

Serum Anti-*Vibrio cholerae* Immunoglobulin Isotype in BALB/c Mice Immunized With ompW-Loaded Chitosan

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Abstract

Background: Chitosan, a linear polysaccharide, is a biocompatible and safe material for the delivery of therapeutic proteins and antigens, particularly via mucosal systems.

Objectives: In this study, the production of antibodies in response to outer membrane protein W (ompW)-loaded chitosan in BALB/c mice was evaluated.

Materials and Methods: Mice were subjected to intraperitoneal injection of ompW or nasal administration of ompW-loaded chitosan on days 1, 14, and 28, and the antibodies were measured on day 42 with ELISA.

Results: The titration of antibodies indicated that the nasal administration of ompW-loaded chitosan was better able to stimulate the immune response compared to intraperitoneal injections. However, the titration of total and IgG isotypes showed a significant difference between intraperitoneal and nasal immunization ($P < 0.01$). A significant difference was also seen in serum IgA isotypes at over 1/80 titrations, but not at lower dilutions ($P < 0.01$). Despite the serum antibodies, the results of lavage fluid analysis revealed that the IgG and IgA isotypes in the mice subjected to nasal immunization with ompW-loaded chitosan were significantly higher than in the other group ($P < 0.01$).

Conclusions: Based on the preliminary results presented in this research, it is suggested that ompW-loaded chitosan could be a suitable choice for nasal application to immunize the host against *Vibrio cholerae*. However, more work is required to determine the efficiency of the antibodies in neutralizing the bacterial toxin or bacterial movement.

Keywords: OmpW, Chitosan, Immunization, *Vibrio cholerae*

1. Background

Cholera is an endemic and life-threatening cause of diarrhea in many parts of the world (1). Infection with *Vibrio cholerae* (*V. cholerae*) results in widespread responses, from serologic to acute purging, and recovery from the infection depends on elimination of the *V. cholerae* bacteria by antibiotics or the patient's own immune response (2). Presence of specific antibodies against the bacteria or the cholera toxin or both of them in the circulation usually induce a resistance to infection (3). The outer membrane protein W (ompW) of *V. cholerae* is expected to stimulate the immune response and induce protective immunity in the host (4, 5). The 22-kDa polypeptide ompW is encoded by a gene located in the small chromosome (Chr II) of *V. cholerae*. Thus, ompW is very immunogenic and can be considered a suitable candidate for vaccine development (6). The induced protection against *V. cholerae* following immunization by 22 kDa ompW protein has been reported

to be mediated by the binding of antibodies to this protein, causing *V. cholerae* immobilization or agglutination. Therefore, these mechanisms interfere with the colonization process that is usually mediated by factors of pilus or non-pilus origin (7). The lipopolysaccharide (LPS) content of *V. cholerae*, in addition to the ompW protein, has been reported to induce a protective immunity in the host (8).

Chitosan, a linear polysaccharide derived from crustacean shells and fungal cell walls, has been used as a biocompatible and safe material for the delivery of therapeutic proteins and antigens, particularly via mucosal systems, during the last decade. Chitosan nanoparticles (NPs) have been reported to be more efficient than the solution form in enhancing drug activity (9-11). Chitosan consists of glucosamine and N-acetyl glucosamine derived through the partial deacetylation of chitin (12, 13). It can interact with mucus and epithelial cells to induce a redistribution of cytoskeletal F-actin, increasing the permeability of the epithelium. Thus, both chemical and physical methods

have been used to prepare chitosan-based particles loaded with proteins; however, major drawbacks are associated with the use of chemical cross-linking methods (14).

2. Objectives

The present study aimed to evaluate the immunization potency of recombinant ompW protein loaded on chitosan NPs. For this purpose, BALB/c mice were immunized, and the production of antibodies from different isotypes was evaluated, based on our previous experiment.

3. Materials and Methods

3.1. Preparation of ompW-Loaded Chitosan and Determination of Loading Efficacy

The recombinant ompW protein was prepared and characterized as previously described (5). Briefly, *E. coli* BL21 (DE3) containing pET28-ompW was inoculated into 100 mL of LB medium containing kanamycin (50 µg/mL), and gene expression was induced by isopropyl-β-D-1-thiogalactopyranoside (IPTG). The recombinant protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) resin and monitored with SDS-PAGE (15).

Next, 6 mg of chitosan was dissolved in 3 mL of 1% acetic acid containing 600 µg of ompW protein and 0.5% (w/w) Tween 80. Aqueous tripolyphosphate (TPP) solution (1.5 mg in 1.5 mL) was added drop-wise to the chitosan-ompW antigen solution while being stirred. Aliquots of 1 mL of the resulting antigen-loaded chitosan NP suspensions were centrifuged for 15 minutes at 10,000 g. The quantity of the protein in supernatant was assayed with the Bradford protein assay for evaluation of loading efficacy. The pellet was re-suspended in 10 µL of phosphate buffer saline (PBS) and stored at -20°C for immunization.

The amount of protein entrapped in the NPs was calculated by the difference between the total amounts added to the loading solution and the amount of non-entrapped protein remaining in the supernatant. A non-loaded NP suspension without Tween 80 was used as a blank to correct any unwanted interference by chitosan. The loading efficacy (LE) and loading capacity (LC) of the ompW-loaded chitosan NPs were calculated by the following equations, respectively:

$$LE(\%) = \left[\frac{(Ag_t - Ag_f)}{Ag_t} \right] \times 100 \quad (1)$$

$$LC(\%) = \left[\frac{(Ag_t - Ag_f)}{\text{nanoparticles dry weight}} \right] \times 100 \quad (2)$$

Where Ag_t = total amount of antigen and Ag_f = free antigen (16).

3.2. In Vitro Release

Recombinant ompW-loaded chitosan NPs were resuspended in 6 mL of 0.1 M PBS (pH 7.4), and kept at 37°C under magnetic stirring (100 rpm). At different time intervals, 0.5 mL of the suspension was taken and centrifuged (18,000 g for 15 minutes). The protein concentration in the supernatant was analyzed with the Bradford protein assay. The same volume of fresh PBS buffer was added to the release medium to reach the original volume. A sample consisting of only non-loaded chitosan NPs resuspended in PBS was used as a blank.

3.3. Immunization of Mice

Twenty-four BALB/c mice, 6 - 8 weeks old and weighing 20 g, were purchased from the Pasture institute of Iran and divided into three groups of eight mice each. All mice were housed in the animal unit for three days before any experiments were begun. Blood samples were collected from all mice before immunization, and the sera were kept at -20°C for further use as a control in each group, respectively.

3.4. Airway Immunization by ompW-Loaded Chitosan

In the first group, each mouse was exposed to 20 µg of ompW-loaded chitosan in 20 µL volumes by nasal administration. Booster immunizations were performed with the same antigen on days 14 and 28 after the first immunization. Blood samples were collected on day 42 after the first immunization and centrifuged at 3,500 g for 5 minutes. The sera were separated and stored at -20°C for measuring antibodies with an enzyme-linked immunosorbent assay (ELISA).

3.5. Intraperitoneal Immunization With ompW

The recombinant ompW protein was mixed with an equal volume of complete adjuvant, and 350 µL of mixed antigen was injected intraperitoneally into each mouse in group two. For booster immunization, ompW was mixed with an equal volume of incomplete adjuvant, then injected at the same time the boosters were given to group one. Blood samples were collected on day 42 of the first immunization and centrifuged at 3,500 g for 5 minutes, then the sera were separated and stored at -20°C for measuring serum antibodies with ELISA. The third group of mice was kept as a control for lavage fluid, under the same conditions but without ompW.

3.6. Preparation of Lavage Fluid

On day 42 of immunization, after the collection of blood samples, the mice were anesthetized and a sized was induced on the chest. Next, 0.5 mL of PBS solution was injected into the lavage through the trachea, then aspirated

three times. The fluid samples were centrifuged and the supernatants were stored at -20°C for measuring lavage antibodies with ELISA.

3.7. Measurement of Anti-ompW Antibody With ELISA

ELISA microplates were coated with $5\ \mu\text{g}$ of lysed *V. cholerae* in $100\ \mu\text{L}$ volumes, and left overnight at 4°C . Non-binding sites were blocked by adding $100\ \mu\text{L}$ of 5% skimmed milk solution to each well for 30 minutes at room temperature. The serum and lavage samples were brought out from 4°C and allowed to come to room temperature. Briefly, $100\ \mu\text{L}$ of different serum dilutions, ranging from 1/10 to 1/320, were added to each lysed *V. cholerae* pre-coated 96-well plate, then incubated for 60 minutes at 37°C . All experiments were done in duplicate.

The microplates were then washed three times with washing buffer, and $100\ \mu\text{L}$ of goat anti-mouse antibody (total antibodies) and goat anti-mouse IgM, IgG, and IgA horseradish peroxidase (HRP) conjugated antibodies (diluted 1/4000) (Sigma Aldrich Co.), were added to the respective wells and incubated for 1 hour at 37°C . The plates were washed again, tetramethylbenzidine substrate (TMB) was added in $100\ \mu\text{L}$ volumes, and the plates were incubated in a dark room for 25 minutes. Stopper solution was then added, and the absorbance was read at 450 nm using Multiscan MS Labsystem (17).

3.8. Statistical Analysis

The results are expressed as the mean of the experiments ($n = 8$) \pm standard error. The paired-samples t-test was used to determine the probability (P) of two sets of data in each group. An independent-samples t-test for the probability (P) between two groups and the ANOVA t-test for the probability (P) among all groups were also performed.

3.9. Human and Animal Rights

All protocols of the study were approved by the institutional animal ethics committee of Baqiyatallah University of Medical Sciences, which follows the NIH guidelines for the care and use of animals.

4. Results

4.1. Serum Antibodies

The ELISA results showed that total serum antibodies against *V. cholerae* in the group of mice subjected to nasal administration of ompW-loaded chitosan were increased in parallel to those receiving intraperitoneal injections of the antigen-adjuvant without chitosan (Figure 1).

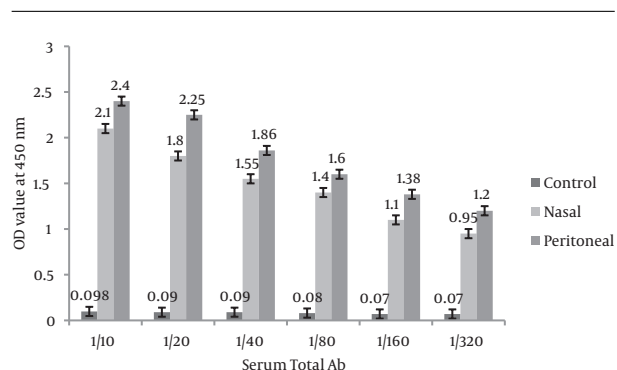


Figure 1. Titration of Mice Serum Total Antibodies Against ompW on day 42 After Immunization, by ELISA

However, there were significant differences between these two groups ($P < 0.01$). Both groups of mice produced high levels of IgM antibodies; however, the level of antibodies in the mice subjected to nasal ompW-loaded chitosan was lower than in the mice who received intraperitoneal ompW without chitosan ($P < 0.01$) (Figure 2).

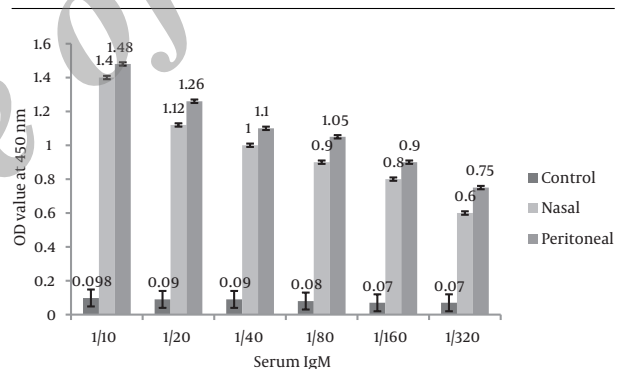


Figure 2. Titration of Mice Serum IgM Against ompW on day 42 of Immunization, by ELISA

In the case of IgG, the results revealed that both groups had high levels of IgG, indicating the efficiency of airway immunization ($P < 0.01$) (Figure 3).

The optical density value of serum IgA also indicated that ompW-loaded chitosan was able to induce a high level of this class of antibody compared to the other groups, and there were no significant differences between them at titers lower than 1/80 dilution (Figure 4).

4.2. Lavage Fluid Antibodies

The results indicated that the levels of IgA in the lavage fluid of both groups were higher than the levels of IgG. The mice subjected to ompW-loaded chitosan also

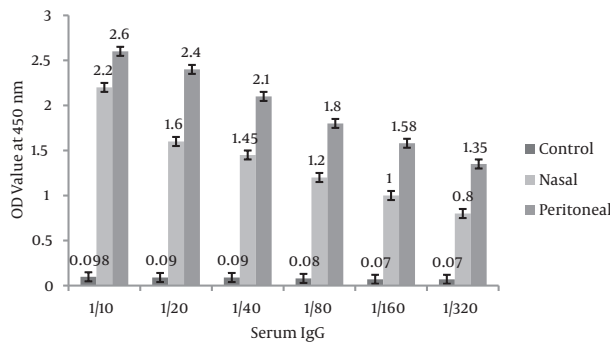


Figure 3. Titration of Mice Serum IgG Against ompW on day 42 of Immunization, by ELISA

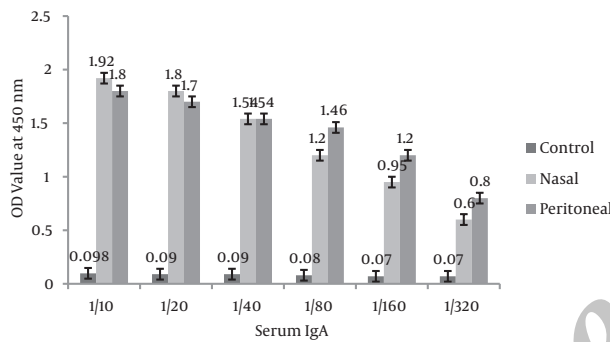


Figure 4. Titration of Mice Serum IgA Against ompW on day 42 of Immunization, by ELISA

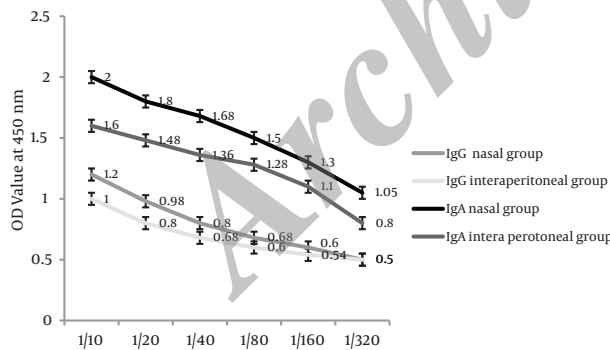


Figure 5. Titration of Lavage IgG and IgA Antibodies Against ompW on day 42 of Immunization, by ELISA

produced more IgA in the lavage fluid, compared to the intraperitoneal-injection group ($P < 0.01$) (Figure 5).

It was also shown that in the ompW-loaded chitosan group, the IgA levels in the lavage fluid were higher than in the serum samples ($P < 0.01$), demonstrating the effect

of airway immunization (Figure 6).

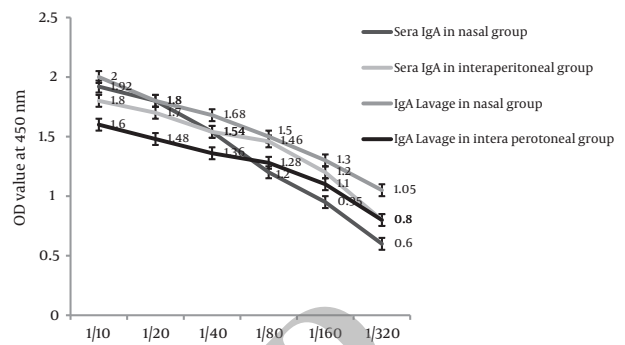


Figure 6. Comparison of Lavage IgA Antibodies Against ompW on day 42 of Immunization, by ELISA

5. Discussion

V. cholerae LPS has been known to induce a protective immune response, and is used as a cholera vaccine. The serum antibodies against *V. cholerae* LPS are elevated following natural disease or oral vaccination, which correlates to a protective ability against cholera (8). OmpW, in contrast to *V. cholerae* LPS, has been studied less. In general, it is known that natural infection with *V. cholerae* induces serum antibodies against a spectrum of cholera antigens that can be detected. Anti-Vibrio antibodies will also be increased by non-oral injection of bacteria. Most antibodies that rise against vibrio O antigen are likely vibricidal, and can kill the bacteria in the presence of complement.

In this study, the total antibodies and IgM, IgG, and IgA isotypes against *V. cholerae* antigen after nasal or intraperitoneal immunization were measured and compared. The enhancement of antibodies revealed that nasal administration of ompW-loaded chitosan was able to stimulate an immune response and produce specific antibodies from different isotypes. However, there were significant differences in the level of serum antibodies between mice subjected to oral administration versus non-oral injection of ompW antigen. Our results are in agreement with those of others who found high levels of IgM, IgG, and secretory IgA in the intestinal mucosal of immune individuals (18-20).

Animal studies may not correlate with the nature of protective antibodies in human cholera. However, based on data from animal experiments, it is possible to suggest that the level of serum antibodies is correlated to human resistance to cholera. It has also been reported that serum IgA levels directly correlate with the induction of protection against *V. cholerae* (21). However, one problem with ac-

quired immunity against cholera is the lack of long-lasting immunity after vaccination, which is one of the most desirable criteria for a candidate vaccine. Natural infection with *V. cholerae* induces at least three years of protection, while immune responses induced by the cholera vaccines developed so far are usually short-lived (20, 22).

The observation that natural infection confers effective and long-lasting immunity against cholera led us to develop a non-oral immunization technique using chitosan as the carrier of ompW in a mouse model. This method of immunization is not strange, as previous vaccinations with whole bacteria by oral injection, as well as oral immunizations with BCG, have been administered (23, 24). However, this might be the first time that a nanoparticle form of chitosan was used to deliver the ompW protein in an animal model to induce an immune response. These findings correlate with those of others that have shown that infection with *V. cholerae* significantly enhances the production of antibodies in the serum and intestines (19, 21). In agreement with our claim, Mokarram and Alonso reported that diphtheria-loaded chitosan was able to enhance anti-diphtheria antibodies following intranasal administration of antigen in BALB/c mice (25). Since all IgM, IgG, and IgA antibodies are known to be protective against *V. cholerae*, these results indicate the efficiency of chitosan in delivering the ompW peptide to antigen-presenting cells and stimulating the production of antibodies.

The IgG isotype usually promotes the lysis of bacteria through complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity, while IgA functions via receptor blockade. Therefore, this study's finding of high levels of IgG and IgA in mice subjected to nasal administration of ompW-loaded chitosan, as well as in those receiving intraperitoneal injections of ompW, suggests that ompW-loaded chitosan could be a suitable choice for inducing protective immunity against *V. cholerae*.

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Footnote

Authors' Contribution: Mahdi Fasihi-Ramandi and Sajjad Ahmadi-Renani contributed to the study design, analyses, and interpretation of the data. Hamideh Ghobadi-Ghadikolaee contributed to the development of the protocol and performed the experiments. Kazem Ahmadi conceived and supervised the project, and wrote the manuscript.

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