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Original Article

Evaluation of the immunogenic property of NT *H. influenza*e protein D with *Neisseria meningitidis* OMV in BALB/c

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Abstract

Introduction: Identifying ideal non typeable *Haemophilus influenzae* (NTHi) vaccine candidates has not been easy due to extensive sequence and antigenic variation among gene products interacting with the immune system. Protein D (PD) is a highly conserved 42 kDa surface lipoprotein available in all *H. influenzae*, including NTHi.

Methodology: In this study, the gene encoding PD was cloned from *H. influenzae* and expressed in *Escheriachia coli* TOPO10 cell in pBAD vector. Arabinose was used to express recombinant protein. In order to purify the protein, Ni-NTA agarose was used to perform affinity chromatography. Purified PD and PD mixed with outer membrane vesicle (OMV) and alum adjuvant were used for subcutaneous immunization in BALB/c mice. After vaccination, IgG responses to PD-OMV, PD-alum, and PD alone were determined by enzyme-linked immunosorbent assay (ELISA).

Results: The recombinant PD containing His6 residues showed a molecular weight of 42 kDa. Anti-PD IgG was detected after first immunization in all groups of mice compared to the negative control group, and it increased after first vaccination, but results showed that the addition of OMV to PD led to a remarkable increase in IgG responses.

Conclusions: Our results suggest an important role for OMV as an adjuvant and show how it could potentially be used when conjugated to *H. influenzae* PD or other safe subunit vaccine candidates.

Key words: vaccine; OMV; PD; *Haemophilus influenzae.*

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Introduction

Haemophilus influenzae is an important pathogen that colonizes the human upper respiratory tract and is also one of the most important causes of respiratory tract infections [1,2]. Prevention of nontypeable *H. influenzae* (NTHi) infections is an urgent goal for public health. Today, vaccines against typeable *Haemophilus influenzae* strains are being used extensively, but they do not protect against infections caused by NTHi strains [3]. Vaccination is the best tool for the prevention of infectious diseases [4,6]. To produce a vaccine against *Haemophilus influenzae* infections, several *H. influenzae* proteins have been studied [7,8]. The identification of NTHi vaccine candidates is not simple, because NTHi display extensive variations among different genes interacting with the immune system [3,9-10]. Therefore, efforts have been made to identify different bacterial components as possible antigens for vaccines. Among them, one promising antigen is the outer membrane protein D (PD) [11].

Previous studies have shown that PD of *H. influenzae* is antigenically conserved [12,13], and the sequence of the *hpd* gene has shown a limited variation [14]. Due to some properties of PD, such as surface exposure, antigenic conservation, and the role of D protein in *H. influenzae* infection, PD is a good candidate antigen for the development of vaccines against NTHi infections [15].

Subunit vaccines are usually combined with adjuvants. Adjuvants of microbial origin are frequently used. One example of an adjuvant of microbial origin is the outer membrane vesicle (OMV) of *Neisseria meningitidis* [16,17].

The aim of the present study was to evaluate immunogenic potential of recombinant protein D by using active immunization and to compare it to antibody responses in the presence of OMV as a

microbial adjuvant and an alum adjuvant as an industrial adjuvant.

Methodology

Bacterial strains and vectors

The NTHi strain (ATCC 49766) was kindly provided by the Pasteur Institute of Iran. The strain was grown overnight at 37°C in brain-heart infusion broth containing hemin (10 mg/mL) (Sigma-Aldrich, St. Louis, USA) and NAD (10 mg/mL) (Sigma-Aldrich, St. Louis, USA).

N. meningitidis serogroup B (ATCC 13090) was obtained from the Pasteur Institute of Iran and grown on chocolate agar medium. *E. coli* TOPO10 (Invitrogen, Carlsbad, USA) host and plasmid pBAD-gIIIA (Invitrogen, Carlsbad, USA) as an expression vector were used for cloning and expression, respectively.

Amplification of hpd

hpd gene was amplified by polymerase chain reaction (PCR) using chromosomal DNA of *H. influenzae* strain (ATCC 49766).

Primers were designed so that the complete gene could be amplified. Additionally, the primers were constructed so that they contained an *Nco*I site at the 5' end of the gene and *Xba*I site at the 3' end of the gene. The sequences of the primers were as follows: forward, 5' CATG**CCATGG**GACTTAAAACTTTAGC 3'; reverse, 5'GATC**TCTAGA**TGTTTTATTCCTTTTAAGAATT CC 3'.

PCRs were carried out in 50 µL volume containing 2 µL of DNA template, 5 µL of 10 × reaction buffer with $MgCl_2$, 2 µL of dNTPs (10 mM), 2 µL of each primer (10 pmol), and 1U of high-quality Pfu DNA polymerase (Thermo Fisher Scientific, Boston, USA). The PCR conditions for *hpd* amplification included initial denaturation for 5 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 45 seconds and extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The PCR product was recovered from the gel and purified using a PCR purification kit (Thermo Fisher Scientific, Boston, USA).

Cloning and expression of protein D

The PCR product was digested with restriction enzymes *Nco*I and *Xba*I and ligated into pBAD-gIIIA vector (Invitrogen, Carlsbad, USA), which was digested with the same enzymes. Ligations were performed by T4 DNA ligase enzyme, and the ligation mixtures were transformed into competent *E. coli* TOPO10. The colonies were selected by double-digestion analysis and DNA sequencing. Then, clones were grown at 37°C in LB broth that included 50 µg/mL ampicillin. Subsequently, arabinose was added at an A650 of 0.5–0.7, to obtain concentrations of 20%, 2%, 0.2%, 0.02%, and 0.002%. Cultures were allowed to grow for another 4 hours. The pBAD plasmids are pBR322-derived expression vectors, designed to regulate the secretion and purification of recombinant proteins. In the presence of L-arabinose, the protein expression controlled by the araBAD promoter is induced, while the absence of L-arabinose leads to a great decrease of protein expression [18].

Purification of recombinant PD

Protein was purified using a Ni-NTA column (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Purity of the eluted protein was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using a conjugated His-tag antibody (Roche, Basel, Switzerland). The purified protein was dialyzed stepwise against 4M, 2M, 1M urea, and phosphate-buffered saline (PBS) (pH 7.2) at 4°C overnight and quantified by the Bradford assay and NanoDrop (Invitrogen, Carlsbad, USA).

SDS-PAGE and Western blotting

SDS-PAGE was used to analyze protein expression. Bacterial pellets were suspended in protein loading buffer, then heated for 10 minutes at 95°C. At the end, 20 µL of each sample were loaded and run. For Western blotting, the proteins were separated by SDS-PAGE and transferred to a poly vinylidene difluoride (PVDF) membrane (Hi-bond Amersham eBioscience, San Diego, USA). Afterward, the PVDF membrane was inserted in nonfat milk 1% in PBS in order to block the membrane. According to the manufacturer's instructions, membranes were incubated with peroxidase-conjugated rabbit (that is, anti-mouse immunoglobulins) at a dilution of 1/6,000.

Endotoxin measurement

Endotoxin (LPS) levels of the purified protein were determined by chromogenic Limulus amebocyte lysate assay (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions.

Immunization of mice

Female BALB/c mice, 7–8 weeks of age, were purchased from the Pasteur Institute of Iran. Mice were divided into four groups (n = 10 per group): group I,

PD; group II, PD-OMV; group III, PD-alum (aluminum hydroxide); and group IV, PBS. The first three groups of mice were immunized subcutaneously three times (days 0, 14, and 28) with 50 µg of purified PD in sterile PBS, 25 µg of purified PD and 25 µg OMV as a microbial adjuvant, and 50 µg of purified PD emulsified in equal volume of alum adjuvant. The control group consisted of animals injected with sterile PBS. Immunization volume was 100 µL. Sera were collected two weeks after vaccination to evaluate the antibody responses.

Measurement of immune response

Total IgG (days 0, 14, 28, and 42) and IgG isotypes (IgG1 and IgG2a) (day 42) responses of immunized mice were measured by enzyme-linked immunosorbent assay (ELISA). Breifly, flat-bottom 96-well plates were coated overnight at 37°C with 5 µg of purified rPD in 100 µL of sterilized PBS. Then, the coated plates were blocked by 5% (w/v) bovine serum albumin (BSA) in PBS and then incubated with antisera (1:10, 1:100, and 1:1000 diluted in PBS/BSA) for 2 hours at 37°C. TPBS (Tween 20 and PBS) was used to wash the plates and then goat anti-mouse peroxidase conjugated with IgG, G1, and G2a diluted 1:6,000 in PBS/BSA were added to TPBS. After incubation at 37°C for 1 hour, plates were washed three times in TPBS, and then ABTS ELISA HRP Substrate (KPL, Maryland, USA) was added to each well. Reaction was stopped after 15 minutes by adding 1% SDS, and the absorbance was evaluated at 405 nm. All assays were done in triplicate.

Serum bactericidal assay

Serum bactericidal assay (SBA) was performed as previously described [19]. Following overnight incubation of *H. influenzae* on chocolate agar, individual colonies were inoculated into Mueller-Hinton broth. The bacteria were grown for approximately 3 hours to early log phase, pelleted by centrifugation, washed and resuspended in Dulbecco's PBS containing 9 mM CaCl2, 4.9 mM MgCl2, and 1% (w/v) BSA. In this study, SBA was performed using pooled serum specimens collected at weeks 0 and 6, and all sera were heat-inactivated for 30 minutes at 56°C. Ten percent (v/v) baby rabbit complement was used as an exogenous complement source [20-22]. Sterile 96-well flat-bottom plates were used for the micro-bactericidal assay. The mixture contained 25 µL of serially diluted sera in PBS, 12.5 µL of PBS containing 300 *H. influenzae* colony-forming units (CFU), and 12.5 µL of complement (20%, v/v). After adding all components to each well, a 10 µL aliquot of each well was spotted onto a chocolate agar plate and incubated overnight at 37°C in an atmosphere containing 5% CO_2. The 96-well plates were incubated for 90 minutes at 37°C, and then a 10 µL aliquot was taken from each well and spotted onto chocolate agar plates. Percentages of viable bacteria were calculated by comparing the respective CFU at 90 minutes with that at time zero in negative control samples [23]. All assays were performed in duplicate.

Statistical analyses

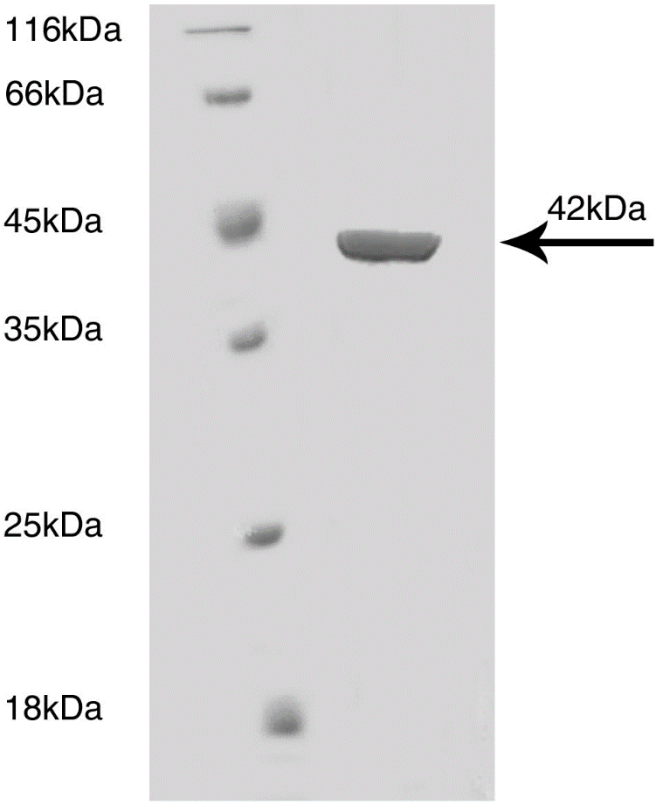
After data normalization, GraphPad Prism 5 was used to do statistical analyses. The data sets were compared with one-way ANOVA and t-tests. P values ≤ 0.05 were considered statistically significant.

Results

Expression and purification of PD protein

The amplified *hpd* PCR product (1,095 bp) was cloned into digested pBAD and the generated plasmid was moved into *E. coli* TOPO10 cells by transformation. Identity of the *hpd* gene in the

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15%, w/v) of purified recombinant protein D (PD) stained with coomassie blue G-250. Lane 1: standard protein size marker; lane 2: purified recombinant PD from Ni2+-sepharose column, molecular weight of 42 kDa.



recombinant construct was confirmed by colony PCR, double digestion using restriction enzymes, and sequencing.

The TOPO10 cells containing the recombinant plasmid were cultured at 37°C in the presence and absence of arabinose. SDS-PAGE with 15% gel was used to do analyses of whole-cell lysates. Following the arabinose induction, one defined band appeared at the 42 kDa position. Arabinose (0.002%) induction at 37°C for 4 hours was the best way to achieve high-level expression of recombinant protein (Figure 1). Ni-NTA affinity chromatography was used to purify the recombinant protein under denaturing conditions and the expression of purified His-tagged protein was confirmed by Western blot. Endotoxin level of the purified proteins was ≤ 0.01 EU/mL.

Serum antibody response after immunization

After vaccination, antibody responses against PD-OMV, PD-alum, and PD only were determined by ELISA. The BALB/c mice immunized with recombinant PD mixed with OMV and alum induced high levels of specific antibody responses (Figure 2). Anti-PD IgG was detected after first immunization in all mice, compared to the control group that increased after the first vaccination. The booster injections significantly increased IgG responses. IgG responses stimulated with PD-OMV and PD-alum were significantly higher than those induced by PD alone, but the difference between IgG response of mice vaccinated with both preparations was significant (Figure 2).

Although there was an increase in antibody titers (IgG) in the PD-recipient group, this response was low compared with the groups immunized with PD-OMV and PD-alum. PD-alum-immunized mice showed the highest response in the second booster, which showed statistically significant differences ($p = 0.0322$) compared with the first booster (Figure 2). In addition, the group treated with D-alum showed statistically significant differences in the responses in all injections, compared with the PD-treated mice. Moreover, the group immunized with D-OMV showed the highest responses in the second booster, which showed significant differences compared with the first booster (second injection) and the first injection ($p = 0.0023$ and 0.0001, respectively). The group of mice immunized with D-OMV depicted significant differences in low level of antibody responses compared with PD group in the first and second booster ($p = 0.0202$ and 0.001, respectively) (Figure 2).

The longevity of IgG responses after 34 weeks was also evaluated. Mice vaccinated with PD only showed

Figure 2. Induction of anti-protein D (PD) IgG responses. Mice immunized three times either with PD only, PD-alum, or PD-outer membrane vesicles (OMV). The control groups were treated with PBS. The bars represent the geometric means of obtained enzyme-linked immunosorbent assay signals. Error bars indicate ± SE in each group. IgG responses stimulated by PD-alum in the first injection and the following two injections (first and second booster) elicited statistically significant differences than that of PD alone and IgG responses stimulated by PD-OMV in the first and second booster elicited statistically significant differences than that of PD alone. A single asterisk indicates statistical significance over PD group ($p < 0.05$) and double asterisk indicates statistical significance over PD group ($p < 0.0001$). The response induced by the second booster of PD-OMV was statistically different compared to the second booster PD-alum ($p = 0.0022$).

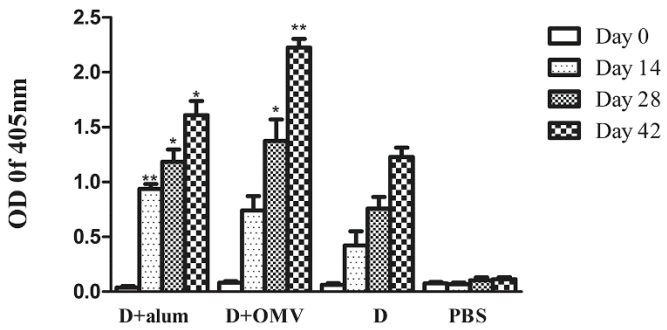
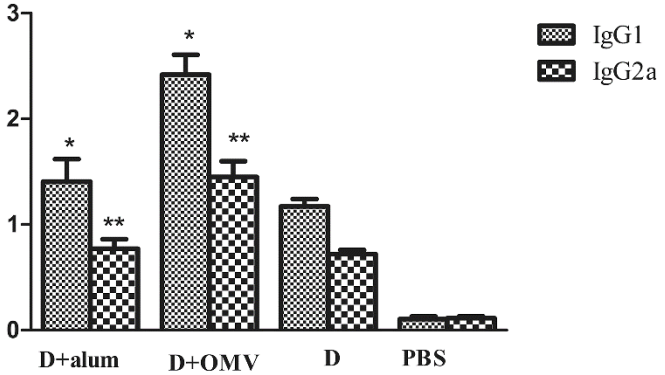


Figure 3. IgG subclasses antibody responses against protein D (PD) were determined by enzyme-linked immunosorbent assay (ELISA) using individual serum samples collected at day 42. The bars represent the geometric means of obtained ELISA signals for optimum dilution (1:1,000). Optimum dilution was determined prior to the comparisons by testing serially diluted sera against the coated antigen. Error bars indicate ± SE in each group. Single asterisks indicate statistical significance of IgG1 responses stimulated by PD-outer membrane vesicles (OMV) ($p < 0.0001$) and PD-alum ($p < 0.05$) over D group and double asterisks indicate statistical significance of IgG2a responses stimulated by PD-OMV ($p < 0.0001$) and PD-alum ($p < 0.05$) over D group.



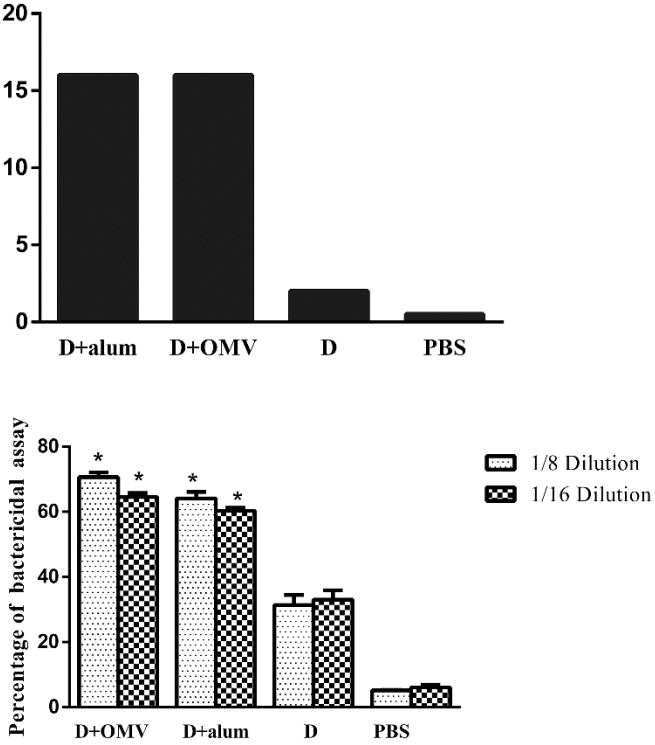
a decrease in the level of IgG, but IgG responses of other mice vaccinated with PD-OMV and PD-alum peaked after the last immunization (data not shown).

Mice sera were collected two weeks after the third vaccination, and IgG1 and IgG2a levels were assessed (Figure 3). Analysis of IgG subclasses displayed significantly higher IgG1 and IgG2a production in mice vaccinated with PD-OMV and PD-alum in comparison with PD-treated and control mice. D-OMV and D-alum treated groups revealed statistically significant differences in IgG1and IgG2a responses compared with the PD-immunized group. Moreover, the D-OMV recipient group elicited a significantly higher response of IgG1 than the D-alum-recipient group. It should be noted that IgG1 response was statistically higher than IgG2a (Figure 3). These results showed that addition of OMV to PD remarkably increased the IgG responses, and also indicated that OMV can act as a potent adjuvant to enhance the antibody responses.

Serum bactericidal assay

Antisera were tested for their ability to increase killing of *H. influenzae* strains *in vitro*. Sera from PD-OMV-vaccinated mice were strongly bactericidal against *H. influenzae* compared to the control group (PBS). Antisera raised against PD mixed with OMV and PD mixed with alum illustrated bactericidal activity against *H. influenzae* with titers ranging from 1/8 to 1/16 (Figure 4).

Figure 4. Bactericidal (BC50) titers are expressed as the reciprocal value of the highest serum dilution that yielded ≥ 50% of bacterial killing, compared to assay controls. Bactericidal titers of the titers on pooled sera collected at weeks 0 and 6. Single asterisks indicate statistical significance of bactericidal responses stimulated by protein D outer membrane vesicles (PD-OMV) (p = 0.0004 and 0.0005 in 1/8 and 1/16 dilution respectively) and PD-alum (p =alue 0.0009 and 0.0008 in 1/8 and 1/16 dilution respectively) over D group. All assays were performed in duplicate.



Discussion

NTHi has been considered an important pathogen in recent years. NTHi strains often establish infection locally in the nasopharynx, followed by bacterial migration to other sections of the upper respiratory tract by a contiguous spread [24]. Effective vaccines against Hib strains have been widely used, but they do not protect children against infections caused by NTHi strains [3]. To develop a vaccine that protects against Hib and NTHi infections, several surface-exposed *H. influenzae* proteins have been intensely studied [25-26]. Today, the mechanisms of action of many adjuvants have been elucidated and it has been shown that the majority of them activate the host immune system via interaction between toll-like receptors (TLRs) and pathogen-associated molecular patterns (PAMPs) [27]. Many studies have revealed a role of OMV as a trigger of the immune system via carriage of NOD1 and NOD2-PAMP [28].

We hypothesized that naturally produced OMVs may play an important role in activating innate immune responses and the mixture of OMV as a PAMP and the protein D as an antigen able to significantly increase the immunogenicity. Among *H. influenzae* antigenic proteins, protein D was reported to be highly immunogenic and in spite of its localization on the surface of all *H. influenzae*, it is genetically conserved [15]. Protein D could be used to design vaccines, since it is one of the most important *H. influenzae* virulence factors.

Our study showed that the IgG responses stimulated by the PD-OMV and PD-alum were significantly higher than those of PD alone. The difference between IgG responses of mice immunized with PD-OMV and PD-alum was significant. The results suggested that the PD could induce high levels of IgG responses after immunization, but IgG responses following stimulation with PD-OMV and PD-alum were significantly higher than PD alone. Our study showed that the level of anti-D IgG in mice immunized with a mixture of D and OMV was significantly higher than that in mice immunized with mixture of D and alum. Thus, these

results indicated that the immunogenicity of PD was enhanced by OMV as the adjuvant. Properties of OMV-derived particles have been demonstrated for potential cancer vaccines [29]. Overall, previous studies showed that the predominant outer membrane proteins (OMPs) (PorB and RmpM) from *N. meningitidis* present in the Cuban meningococci B vaccine had different capacities to prime the immune system [30].

Evaluation of IgG1 and IgG2a levels revealed significantly higher IgG1 and IgG2a production in mice immunized with PD-OMV and PD-alum compared to negative controls and PD alone. PD only induced higher levels of IgG1, but the addition of OMV to PD significantly increased both IgG1 and IgG2a responses. However, IgG1/IgG2a ratio indicated that both combinations directed immune responses toward Th2. These results suggest that OMV can act as a proper adjuvant.

Conclusions

The data obtained in this study indicated that conjugated PD with OMV as a vaccine candidate has the advantage of inducing strong humoral responses. We demonstrated that purified rPD with OMV elicits antibodies capable of inducing bactericidal activity against *H. influenzae*, which makes its potential to be a vaccine candidate against NTHi promising.

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