia XK 16) column of Sephacryl S-400 (1.6 × 30 cm) equilibrated in PBS, pH 8–6. Eluted fractions were assayed for uronic acid content, and positive fractions eluting near the void volume of the column were pooled, concentrated, passed through a 0.45 μm pore-size filter and stored at 4 °C.

Derivatization and conjugation of alginate. The alginate was derivatized as follows. Alginate (10 mg) and adipic acid dihydrazide (ADH) (0.5 M final concentration) were dissolved in 5 ml 0.05 M PBS buffer, pH 7–4, and the pH was adjusted to 5–6 by adding 0.3 M HCl. After stirring at 4 °C for 4 h, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) was added (0.2 M final concentration), and the reaction mixture was stirred at 4 °C for 18 h while the pH was maintained between 4–9 and 5–1. The reaction mixture was dialysed exhaustively against distilled H₂O at 4 °C.

A total of 10 mg TT was added to the adipic hydrazide (AH) derivative of alginate, and coupling was done with 0.1 M EDAC for 1 h at room temperature and 24 h at 4 °C. The reaction mixture was passed through a Sepharose CL-4B column with PBS, pH 7–4, used as running buffer (flow rate 30 ml h⁻¹). Void-volume fractions that assayed positive for both protein and uronic acid were designated polysaccharide–protein conjugate and were pooled, passed through a 0.45 μm pore-size filter and stored at 4 °C.

Chemical analysis of alginate–TT conjugate. The amounts of protein and uronic acid present in the conjugate were quantified by the Folin–Lowry assay with BSA as the standard (Lowry et al., 1951), and the carbazole-borate assay with sodium alginate as the standard, respectively.

Pyrogenicity determination. New Zealand White rabbits (2–2.5 kg each), three in each group, were used. Alginate–TT antigen was administered intravenously at 1 ml per kg rabbit body weight. Rectal temperatures were recorded at 15 min intervals for 3 h after challenge.

Toxicity test. The lethal effect of alginate–TT conjugate was evaluated in five female mice (weight 22 g) and two guinea pigs (weight 250 g). One human dose (10 μg ml⁻¹) of conjugate was administered intraperitoneally (i.p.). Animals were observed for 5 days post-challenge.

Stability test. Alginate–TT conjugate was placed at 37 °C for 1 week, and rerun over the same size-exclusion chromatography column. The profiles of the protein and polysaccharide were determined to check stability.

Immunization of mice. Female BALB/c mice, 6–8 weeks old, were injected i.p. in groups of five on days 0, 14 and 28 with either purified alginate or alginate–TT conjugate suspended in PBS. Blood was obtained from the orbital sinus on days 14, 28 and 42. All immunizations were done without adjuvant. Alginate-specific IgG was determined by ELISA.

ELISA. ELISAs were performed as follows. Microtitre plates were coated with alginate derived from P. aeruginosa strain 8821M (6 μg ml⁻¹ in PBS, pH 7–4), and kept overnight at 4 °C. Between incubation steps, plates were washed three times with PBS containing 0–0.5 % Tween 20 (PBS-Tw). Individual mouse sera were diluted in the blocking buffer (1/50) and assayed in triplicate. Incubation was performed for 1–5 h at 37 °C. Bound antibodies were allowed to react with a horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:8000 as secondary antibody, for 1 h at 37 °C, o-Phenylenediamine dihydrochloride (Sigma; 0.4 mg ml⁻¹ in 0.2 M Na₂HPO₄·0.1 M citric acid, pH 5) and 10 μl H₂O₂ was used as substrate. After 15 min incubation in the dark, the reaction was stopped by the addition of 0.05 ml H₂SO₄ (20 %), and A₄₅₀ was measured.

Opsonophagocytosis assay. Opsonophagocytic killing was determined by using 100 μl heat-inactivated mouse serum diluted 1:10, 100 μl mouse macrophages at 1 × 10⁶ ml⁻¹, 100 μl % fresh infant rabbit serum as a complement source, and 100 μl mucoid P. aeruginosa 8821M at 1 × 10⁶ ml⁻¹. These components were mixed in sterile microtube tubes. Control tubes, from which antibody, complement or macrophages were omitted, and 100 μl RPMI medium/fetal calf serum was substituted, were run with each assay. For all assays involving mouse sera, pooled serum from members of the respective immunization groups was used. The tubes were held at 37 °C for 90 min with gentle shaking and a 10 μl sample was removed, diluted in saline, and plated for bacterial counts. The plates were incubated overnight at 37 °C, and mucoid colonies were counted. The percentage kill was calculated as follows:

\[
\text{Percentage kill} = \left[1 - \frac{\text{c.f.u. of immune serum at 90 min}}{\text{c.f.u. of preimmune serum at 90 min}}\right] \times 100
\]

Active protection. Mice were divided into three groups, A–C, each containing six mice. Groups A and B were immunized i.p. three times (on days 0, 7 and 14) with 4 μg MEP–TT and MEP (in 0.1 ml PBS, pH 7–4), respectively. Group C contained six unimmunized control mice. Two weeks after the last immunization, mice were challenged i.p. with 3 × 10⁶ c.f.u. (4 × 10⁵ LD₅₀) of the heterologous strain of mucoid P. aeruginosa suspended in sterile PBS, pH 7–4; the inoculum was given in a volume of 1 ml. Mice were observed for 7 days, and mortality was recorded.

Statistical analysis. The statistical analysis was performed using SPSS version 11.5 (SPSS). Differences in the mean ELISA absorbance and the mean percentage of opsonic killing were compared by analysis
of variance (ANOVA) by using a post hoc multiple comparison (Bonferroni correction) test. The chi square test was used to analyse the survival data from the protection experiment. $P \leq 0.05$ was considered significant.

**RESULTS AND DISCUSSION**

**Characterization of alginate**

The purification of the alginate produced by *P. aeruginosa* should ensure removal or inactivation, or both, of other immunogenic or biologically active substances, such as proteins, toxins and LPS. At the same time, purification should not result in any significant change in the structure and properties of the alginate.

On a Sephacryl S-400 column, sodium alginate eluted at a lower volume than purified alginate, indicating a somewhat larger molecular size. Chemical analysis showed that the purified antigen contained 91·6% (w/w) uronic acid, <7·6% protein, <0·0061% LPS, and 0·7% nucleic acid.

Only the large-size polymer fractions of alginate were collected, because it has been shown that only the highest-molecular-size polymers of alginate are able to induce opsonic antibodies in mice with pre-existing levels of non-opsonic antibodies (Pedersen & Kharazmi, 1990). Non-opsonic antibodies are frequently seen in healthy individuals as well as in most patients with CF, even before the onset of detectable infection (Pedersen & Kharazmi, 1990).

**Efficiency of coupling reaction**

The conjugation of capsular polysaccharide (CP) or other bacterial polysaccharide-based vaccines to a carrier protein is a well-established approach to increase the immunogenicity of the former (Fattom et al., 1995). Because of the very large molecular mass of alginate, conjugating it to carrier proteins to produce immunogenic vaccines has proven difficult. Cryz et al. (1991) constructed a conjugate vaccine of depolymerized alginate with exotoxin A of *P. aeruginosa*. This approach, however, has some potential disadvantages, as conformational epitopes of bacterial polysaccharides are often stabilized by polymer length, which can be destroyed by depolymerization (Watson et al., 1992). Another alginate conjugate vaccine was synthesized and evaluated by Theilacker et al. (2003), who bound thiolated alginate to KLH by using succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) as a linker. Using this technology, they were able to construct a water-soluble conjugate of native, large-molecular-weight alginate. A potential disadvantage of the chosen carrier protein and conjugation chemistry is that neither KLH nor the cross-linker SMCC have yet been used in bacterial polysaccharide conjugate vaccines injected into humans.

We used TT as the carrier protein because in practice, only a handful of proteins of bacterial origin, such as TT and diphtheria toxoid, have been used for the preparation of the conjugate vaccines that have been licensed for human use or are currently under development (Sood et al., 1996). The TT molecule ($M_r 150,000$) is a more effective carrier protein than diphtheria toxoid ($M_r 58,000$), perhaps owing to its size (Watson et al., 1992). However, its use as a universal carrier may prove to be undesirable, as its frequent use may overload the immune system with large doses of this protein in combined vaccines, and therefore result in a higher frequency of adverse reactions due to pre-existing antibodies in targeted populations (Peters et al., 1991; Barington et al., 1994). Overall, diversifying our carrier-protein pool may prove to be crucial for the development of human conjugate vaccines.

Our initial attempts to conjugate native, non-depolymerized alginate to TT were not successful. We found that ADH coupling via EDAC had to be repeated once or twice to obtain sufficient ADH bound to the alginate, or alternatively that the reaction time of ADH, alginate and EDAC needed to be increased.

In preliminary studies, the concentration of the reactants during the coupling reaction, and the reaction time, influenced the yield of conjugate. The coupling reactions
Serum antibody response to native and conjugated alginate

Purified alginate failed to induce a significant rise in IgG antibodies to alginate antigen (Fig. 2). The first immunization with alginate alone elicited low levels of IgG antibody, but this did not increase significantly after second and third immunizations ($P=0.214$ and $P=0.133$, respectively). The first immunization with conjugate also induced low levels of IgG. No significant rise in alginate-specific IgG was observed 14 days after the second vaccine dose ($P=0.963$), but a third immunization boosted antibody levels significantly ($P<0.0001$). As shown in Table 1, immunization with the alginate–TT conjugate also induced IgG against the carrier protein. The second immunization with conjugate induced high levels of TT-specific IgG compared to the first immunization ($P=0.007$), but no significant rise was observed 14 days after the third immunization ($P=0.10$). Native alginate therefore acted as a typical T-cell-independent antigen, whereas a T-cell-dependent response was observed with the conjugate.

Induction of IgG against the components of the alginate–TT conjugate over a period of 42 days is shown in Fig. 3. Mice immunized with conjugate not only elicited IgG against the two components of the conjugate, but also induced significant levels of IgG antibodies to the complete vaccine. A considerable rise in alginate–TT-specific IgG was observed after the second and third vaccine dose ($P<0.0001$).

Opsonic activity of mouse sera against mucoid

P. aeruginosa

High levels of opsonic antibodies to alginate correlate with clearance of mucoid $P. aeruginosa$ from the lung in rodent models of chronic infection (Pier et al., 1990). We therefore compared the potential of native and conjugated alginate to induce opsonic antibodies specific for alginate. The experiment was repeated three times and the results are shown in Fig. 4. In the opsonophagocytic killing assay, antisera from mice immunized with the conjugate mediated phagocytic killing (86·3 %) against the vaccine strain on day 42, but antisera from mice primed with native alginate was less efficient in mediating phagocytic killing (68·5 %). There was no phagocytic killing when antibody, complement or macrophages were omitted.

Table 1. Antibody responses of mice immunized with alginate–TT conjugate or alginate alone

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Target antigen</th>
<th>Isotype</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate–TT conjugate</td>
<td>Alginate–TT</td>
<td>IgG</td>
<td>0·357±0·03</td>
<td>0·770±0·14</td>
<td>0·888±0·05</td>
</tr>
<tr>
<td>Alginate–TT</td>
<td>Alginate</td>
<td>IgG</td>
<td>0·226±0·07</td>
<td>0·248±0·10</td>
<td>0·725±0·30</td>
</tr>
<tr>
<td>Alginate–TT</td>
<td>TT</td>
<td>IgG</td>
<td>0·368±0·04</td>
<td>0·628±0·08</td>
<td>0·798±0·04</td>
</tr>
<tr>
<td>Alginate–TT</td>
<td>Alginate</td>
<td>IgG</td>
<td>0·268±0·04</td>
<td>0·349±0·17</td>
<td>0·258±0·04</td>
</tr>
</tbody>
</table>

BALB/c mice were immunized with two candidate vaccines, as indicated, i.p. in PBS. Values show the mean±SEM of $A_{492}$.
Although IgG titres determined by ELISA are useful to screen sera for serologic responses, the levels of opsonic antibodies are the best predictor of protective efficacy in animal models (Pier et al., 1990), and are associated with the resistance of CF patients to chronic mucoid P. aeruginosa infection (Pier et al., 1983). In the opsonophagocytic killing assay, alginate conjugated to TT was superior to the native polysaccharide in its ability to induce opsonic antibodies in mice. In contrast to the result found here, Theilacker et al. (2003) did not report the induction of opsonic antibodies in mice after their immunization with native alginate. A possible explanation for these conflicting results may be the different composition of the alginate preparations used in the two studies: the alginate antigens differed in $K_d$, the ratio of mannuronic acid to guluronic acid, and acetate content (Garner et al., 1990).

**Active protection**

Three immunizations of mice with 4·0 μg conjugate per dose showed significant protection ($P<0.01$) against intraperitoneal challenge with $4 \times LD_{50}$ of wild-type mucoid P. aeruginosa. This challenge dose killed 6/6 of mice that were uninoculated, 1/6 of mice immunized with conjugate and 3/6 of mice immunized with alginate alone. There was no significant difference in the rates of survival of mice immunized with alginate and those of uninoculated mice ($P=0.54$).

A key feature of any vaccine is its ability to protect against infection with strains of the organism heterologous to the one from which the vaccine was derived. For alginate, with its considerable variation in the ratio of mannuronic to guluronic acid and degree of $O$-acylation, it is crucial to establish that vaccination with a single preparation induces antibodies reactive against heterologous mucoid P. aeruginosa strains (Sherbrock-Cox et al., 1984). Our data suggest that immunization of mice with the alginate–TT conjugate results in an increased LD$_{50}$ after heterologous-type challenge, and IgG induced by the conjugate was cross-reactive to the heterologous mucoid strain. In contrast to our results, in another study, cross-reactive IgG was virtually absent after immunization of rats with an alginate–exotoxin A conjugate (Johansen et al., 1994, 1995). These data suggest that extensive depolymerization and/or de-$O$-acylation may lead to the loss of epitopes shared between heterologous strains.

In conclusion, conjugation of alginate to TT utilizing ADH coupling via EDAC yielded an alginate-based conjugate rich in protein and uronate, which was non-toxic, non-pyrogenic, and elicited high titres of alginate-specific IgG. Antisera raised against the conjugate had high opsonic activity against the vaccine strain. The alginate conjugate was also able to protect mice against a lethal dose of mucoid P. aeruginosa. These data indicate that an alginate-based vaccine has significant potential to protect against chronic infection with mucoid strains of P. aeruginosa in the CF host.
ACKNOWLEDGEMENTS

Special thanks to Dr Gerald B. Pier (Professor of Medicine, Microbiology and Molecular Genetics, Harvard Medical School) for his invaluable guidance. Support was provided by the Department of Bacteriology, School of Medical Sciences, Tarbiat Modares University. The technical expertise was donated by the Iranian Blood Transfusion Organization, Research Center. The experiments comply with the current laws of the country in which they were performed.

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