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Immunological evaluation of an alginate-based conjugate as a vaccine candidate against *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic pathogen that causes serious infections, is usually resistant to antimicrobial agents, and is the leading cause of morbidity and premature mortality in patients with cystic fibrosis (CF). Mucoid strains of *P. aeruginosa* produce a virulence factor known as alginate. Developing a strategy to raise opsonic antibodies against alginate could be promising for the treatment of *P. aeruginosa* infection in CF patients. Conjugation of alginate to a carrier protein is a good method for increasing the immunogenicity of alginate. We conjugated alginate to the outer membrane vesicle (OMV) of *Neisseria meningitidis* serogroup B, which is a safe carrier protein, and evaluated its efficacy in mice. To evaluate the immune response, total IgG, IgG1, IgG2a, and IgG2b titers were analyzed. Immunization of mice with the alginate–OMV conjugate raised the levels of opsonic antibodies, and the vaccinated mice were protected when challenged intranasally with *P. aeruginosa*. Further studies showed that the conjugated vaccine could eliminate *P. aeruginosa* from the lungs of infected mice. This study supports the proposal that immunization of mice with an alginate–OMV conjugate vaccine could be safe and protective against *P. aeruginosa* infection.

Key words: *Pseudomonas aeruginosa*; alginate; conjugate vaccine.

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Pseudomonas aeruginosa participates in the development of infection in immunocompromised individuals, such as patients suffering from cancer, AIDS, and especially cystic fibrosis (1, 2). Resistance to many antibiotics by *P. aeruginosa* isolated from hospitalized patients has reached a critical point (3). *P. aeruginosa* infection of the lungs of CF patients initiates by non-mucoid strains, but, after the increase in the *P. aeruginosa* population, new traits emerge yielding mucoid strains (4) due to the overproduction of an exopolysaccharide known as alginate (5). Alginate is a non-repeating copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G) that are linked by 1–4 linkages and is acetylated at positions O-2 and/or O-3 of the M residues (6). Alginate protects *P. aeruginosa* against host immune defense mechanisms (7–9) and may establish a barrier against antibiotic penetration into biofilm colonies (10) or confer resistance to reactive oxygen intermediates (11). The two main causes of shifting from non-mucoid to mucoid variants has been identified as the lack of sufficient oxygen in the mucus and the release of reactive oxygen species from polymorphonuclear cells (PMNs) in the lung (12, 13). Several studies showed that alginate elicits opsonic antibodies and has conserved epitopic sites in the most mucoid strains (14–16). The safety of alginate has been proven at various dosages by some human trials (14), and a direct relationship between the molecular size of alginate and the induction of opsonic antibodies has been observed (17). In spite of the safety of alginate as a vaccine candidate to confer immunity against *P. aeruginosa* infection, it does not have sufficient immunogenicity to provoke long-lived opsonic antibodies (14). The most successful method to overcome this issue and increase the immunogenicity of alginate is conjugation, whereby surface polysaccharides are conjugated to a carrier

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protein (18, 19). Coupling of alginate to keyhole limpet hemocyanin (KLH), detoxified exotoxin A, tetanus toxoid (TT), and flagellin has been investigated to assess the immunogenicity of these conjugates (20–23). An approved carrier protein that is wildly used in conjugate vaccines is the outer membrane vesicle (OMV) of *Neisseria meningitidis* serogroup B (24–26). OMVs extrude from the outer membrane of gram-negative bacteria and consist of spherical lipids with an average diameter of 50–250 nm (27). OMVs are very immunogenic, which makes them useful as carrier proteins due to their ability to stimulate protective humoral immune responses (28). Another advantage of OMVs is their stability for many months at 4 °C and after lyophilization (29). The objectives of the present study were to synthesize an alginate–OMV conjugate using purified alginate and OMV and evaluate the antibody response in mice treated with the conjugate. We also examined cross protection by opsonophagocytic activity.

MATERIAL AND METHODS

Bacterial strains

Two mucoid *P. aeruginosa* strains were used in this study: *P. aeruginosa* 8821M (21) and a clinical isolate, *P. aeruginosa* 6494, which was obtained from CF patients.

Extraction and purification of alginate

Alginate was obtained from *P. aeruginosa* strain 8821M as previously described with slight modifications (22). The bacteria were grown for 72 h at 37 °C in Mian's medium, repeated centrifugation (90 min at 15500 × *g* at 4 °C) was used to remove the bacterial cells, and alginate was precipitated using cold 90% ethanol (final concentration) at 4 °C for 60 min. The precipitated alginate was collected by centrifugation at 4000 × *g* for 20 min, washing twice in ethanol, dialyzing against deionized water (dH_2O) for 24 h and finally lyophilization. The lyophilized alginate (2 mg/mL) was dissolved in phosphate-buffered saline (PBS) supplemented with 10 mM $MgCl_2$ and 1 mM $CaCl_2$. DNase I and RNase A, each at 150 μg/mL (Fermentas, Pittsburgh, PA, USA), were used to remove any possible DNA and RNA contamination. Proteinase K was added (200 μg/mL, Fermentas) to the mixture for 6 h at 56 °C, and the mixture was heated for 30 min at 80 °C to inactivate and digest the enzymes. Ethanol precipitation was repeated, and the precipitate was dialyzed three times against dH_2O and then lyophilized, and size exclusion chromatography was used to purify the alginate. The alginate was then dissolved at 2 mg/mL in phosphate buffer and passed through a XK 16 column (1.6- by 70-cm) packed with Sephacryl S-400. The uronic acid content from the eluted fractions was examined, and the positive fractions that eluted just past the void volume were collected. Eventually, the fractions containing uronic acid were pooled, dialyzed against dH_2O, and lyophilized (21, 22).

Biochemical analyses

The protein content of the OMVs, alginate, and the conjugate were determined using the Bradford assay (30). The uronic acid content in the purified alginate was assayed by the carbazole-borate method using seaweed alginate (Sigma, St. Louis, MO, USA) as the internal standard (31). The DNA content was assayed by determining the absorbance at 260 nm, and the biological activity of the endotoxin was estimated using the Limulus Amebocyte Lysate (LAL), with *Escherichia coli* endotoxin as the standard.

Preparation of OMVs

The OMVs were prepared as previously described (32). Briefly, *N. meningitidis* serogroup B strain (CSBPI, G-245) was grown in a 40-L fermentor containing modified Frantz medium at 37 °C for 24 h to the early stationary phase (33). The cells were inactivated at 56 °C for 30 min, washed twice with PBS (pH = 7.2), and then suspended in NaCl buffer. After homogenization for 30 min, the wet weight of the cells was determined, and the suspension was centrifuged for 1 h at 3000 × *g*. The OMVs were extracted using 1 M Tris-HCl buffer, pH 8.6, containing 10 mM EDTA and 0.5% w/v deoxycholate, and repeated centrifugation at 20 000 × *g* for 60 min followed by ultracentrifugation at 125 000 × *g* for 2 h.

Alginate–OMV conjugate synthesis

Alginate (12 mg) was dissolved in dimethyl sulfoxide (Merck, Darmstadt, Germany) that was activated with cyanogen bromide at pH 12, dialyzed against distilled water and coupled to the spacer adipic acid dihydrazide (ADH, Sigma) at a final concentration of 0.6 M. The pH was brought to 5.2 using 0.2 M HCl; and this mixture was stirred at room temperature for 10 min, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC, Sigma) was added to a concentration of 0.2 M, the pH was maintained at 5.6 with 0.1 M HCl, and the solution was stirred overnight at room temperature. The product of this reaction was dialyzed against dH_2O at 4 °C for 20 h. To conjugate the product to the carrier protein, 10 mg of OMVs, EDAC (0.2 M final concentration) and 1.5 mg of N-hydroxysulfosuccinimide sodium (Sigma) were added to the dialyzed solution. Finally, this mixture was incubated with gentle stirring at 4 °C for 48 h and then for 2 h at room temperature.

After dialyzing against dH_2O_2, the reaction mixture was subjected to gel filtration through a Sepharose CL-4B column. The void volume fractions that were positive for protein and uronic acids were determined, assumed to be polysaccharide–protein conjugates and were pooled, dialyzed, and lyophilized.

Safety of the conjugate

The pyrogenicity test and toxicity of the conjugate was analyzed as previously described (21).

Immunogenicity studies

BALB/c mice (10/group), 6–8-week old were injected subcutaneously three times at biweekly intervals with 10 μg

of alginate in the conjugate vaccine (Alg–OMV), 10 μg of alginate and 20 μg of OMV in the mixture of alginate and OMV (Alg/OMV), purified alginate (10 μg), and PBS. All the vaccines and also PBS were suspended in incomplete Freund's adjuvant (IFA, Sigma, USA), except for the mixture of alginate and OMV, to assess the adjuvant effect of OMV. The mice were bled prior to injection on days 14, 28, and 42 after the first immunization, and the obtained serum samples were stored at −20 °C until use.

ELISA

The enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of anti-alginate antibodies in the sera of the immunized mice. Alginate (purified from *P. aeruginosa* 8821M) was coated on a 96-well plate at a concentration of 10 μg/mL in PBS, pH 7.4, and incubated for 24 h at 4 °C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) at incubation intervals. PBS containing 2% BSA was used as the blocking buffer for 2 h at 37 °C. The mice sera were diluted in blocking buffer (1:40) and incubated for 90 min at 37 °C. After washing five times, HRP-conjugated anti-mouse IgG (Sigma) diluted 1:10000 was used as secondary antibody for 90 min at 37 °C.

Tetramethylbenzidine (TMB, Sigma, USA) was used as a substrate to develop the reaction in the dark for 30 min. The reaction was stopped with 100 μL of H_2SO_4, and the results were read at an optical density of 450 nm (OD450) by an ELISA reader (Biotek Elx 800) (22, 23). The IgG isotypes (IgG1, IgG2a, IgG2b) were also assayed to determine the types of immune response, i.e., humoral or cellular. All of the steps of the ELISA were the same as those mentioned above, except for the addition of isotypes after the serum incubation step (34).

Opsonophagocytosis assay

The opsonic activity of the antibodies was evaluated as previously described (35, 36). Briefly, 100 μL of diluted serum samples (1:4 to 1:64) were mixed with 100 μL bacterial suspension (10^6 CFU/mL of *P. aeruginosa* 8821M or the clinical isolate) and incubated at 37 °C for 30 min. After washing with PBS (pH 7.4), 100 μL of mouse macrophages at 10^6/mL and 100 μL of 10% fresh infant rabbit serum, which served as a complement source, were added to the mixture and incubated at 37 °C for 90 min. Control tubes were run with each assay by omitting antibody, macrophages, or complement and substituting RPMI Medium (Capricorn Scientific, Ebsdorfergrund, Germany). Finally, a 100-μL aliquot was removed, diluted in PBS, and plated for bacterial counts. The opsonic activity of the serum was calculated as follows:

[1 − (CFU immune serum at 90 min/CFU of preimmune serum at 90 min)] × 100.

Protection and challenge studies

P. aeruginosa strains (8821M and the clinical isolate) were grown on LB broth for 24 h without shaking, and then 1 mL of this bacterial suspension was transferred to 9 mL of LB Broth and incubated with shaking at 37 °C until an OD650 of 0.6 was obtained. Bacterial cells in the mid-exponential growth phase were harvested by centrifugation at 10 000 rpm at 4 °C for 15 min, resuspended in PBS, and washed twice. The number of bacterial cells that were injected into animals was determined by plating on LB agar medium immediately before the mice were challenged (37). The mice (6/group) were infected 3 weeks after the last immunization. The mice were anesthetized by administering (intraperitoneally) a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) and then inoculated with 15 μL of bacterial suspension (2LD50) into each nostril in an upright position, and the animals were followed up to day 7. After the observation of moribund symptoms such as ruffled fur and non-responsiveness to stimuli, the mice were sacrificed and considered non-survivors.

Bacterial load in lung

To quantify the *P. aeruginosa* in the lung, the mice (4/group) were inoculated intranasally and sacrificed 18 h after challenge. The lungs were then removed, weighed, homogenized, diluted in PBS and finally plated on LB agar at 37 °C for 24 h. The number of colonies was manually counted to determine the CFU/g of the lung tissue (23).

Statistical analysis

For statistical analysis, the SPSS 18.0 software was used. Survival data for the different mouse groups were analyzed using one-way analysis of variance (ANOVA) and a p-value less than 0.05 was considered significant.

RESULTS

Purified alginate properties

We used enzymatic digestion to remove contamination of DNA, RNA, and protein. To select high molecular-sized alginate, size exclusion chromatography using Sephachryl S-400 was performed, and fractions containing uronic acid that eluted near the void volume were collected (Figure S1). The chemical constituents of the purified alginate indicated negligible contamination (<4% protein, <0.0042% LPS, and <0.6% nucleic acid), and this alginate sample mostly contained uronic acid (94.3% wt/wt).

OMV Characterization

After extraction and purification of the OMVs, the total protein yield was determined as 320–740 mg in the final product. The integrity of the vesicles was confirmed by electron microscopy, and the size of these vesicles was determined to be between 80 and 120 nm (Figure S2). The outer membrane vesicles of *N. meningitidis* serogroup B mainly consists of a 45-kD PorA protein. The expression of PorA in the OMV preparations was assessed using SDS-PAGE (Figure S3).

Characterization of the alginate–OMV conjugates

A major, broad peak that contained uronic acid and protein was found after gel filtration through a Sepharose CL-4B column near the void volume (Fig. 1). Therefore, these fractions were presumed to contain conjugated molecules, and only fractions eluting at the void volume were presumed to be free of non-conjugated polysaccharide and were used as the Alg–OMV conjugate. Some other samples also contained free protein or unconjugated uronic acid. The alginate–OMV conjugate was composed of 78% protein and 22% uronic acid by weight. The yield, in terms of protein incorporation, of the conjugation was 24%. Using ^{1}H-NMR and Zeta potential tests, we confirmed the presence of a covalent bond, which was an indicator of a successful conjugation (Figures S4 and S5). This conjugate was not pyrogenic or toxic after i.p. injection into animals.

Anti-alginate humoral responses

To assess the antibody response against alginate, mice sera were collected, and their antibody titers were assessed using indirect ELISA. Fourteen days after the first immunization, low-level production of IgG was observed in the first group, which received purified alginate. The first booster slightly increased the IgG titers ($p < 0.0001$), but the second booster did not change the pattern of the IgG titers ($p = 0.258$) (Fig. 2A). In the second group of mice, which received a mixture of alginate and OMV, the titers of anti-alginate antibodies were not increased after the second immunization ($p = 0.135$). In contrast to these two groups, immunization with the alginate–OMV conjugate elicited a high level of antibodies to alginate after the first and second boosters ($p < 0.0001$). The isotype response in the immunized groups showed that IgG1 was dominant compared to the other IgG subclasses (Fig. 2B), but no significant difference between IgG 2a and IgG 2b in all immunized groups was observed ($p > 0.1$). These results showed that the immune response against alginate is a Th2-dependent response due to a high level of IgG1 in the immunized groups.

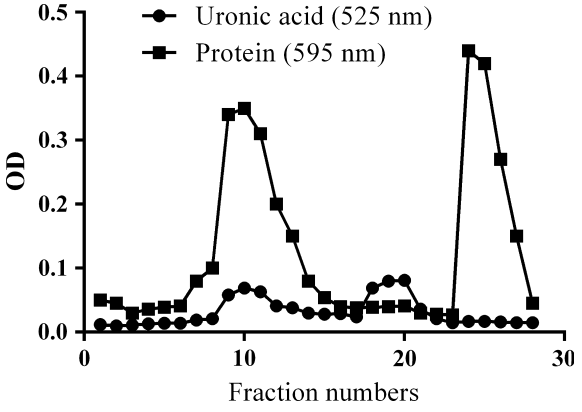

Fig. 1. Gel filtration of the alginate–OMV conjugate through CL-4B Sepharose. The protein content of the fractions was measured using the Bradford assay at 595 nm, and uronic acid was measured using the carbazole assay (525 nm). Fractions 9–13 contained protein and uronic acid components and were presumed to contain conjugate.

Opsonophagocytic killing activity

An opsonophagocytic killing assay was used to determine the functional activity of the antibodies raised against alginate in the presence of mouse macrophages and rabbit complement. At all dilution ranges, the opsonic activity of the conjugate group was significantly ($p < 0.05$) higher when compared to native alginate or the mixture of alginate and OMV groups (Fig. 3). At serum dilutions ranging from 1:2 to 1:64, the sera of mice immunized with the alginate–OMV conjugate showed 90% to 51% opsonic killing activity, respectively (*P. aeruginosa* 8821M).

Protection efficacy

The BALB/c mice were challenged intranasally with *P. aeruginosa* 8821M (2LD50) 21 days after the last immunization, and the mice were monitored for 7 days (Fig. 4). Unlike the conjugated group with 100% protection, only 33% of the mice in the native alginate or alginate/OMV mixture-immunized groups survived (Fig. 4A). Further studies were performed to assess the protection of the vaccinated groups by challenging the mice with *P. aeruginosa* 6494 (clinical isolate). The results showed that the protection rate was 83% in the group of mice immunized with the conjugated vaccine (Fig. 4B).

Pulmonary clearance of *P. aeruginosa* mucoid strains from the lungs of BALB/c mice

To assess the ability of the alginate–OMV conjugate vaccine to eliminate *P. aeruginosa* infection, we examined the clearance rate of viable bacteria from the lungs of vaccinated mice 18 h after intranasal challenge with the *P. aeruginosa* 8821M or 6494 strains. The results showed that immunization with conjugate vaccine resulted in a decrease in the bacterial load in the lung compared with the control group ($p < 0.05$). Vaccination with the alginate–OMV conjugate in the mice challenged with

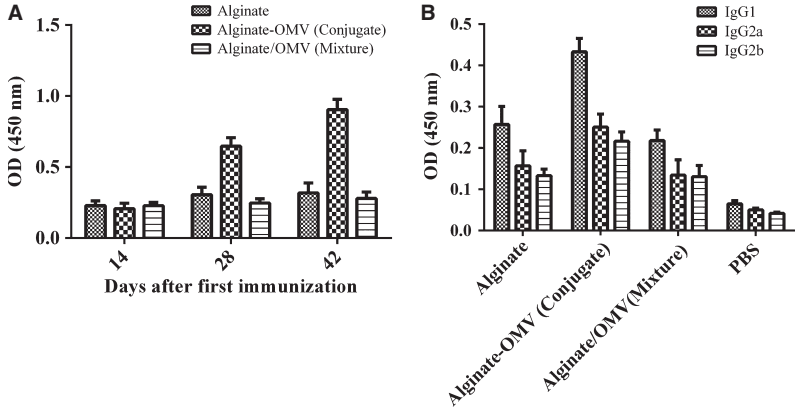

Fig. 2. The total anti-alginate-IgG titer was assayed using indirect ELISA, and the OD measured at 450 nm (A). Titers of specific IgG antibody subtypes in four immunized group of mice (B). All sera were analyzed 56 days after the first immunization. Sera (six mice per group) were collected from the orbital sinus following primary and booster immunizations. In the conjugated group, the anti-alginate antibody titer was higher than those of the other immunized groups. Bars represent the means of triplicate determinations, and the error bar represents S.D. The results were accepted to be significant at $p < 0.05$.

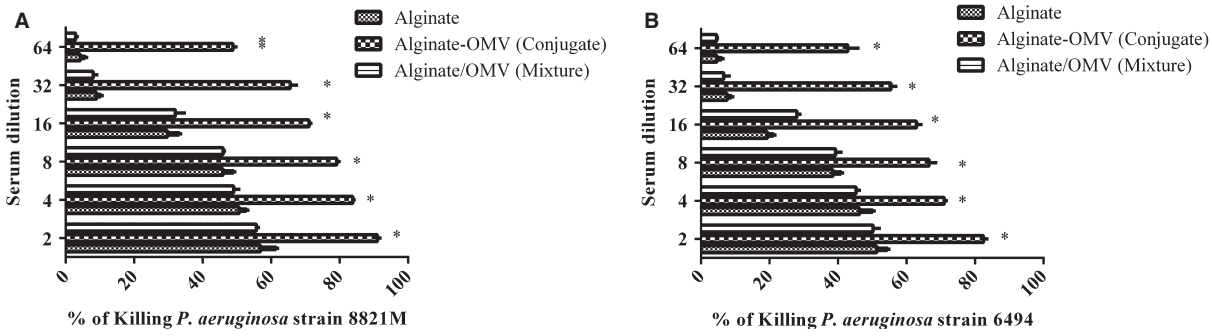

Fig. 3. The opsonic killing activity of different dilutions of anti-alginate IgG against *P. aeruginosa* strain 8821M 2 weeks after the final immunization with conjugate, native alginate, or the mixture of alginate and OMV (A). The opsonic killing activity of different dilutions of anti-alginate IgG against *P. aeruginosa* strain 6494 2 weeks after the final immunization (B). Bars represent the means of triplicate determinations, and error bars indicate the S.D. *$p < 0.001$; **$p < 0.05$ by ANOVA *post hoc* test.

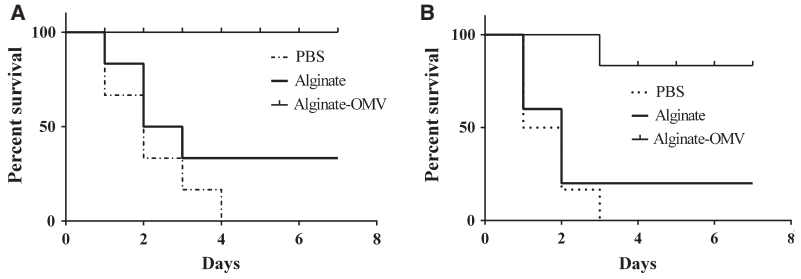

Fig. 4. Survival likelihood of mice following intranasal challenge with the *P. aeruginosa* 8821M or 6494 strains. Mice immunized with alginate–OMV conjugate or native alginate and challenged with *P. aeruginosa* 8821M [~6.4×10^8 CFU/mouse; log-rank test: alginate–OMV vs PBS (control group), $p = 0.0007$; alginate–OMV conjugate vs native alginate, $p = 0.0194$; native alginate vs PBS, $p = 0.2380$] (A), Mice immunized with alginate–OMV conjugate or native alginate and challenged with *P. aeruginosa* 6494 (~8.8×10^7 CFU/mouse; log-rank test: alginate–OMV conjugate vs PBS, $p = 0.0012$; alginate–OMV conjugate vs native alginate, $p = 0.0197$; native alginate vs PBS, $p = 0.5128$) (B).

strain 8821M caused an 81% reduction ($p < 0.05$) of the bacterial load in the lungs (Fig. 5A), and strain 6494 caused a 66.0% reduction ($p < 0001$) (Fig. 5B). The mice immunized with native alginate or the mixture and then challenged could not effectively eliminate the bacteria from their lungs.

Signs of macroscopic pathology in the lungs (hemorrhages and atelectasis) were also observed for the unimmunized groups as compared with the vaccinated group with alginate–OMV conjugate (Figure S6).

DISCUSSION

P. aeruginosa is the major cause of morbidity and mortality in CF patients (21). Alginate is overproduced by mucoid strains and has a major role in the infection process in CF patients by specialized mechanisms (38). It has been demonstrated that alginate can be a good antigen target for developing a vaccine due to its conserved epitope in mucoid strains (39, 40). Alginate, like most capsular polysaccharides antigens, is not a good immunogen. To overcome this problem, conjugation methods have been widely used (20–23). Therefore, we conjugated a strong and safe carrier protein to alginate by optimizing the conjugation process. We purified high molecular-weight alginate because previous studies showed that the high molecular size could elicit an antibody response against alginate (17, 22).

Selecting a suitable carrier protein is a key success factor for the efficacy of a conjugated vaccine. Depolymerized alginate was conjugated to exotoxin A of *P. aeruginosa* by Cryz et al. (20); however, the disadvantage of this conjugate has been explained previously (21, 22). Theilacker and his collogues conjugated underpolymerized alginate to keyhole limpet hemocyanin (KLH), but this carrier protein and the linker that was used for conjugation has never been used in humans as a bacterial conjugate vaccine (22). Kashef et al. conjugated tetanus toxoid (TT) to alginate. The disadvantage of such a conjugate was the frequent use of TT in other human vaccines, which could overload the immune system and might be an obstacle to the universal use of this carrier protein.

In this study, we used the outer membrane vesicle (OMV) of *N. meningitidis* serogroup B as the carrier protein due to its license for use in human vaccines (41). The efficacy, tolerability, and safety of OMV vaccines have been well proven (26).

In the present study, unconjugated alginate did not elicit a strong protective immune response, as expected (21, 22). Others previously reported that the combination of alginate with carrier proteins (unconjugated), such as KLH and type A flagellin of *P. aeruginosa,* could not raise protective IgG titers against alginate (22, 23). In this study, we expected to observe a significant rise in the IgG titers in the alginate/OMV (mixture)-vaccinated group against alginate because some studies (33, 42, 43) showed that the adjuvant effect of OMV could induce high titers of antibody against the targeted antigen. In spite of these findings, we found that immunizing the mice with the mixture of alginate and OMV did not increase the levels of protective IgG titers. A possible explanation for these conflicting results may arise from the composition of the vaccines. IFA, a water-in-oil emulsion, is one of the most commonly used adjuvants in research. IFA induces a predominantly Th2-biased response

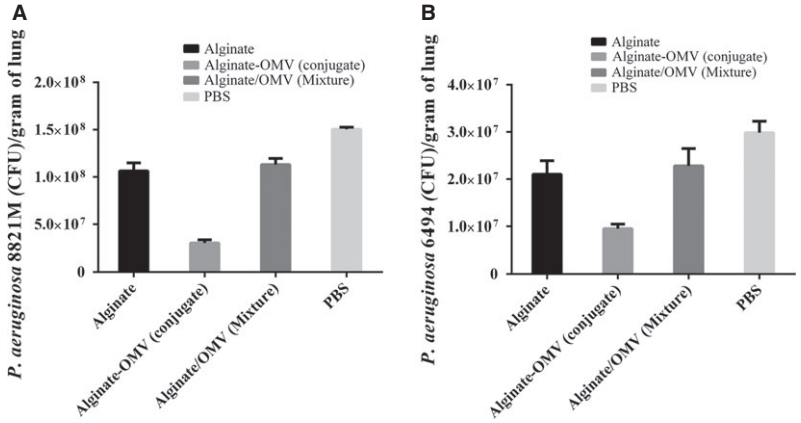

Fig. 5. Number of viable bacteria in lung homogenates of immunized mice. Mice were sacrificed as described above 18 h after infection, and then the bacterial loads in the lung homogenates were determined. Clearance of strain 8821M (~8×10^7 CFU/mouse) in vaccinated groups (A), Clearance of strain 6494 (~6.4×10^6 CFU/mouse) in vaccinated groups (B). The CFUs were calculated after enumerating the number of colonies on each LB plate. Bars represent the mean, and the error bars show the S.D. The results were accepted to be significant at $p < 0.05$.

through the formation of a depot at the injection site and slow release of the antigen with stimulation of antibody-producing plasma cells (44). IFA has been used in some human vaccine formulations (44). However, it is not currently used in humans, due to its side effects, which includes granulomas and abscesses at the site of injection (45). One potential limitation of IFA is that it does not augment the types of cellular immune responses that are thought to be critical to the control of many viral infections and tumors. OMV would be a relatively safe adjuvant with high potency for inducing a typical secondary response and also increasing the expression of co-stimulatory molecules on murine B cells. Adjuvants used in this study could potentially cause an increase in antibody response in both intensity and duration. Although the main aim of this work was not to determine the effect of adjuvants on the immune response against the alginate, we nevertheless found that using OMV (in unconjugated form) and IFA could not increase the antibody titer against the alginate when compared to alginate that was conjugated to OMV. This means that the adjuvant's effect on OMV cannot induce protection against a *P. aeruginosa* infection, unless it was coupled to alginate and acted as a carrier protein. Thus, based on these results, IFA might protect the conjugated alginate from both dilution and rapid degradation and elimination by the host. Campodo′nico (23) used Specol as the adjuvant for immunizing mice with polymannuronic acid–flagellin type of a conjugate. Further studies may be needed to explain the role of different adjuvants in the immune response to our conjugated vaccine candidate. These data showed that the covalent bond between alginate and OMV is the most important factor for provoking the immune system and shifting alginate from a T-cell-independent to a T-cell-dependent antigen when in its conjugated form.

Despite the high titers of antibody against alginate in CF patients, these patients are unable to eliminate lung colonization by *P. aeruginosa*. Therefore, it is assumed that antibodies to alginate are unable to mediate the killing of mucoid *P. aeruginosa*. By conjugating alginate to OMV, a high titer of opsonic antibodies was induced. When we tested the opsonic activity of the antibodies raised against alginate in the group of mice immunized with the conjugate vaccine, a strong opsonic activity against *P. aeruginosa* 8821M and the clinical isolate was found.

The results showed 100% protection in the group of mice hyperimmunized with the alginate–OMV conjugate compared with the control group. The protection rate was decreased to 83% after challenge with a clinical isolate (*P. aeruginosa* 6494), as expected, because fresh clinical isolates are usually more aggressive than standard strains. The bacterial load in the lung of the vaccinated mice (conjugate group) significantly decreased after nasal challenge with the *P. aeruginosa* 8821M and clinical isolate strains. However, it is not known if the opsonic antibodies can mediate the pulmonary clearance of *P. aeruginosa* or other immunological processes involved because Sukumarsaha reported that increased numbers of neutrophils and macrophages and high levels of IFN-gamma were responsible for the bacterial clearance from the lungs of mice immunized with a multivalent DNA vaccine (37).

A major surface component of all mucoid strains of *P.aeruginosa* is mucoid exopolysaccharide (MEP, also called alginate). Our vaccine candidate could elicit protective and opsonic antibodies against alginate derived from *P. aeruginosa* 8821M; additionally, further studies have shown that it could protect mice, not only mice challenged by *P. aeruginosa* 8821M but also against mice challenged by a clinical isolate strain (*P. aeruginosa* 6494). As most of the CF patients were infected by mucoid strains and most of these strains secreted the alginate, we can assume that this conjugate molecule may offer protection against most of the mucoid strains, although more studies are needed that focus on other mucoid strains.

In conclusion, using conjugation technology, we designed and synthesized an alginate–OMV conjugate vaccine that could protect mice against *P. aeruginosa* infection by eliciting high titers of opsonic, anti-alginate antibodies. It seems that this conjugate vaccine may protect CF patients from the colonization of *P. aeruginosa*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Gel filtration profile of *P. aeruginosa* 8821M alginate. Alginate passed through Sephacryl S-400 at an absorbance of 206 nm. Uronic acid was detected between fractions 11–17.
Figure S2. Electron micrograph of OMV. The vesicle diameter is approximately 80–120 nm (100 nm on average).
Figure S3. OMV preparation from *N. meningitidis* serogroup B separated in a 10% SDS-PAGE gel.
Figure S4. ^{1}H-NMR spectra for the solutions of alginate–OMV conjugate. Signal assignments are given in the text.
Figure S5. Zeta potential of alginate (A), OMV (B), and alginate–OMV conjugate (C). The potential of the conjugate was decreased to −19 mV after conjugation occurred between the surface groups of alginate and OMV.
Figure S6. Macroscopic illustrations of lungs infected with mucoid *P. aeruginosa* 8821M at 18 h post-infection. Picture of lung from a placebo control mouse indicating small atelectasis and moderate hemorrhagic areas (A). Lung section from a vaccinate mouse (alginate–OMV conjugate) without significant affected areas (B).