

Expression, Purification and Characterization of Three Overlapping Immunodominant Recombinant Fragments from *Bordetella pertussis* Filamentous Hemagglutinin

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Abstract

Background: Filamentous hemagglutinin (FHA) is one of the most important immunoprotective antigens of *Bordetella pertussis* (*B. pertussis*) and a major component of the acellular pertussis vaccine. In the present study, three overlapping recombinant fragments from the immunodominant region of FHA were produced and their immunogenicity was investigated.

Methods: Three overlapping coding sequences of FHA antigen were amplified from *B. pertussis* genomic DNA by PCR. Amplified fragments were expressed in *Escherichia coli* (*E. coli*) BL21(DE3) strain and purified through His-tag using Nickel-based chromatography. Purified fragments were characterized by SDS-PAGE and Western blotting techniques. In vitro peripheral blood mononuclear cells (PBMC) proliferation and IFN- γ production were assessed in a limited number of healthy adults vaccinated with a commercial acellular pertussis vaccine in response to all purified FHA fragments by H3-Thymidine incorporation and ELISA, respectively.

Results: Recombinant FHA segments were successfully cloned and produced at high levels in *E. coli* BL21(DE3). SDS-PAGE and Western blot analyses confirmed their purity and reactivity. All three recombinant fragments together with a commercial native FHA were able to induce in vitro PBMC proliferation and IFN- γ production.

Conclusion: Our preliminary results suggest that these overlapping recombinant FHA fragments are immunogenic and may prove to be immunoprotective.

Avicenna J Med Biotech 2013; 5(1): 20-28

Keywords: *Bordetella pertussis*, Filamentous hemagglutinin, Immunodominant, Prokaryotic expression, Recombinant antigen

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 Received: 11 Aug 2012
 Accepted: 9 Sept 2012

Introduction

Whooping cough, pertussis, is a highly contagious acute respiratory disease caused by a

gram negative coccobacillus, *Bordetella pertussis*¹. Before introduction of pertussis

childhood vaccination program in 1950's, pertussis was one of the major causes of infant death ². Global vaccination program of children with inactivated killed whole cell bacteria significantly reduced disease related mortality, but reactogenicity of the initial whole cell vaccines (Wc) led to generation of acellular pertussis subunit vaccines (Ac) composed of the main immunoprotective pertussis antigens, including Pertussis Toxin (PT), Filamentous Hemagglutinin (FHA), pertactin and fimbriae 2-3 antigens ³. Although Ac vaccines have been adapted in many developed countries, most developing countries still use Wc vaccines in their routine vaccination programs mainly due to the high cost of Ac vaccines ⁴.

Despite global vaccination coverage, pertussis re-emerged in the last two decades in both industrial and developing countries with an annual incidence of 40-50 millions resulting almost in 400,000 deaths according to the WHO report ³. Waning of protective immunity after natural infection or vaccination and shifting from reactogenic Wc vaccines to safer but less effective Ac vaccines are some reasons for pertussis resurgence ³. Both humoral and cellular arms of the immune system are involved in the protection against pertussis and interestingly the nature of the protective immunity is different between naturally infected patients and immunized people with either Wc or Ac vaccines ⁵. Therefore, employment of improved subunit components for Ac vaccine formulations and enhancement of their immunogenicity could boost the potency of Ac vaccines.

Filamentous hemagglutinin (FHA) is the major adhesion factor of Bp which is originally synthesized as a large 367 kDa precursor molecule ⁶. This precursor is modified to a mature 220 kDa secreted protein after proteolytic truncations ⁶. FHA is one of the major virulence factors involved in bacterial pathogenesis and an important immuno-protective component of Bp ^{5,7,8}. Using various approaches, a number of binding domains, and also B-cell and T-cell immuno-dominant epi-

topes have been identified within this large protein ⁹⁻¹¹. It has also been shown that the C-terminal region of mature FHA has the major immunodominant epitopes involved in bacterial immunogenicity and protective immunity ¹²⁻¹⁴.

In the present study, three recombinant overlapping fragments from the C-terminal region of FHA were produced at high levels in a prokaryotic expression system. Our preliminary *in vitro* results suggest that these recombinant fragments are immunogenic and resemble the native FHA fragments.

Materials and Methods

Bacterial strains

E. coli strains JM109 and BL21 (DE3) were purchased from Novagen (Merck KGaA, Darmstadt, Germany) and cultured in LB agar containing 1% w/v peptone (Merck KGaA, Darmstadt, Germany), 0.5% w/v yeast extract (Merck KGaA, Darmstadt, Germany), 0.6% w/v NaCl and 1.5% w/v agar (Merck KGaA, Darmstadt, Germany). LB broth medium constituents were the same as LB agar without agar.

Construction and expression of the recombinant proteins

Three overlapping regions from FHA coding sequence were selected and amplified from Bp genomic DNA for construction of the recombinant proteins designated as rFHA1-3 (Figure 1). Polymerase chain reaction (PCR) was performed for amplification of rFHA1-3 segments using specific primers containing EcoRI and HindIII restriction sites in both ends (shown as bold sequences): 5'-GAATTC TCGTCCGCAGATCACCGACGCGGT-3' as sense and 5'-AAGCTTATCGTGGCCTGCC

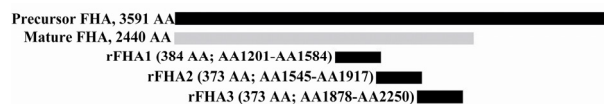


Figure 1. Schematic representation of precursor FHA, mature native FHA and the location of the three overlapping recombinant FHA fragments (rFHA1-3) employed in this study. The amino acid lengths and positions of all proteins are shown in parentheses

TTCAGG CTGC-3' as anti-sense for rFHA1, 5'-GAATTCTGACATCGTCATCAAGACG GAACAG-3' as sense and 5'- AAGCTTCTT GAAATACTCCATGGCGG ACAC-3' as anti-sense for rFHA2, 5'-GAAT TCTGACGAA CATCGCCATCTGCTCAAT -3' as sense and 5'-AAGCTTGCCGTCGGCAAGGGATGTC TGAG-3' as anti-sense for rFHA3. Twenty five μ l reaction mixture of PCR was prepared using 2.5 μ l 10 $\times$ PCR buffer, 10 pm of each primer, appropriate concentrations of MgCl₂ (1 mM for rFHA1 and rFHA2 and 3 mM for rFHA3), 2 μ l DMSO, 1 μ l of 10 mM dNTPs (Roche, Mannheim, Germany), 1 unit/reaction pfu DNA polymerase (Fermentas, Moscow, Russia) and 1 μ l Bp DNA as template. Each amplification reaction underwent initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 2 min and final extension was also performed at 72°C for 10 min. Taq DNA polymerase was added to the PCR reaction in the last five minutes of PCR program to add Adenosine to the end of PCR products for ligation into pGEMT-easy T-vector. Amplified products were visualized by ethidium bromide, purified from agarose gels (Fermentas, Moscow, Russia), directly cloned in pGEMT-easy cloning vector (Promega, Madison, USA) and transformed into *E. coli* JM109 competent cells.

After confirmation of the selected clones by sequencing, inserts were digested with restriction endonucleases EcoRI and HindIII (Fermentas, Moscow, Russia) and subsequently ligated in pET22b(+) expression vector (Merck Millipore, Darmstadt, Germany). *E. coli* BL21 (DE3) competent cells were transformed with pET22b(+)-rFHA1-3 constructs and positive clones were selected by colony-PCR.

The colony-PCR conditions for all FHA recombinant fragments were similar to their original PCR conditions, except it was run in 25 cycles using Taq DNA polymerase instead of pfu DNA polymerase. After confirmation by nucleotide sequencing, transformed cells were grown in LB broth containing 100 μ g/ml ampicillin. Finally, 1 mM IPTG was added when OD_{600 nm} was 0.6. After 3-4 hr incuba-

tion at 37°C, cells were harvested using centrifugation at 2000 g for 30 min at 4°C.

Purification of the recombinant proteins

Recombinant protein purification was performed in a denaturing condition. Harvested bacterial pellets containing inclusion bodies were solubilized in 20 ml of lysis buffer (100 mM NaH₂PO₄, 100 mM NaCl, 30 mM Tris-HCL, pH=8) and incubated on ice for 1 hr. This solution was continuously sonicated at 70% amplitude for 15 min for cell destruction and then centrifuged at 12000 g for 10 min. Supernatants were discarded and pellets were resuspended in buffer A (100 mM NaH₂PO₄, 50 mM NaCl, 10 mM Tris-HCL, 30 mM imidazole, 8 M urea, pH=8) and incubated at room temperature for 1 hr. After centrifugation at 12000 g, pellets were discarded and supernatants containing His-tagged recombinant proteins were applied as starting materials on Ni-NTA agarose (Qiagen, Germantown, Maryland, USA) column equilibrated with buffer A. Following the binding of recombinant proteins to column, the protein refolding process was performed by applying a descending continuous gradient of urea concentration from 8 M to zero for 3 hr. After refolding, elution was started by applying buffer B (100 mM NaH₂PO₄, 50 mM NaCl, 10 mM Tris-HCL, 80 mM imidazole, pH=8) to detach non-specific proteins from the column. Then, target proteins were eluted by applying buffer C (100 mM NaH₂PO₄, 50 mM NaCl, 10 mM Tris-HCL, 500 mM imidazole, pH=8). The purity of selected fractions was checked by SDS-PAGE and the proteins were then dialyzed against PBS. All fractions were concentrated using Amicon Ultra-4 centrifugal filter units (Merck KGaA, Darmstadt, Germany) followed by filtration with 0.2 μ m membrane filter (Merck KGaA, Darmstadt, Germany). Protein concentrations were determined by BCA colorimetric assay (Pierce, Rockford, IL, USA).

Western blotting analysis

Purity of the all recombinant proteins was checked by SDS-PAGE. For Western blot

analysis, 2 $\mu\text{g}/\text{well}$ of each purified recombinant rFHA1-3 was loaded on polyacrylamide gel, and after electrophoresis, all proteins were transferred to PVDF membranes (Merck KGaA, Darmstadt, Germany) using an electro-blot system (BioRad, Hercules, California, USA) at 100 V for 45 min. Membranes were blocked in PBS containing 0.05% Tween 20, and 3% skim milk at 4°C for 16 hr. The membranes were washed 3 times with washing buffer (PBS containing 0.05% Tween 20) and then incubated with appropriate concentrations of two different polyclonal antibodies including rabbit anti-FHA (produced in our lab) and rabbit anti-His tag (Abcam, Cambridge, UK) for 90 min at room temperature in a shaking condition. After 3 times washing, HRP-conjugated secondary antibody (produced in our lab) was added to membranes and incubation was performed under the same conditions of the primary antibodies. Finally, all membranes were detected using ECL system (GE Healthcare Life Sciences, Uppsala, Sweden).

***In vitro* assessment of human cell-mediated immunity to recombinant FHA fragments**

To evaluate the *in vitro* immune response against purified recombinant FHA fragments, four healthy volunteers (mean age 30 years and range between 26 to 33 years) were vaccinated with a single dose of Infanrix (GlaxoSmithKline Biologicals, Rixensart, Belgium), an FDA-approved commercial acellular pertussis vaccine containing pertussis toxin, FHA and pertactin. Heparinized peripheral blood samples were collected before immunization and at 1 week and 1 month after vaccination from all subjects. Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque (Sigma, ST Louis, USA) density-gradient centrifugation as described

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After twice washing with RPMI-1640 medium (Gibco-Biotech, Paisley, Scotland), separated PBMC were resuspended in preservation medium (RPMI supplemented with 10% dimethyl sulfoxide and 30% fetal calf serum [Gibco-Biotech, Paisley, Scotland]) and frozen

in liquid nitrogen until performance of the experiments. Proliferation responses of all pre- and post-vaccination samples from each subject were tested in the same run. Cell proliferation was measured by culturing 2×10^5 PBMC in 0.2 ml of complete culture medium (RPMI medium supplemented with 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 10% fetal calf serum) in flat-bottom 96-microwell culture plates (Nunc, Roskilde, Denmark). All cultures were performed in triplicate.

Purified recombinant FHA fragments (5 $\mu\text{g}/\text{ml}$) together with a commercial native FHA (2 $\mu\text{g}/\text{ml}$) (NIBSC, Hertfordshire, UK) as a control, and phytohaemagglutinin (PHA) (5 $\mu\text{g}/\text{ml}$) as a mitogen were added to PBMC culture and the plates were incubated at 37°C in a humidified atmosphere with 5% CO₂. After 48 hr, supernatants were harvested for measurement of secreted IFN- γ and fresh culture medium was added. H3-Thymidine (Perkin Elmer, Massachusetts, USA) was added to culture at 1 $\mu\text{Ci}/\text{well}$ on day 2 for PHA-stimulated wells and on day 6 for antigens-stimulated wells and after 18 hr incubation, cells were harvested. The level of radioactivity (counts per minute-CPM) of all wells was measured by a beta counter system (Beckman Coulter, Brea CA, USA) and stimulation index was calculated by dividing the mean CPM of cells exposed to the FHA antigens or PHA by the mean CPM of unstimulated cells incubated with medium alone.

ELISA for IFN- γ determination

Levels of IFN- γ in culture supernatants of stimulated PBMC were measured by Sanquin IFN- γ ELISA kit (Sanquin, Amsterdam, Netherlands) according to the manufacturer's instructions. The kit sensitivity for IFN- γ detection was 5 pg/ml.

Statistical analysis

Data analysis was performed using SPSS 15.0 for Windows. Paired sample t-test or Wilcoxon test was appropriately used for statistical analysis. The p-values less than 0.05 were considered significant.

Results

Construction and expression of the recombinant proteins

The coding sequences of rFHA1, 2 and 3 segments were amplified from Bp Tohama-I genomic DNA by PCR. Agarose gel electrophoresis of the amplified PCR products confirmed their 1152, 1119 and 1119 *bp* sizes, respectively (Figure 2A). Sequencing of all

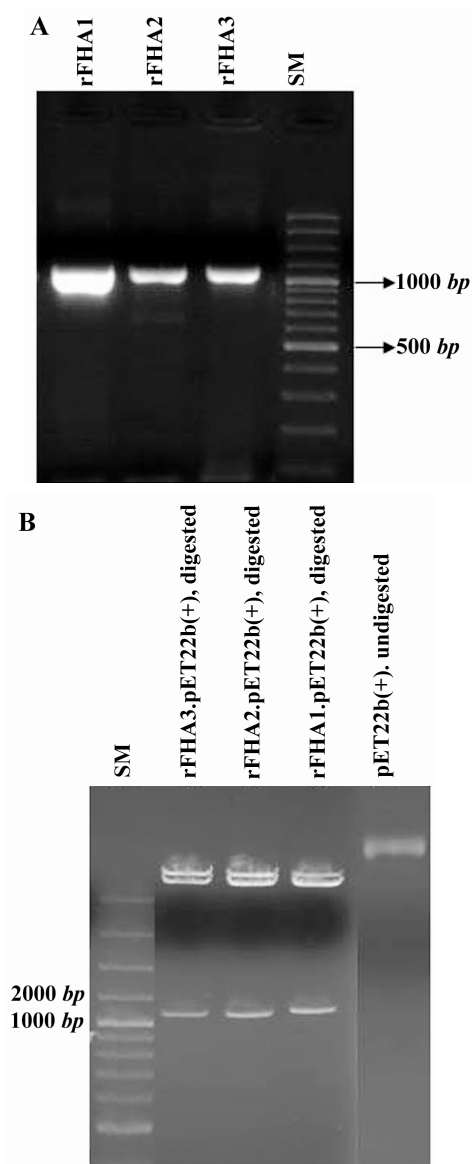


Figure 2. Representative results of PCR amplification; A) and restriction enzyme digestion; B) of the three FHA coding sequences. Agarose gel electrophoresis of amplified PCR products of rFHA1-3 fragments confirms their 1152, 1119 and 1119 *bp* sizes, respectively. Double digestion of pET22b(+)-rFHA1-3 constructs with EcoRI and HindIII endonucleases indicates the proper insertion of rFHA1-3 DNA segments into the expression vector. SM: DNA size marker

three gene segments after cloning in pGEMT-easy cloning vector demonstrated complete homology with the reference genome sequence of *B. pertussis* Tohama-I strain (NCBI GeneBank accession number: M60351) (data not presented). Insertion of the rFHA1-3 segments into pET22b(+) expression vector was verified by restriction analysis using EcoRI and HindIII restriction endonucleases (Figure 2B) and confirmatory sequencing was performed on pET22b(+)-rFHA1-3 constructs before transformation into *E. coli* BL21(DE3) expression host. For optimization of induction of all three recombinant proteins, a final concentration of 1 *mM* IPTG, $OD_{600\text{ nm}}=0.6$ and 3-4 *hr* incubation at 37 °C after IPTG induction were set up. Upon IPTG induction, all three recombinant FHA proteins were expressed at high levels (20-35% of total bacterial extract protein) in *E. coli* BL21(DE3) with approximately 40 *kDa* molecular weight (Figures 3A, B and C).

Purification and structural characterization of the recombinant proteins

SDS-PAGE analysis of final products purified by Ni-NTA agarose showed highly pure preparations for all three segments (Figure 4A). For final confirmation of the identity of recombinant proteins, immunoblotting analysis was performed using an anti-FHA antibody together with an anti-His tag antibody (Figure 4B).

Functional characterization of the purified recombinant FHA fragments

To assess immunogenicity of the recombinant FHA fragments, their abilities to induce *in vitro* proliferation of PBMC isolated from healthy individuals immunized with a commercial Ac pertussis vaccine containing pertussis toxin, FHA and pertactin was evaluated. Along with the recombinant fragments, a commercial native FHA antigen as a positive control and PHA as a polyclonal activator were also included. Proliferation results indicated that all samples strongly responded to PHA (Figure 5A). Compared to pre-vaccination samples, proliferative responses of

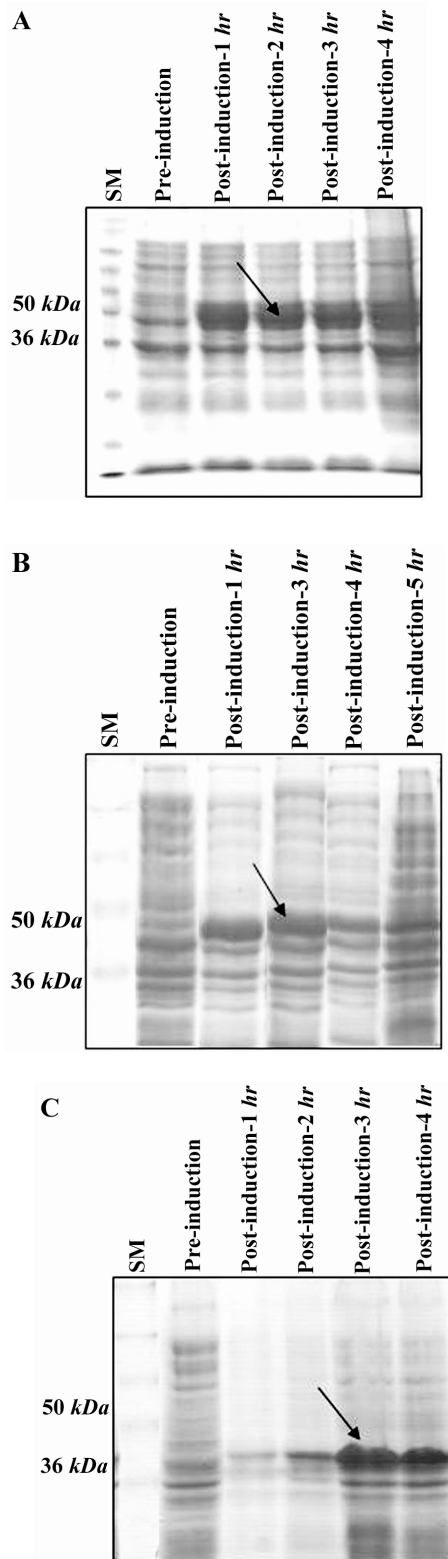


Figure 3. Induction of rFHA1; A), rFHA2; B) and rFHA3; C) proteins expression in *E. coli* BL21 (DE3). IPTG was added to a logarithmic liquid culture of transformed bacteria at 1 mM concentration when OD_{600nm} was 0.6. Pre-induction and post-induction samples were collected at different time points and run on 12% SDS-PAGE followed by Coomassie blue staining. The arrow in the middle of the gel shows the expected target protein molecular weight (~ 40 kDa); SM: protein size marker

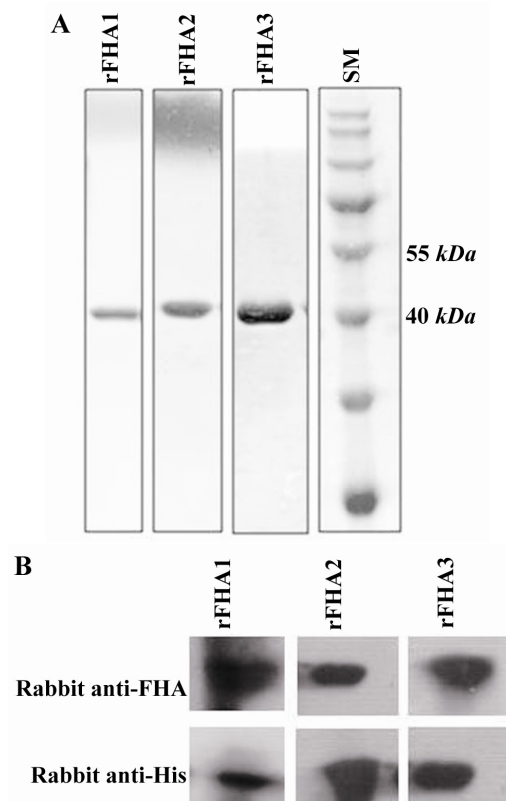


Figure 4. Analysis of the purified recombinant FHA fragments by SDS-PAGE; A) and Western blotting; C). All proteins were purified through His-tag by Ni-NTA column. SDS-PAGE analysis was performed using a 12% polyacrylamide gel followed by Coomassie blue staining. Western blot analyses of rFHA1-3 fragments were performed using two different preparations of detecting antibodies, including rabbit anti-FHA produced in our lab and a commercial rabbit anti-His tag. SM: protein size marker

PBMC in 4 weeks post-vaccination samples were significantly increased for all native and recombinant FHA proteins ($p < 0.05$) (Figure 5A). Although the proliferative response of 1 week post-vaccination samples was higher than that of pre-vaccination for all native and recombinant FHA, it was only significant for native FHA and rFHA2 ($p < 0.05$). Production of IFN- γ was significantly higher in 4 weeks post-vaccination as compared to pre-vaccination samples for all native and recombinant FHA antigens ($p < 0.05$) (Figure 5B). Similar to the proliferative response, the levels of IFN- γ in 1 week post-vaccination samples were higher than those of the pre-vaccination counterparts, but due to the limited number of samples tested, the difference was not statistically significant. PHA induced several folds

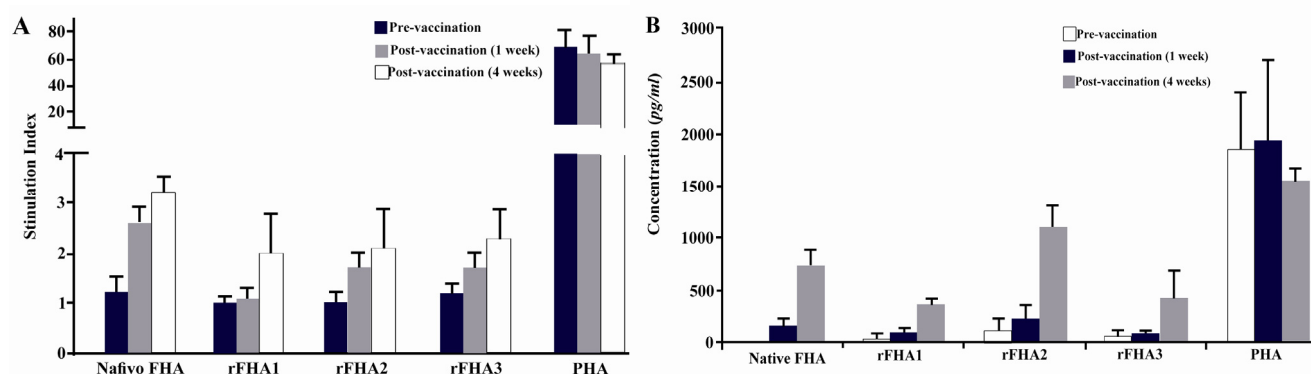


Figure 5. *In vitro* cell proliferation; A) and IFN- γ production; B) induced by native and recombinant FHA proteins. Peripheral blood mononuclear cells (PBMC) were collected from four healthy adults before and at 1 and 4 weeks after vaccination with a commercial acellular pertussis vaccine. 2×10^5 cells were stimulated with appropriate concentrations of native commercial FHA ($2 \mu\text{g/ml}$), three recombinant FHA fragments ($5 \mu\text{g/ml}$) and PHA ($5 \mu\text{g/ml}$). After 2 days stimulation for PHA and 6 days stimulation for FHA antigens, H3-Thymidine was added and the cells were harvested after 18 hours stimulation. Stimulation indices (SI) were calculated by dividing the mean counts per minute (CPM) of cells exposed to the FHA or PHA to the mean CPM of unstimulated cells incubated with culture medium alone. For IFN- γ assay, culture supernatants were harvested 48 hours after stimulation. For both proliferation and IFN- γ production, mean values $\pm$ SEM obtained from four subjects are indicated

higher levels of IFN- γ as compared to native or recombinant FHA proteins.

Discussion

Currently in many developed countries, the reactogenic Wc pertussis vaccines have been replaced by safer Ac subunit vaccines^{3,16}. The traditional Wc pertussis vaccines are still employed for mass vaccination of children in many countries, mainly due to the high cost of Ac vaccines. Application of recombinant DNA technology to produce components of acellular vaccines, which reduce the production costs, can be a solution to shift from Wc to Ac pertussis vaccines in developing countries. Production of recombinant pertussis antigens, especially the main components of acellular vaccines, including PT, FHA and pertactin has long been studied by many investigators^{4,8,17-20}, but to date there is no approved recombinant pertussis subunit vaccine.

In this study three overlapping recombinant fragments from the immunodominant region of FHA, an important immunoprotective antigen of Bp and a main constituent of most Ac vaccines, were produced at high levels and their capabilities to induce *in vitro* PBMC proliferation and IFN- γ production were preliminary assessed in a small number of samples.

Mature FHA is secreted as a large protein with 220 kDa molecular weight and nearly 2370 amino acids length from the 367 kDa precursor after C-terminal proteolytic cleavage^{6,21}. To date at least three binding motifs or domains, including RGD motif (amino acids 1097-1099)⁹, carbohydrate recognition domain (CRD) (amino acids 1141-1279)¹⁰ and heparin binding domain (HBD) (amino acids 442-863)¹¹ have been identified in this large molecule. In addition, multiple reports have been published on identification of the immunodominant T- and B-cell epitopes of FHA using different approaches^{12,13,22-26}.

Most published studies have demonstrated that B-cell recognizing epitopes are located within the C-terminal part of FHA^{13,22,24,25}. In a comprehensive study, Leininger *et al* have investigated the reactivities of different native and recombinant FHA preparations with a panel of monoclonal anti-FHA antibodies as well as sera from convalescent patients and samples from subjects vaccinated with Wc or Ac vaccines¹³. They found a region of FHA spanning amino acids 1653-2111 designated as type I region as the most immunodominant part of FHA for both human and mouse B-cells¹³. Later on they confirmed their results by antigenic analysis of FHA with fragments obtained from phage display libraries and

polyclonal anti-FHA antibodies²⁴. In agreement with these studies, the reactivity pattern of seventeen serum samples from convalescent pertussis patients was investigated with twelve FHA recombinant fragments. The results showed that the B-cell epitopes are mostly located in a region between amino acids 1760-2267²⁵. Furthermore, FHA specific T-cells were cloned from a patient recovered from whooping cough and a subject vaccinated with Wc vaccine. These clones were then studied to assess their proliferative response to different recombinant FHA fragments¹². This study defined a fragment of FHA spanning amino acids 1653-2347 as the T-cell immunodominant region¹².

Considering these findings, we produced and characterized three overlapping fragments containing the immunodominant epitopes of FHA including rFHA1 (from aa 1201 to aa 1584- a total of 384aa), rFHA2 (from aa 1545 to aa 1917- a total of 373aa) and rFHA3 (from aa 1878 to aa 2250- a total of 373aa). All three fragments were successfully expressed in *E. coli* BL21(DE3) and efficiently purified by NiNTA chromatography. All these recombinant fragments were able to induce a proliferative response and IFN- γ production in PBMC from healthy individuals vaccinated with a commercial Ac pertussis vaccine, suggesting their potential capability to trigger a Th1 response. The responses obtained for post-vaccination samples, particularly PBMC samples collected 4 weeks after vaccination were significantly higher than those of the pre-vaccination samples. Significance of our results in terms of both stimulation index and IFN- γ production is very encouraging and important given the limited number of samples tested.

Despite the overall consistency observed between the results obtained from the two assays regarding the responsiveness of the post-vaccination PBMC samples compared to the pre-vaccination samples, the magnitude of the response is not proportional and differs between the two assays, particularly for rFHA2 fragment. Such differences are not very unex-

pected given the fact that they measure two different biological parameters of the stimulated PBMC, and also the small number of samples tested. Secretion of IFN- γ in response to rFHA2 was higher than the other two fragments, which implies higher immunogenicity of this fragment.

Assessment of immunogenicity and antigenicity of these fragments in a larger number of subjects is being planned. We are also embarking on assessment of immunoprotectivity of these recombinant fragments in a mouse intranasal challenge model of pertussis. Since FHA is an important component of all Ac pertussis vaccines, identification of immunogenic and protective properties of rFHA1-3 fragments may pave the way for their use in the new Ac pertussis vaccine formulations.

Acknowledgement

This study was supported in part by grants from the Ministry of Health and Medical Education of Iran and Avicenna Research Institute. The authors thank Mr Fathollah Tahmasbi and Mrs Akbari for their technical assistance.

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