ARTICLE

A recombinant hybrid peptide composed of AAF adhesin of enteroaggregative *Escherichia coli* and Shiga toxin B subunit elicits protective immune response in mice

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Abstract Shiga toxin producing *Escherichia coli* (STEC) are a group of diarrheagenic *Escherichia coli* (*E. coli*) whereby Shiga toxin is the main virulence factor. It is composed of an A subunit, which mediates toxicity, and a B subunit (StxB), which is a nontoxic homopentameric protein responsible for toxin binding and internalization into target cells by interacting with the glycolipid, globotriaosylceramide (Gb3). Enteroaggregative *Escherichia coli* (EAEC) are a group of *E. coli* with aggregative adherence to epithelial cells, which play an important role in its pathogenesis. EAEC are the cause of diarrhea in developing countries and in the developed world. Aggregative adherence fimbria (AAF) of EAEC represents the adhesin that confers the presence of aggregative adherence (AA) phenotype on EAEC strains. The gene encoding non-toxic B subunit of Shiga toxin (StxB) was coupled to aggregative adherence fimbriae (AAF) of the EAEC structural gene. The resulting polypeptides (B-AAF/I, B-AAF/II) were designed to elicit immune response in immunized mice with recombinant peptides. The antibody, hence obtained, inhibited the adherence of prototype EAEC strains to HeLa cells and, on the other hand, protected the immunized mice against a lethal dose of Shiga toxin. Therefore, this promising data could indicate that this kind of polypeptide strategy is a good candidate for any probable vaccine design against diarrheal infection.

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Introduction

Antibacterial vaccines have been used for bacterial protection. Efficacy of these vaccines mainly depends on their ability to elicit protective antibodies. In most cases peptide vaccines fail to elicit efficient immune and clinical responses [1].

Shiga toxin from STEC is composed of an A subunit, which mediates toxicity, and a B subunit (StxB), which is a non toxic homopentameric protein responsible for toxin binding and internalization into target cells by interacting with the glycolipid globotriaosylceramide (Gb3) [2]. It has been recognized that *E. coli*-producing Stx and, as now known, other putative virulence factors, are the major causes of pediatric Hemolytic uremic syndrome (HUS) [3]. On the other hand, the EAEC-defining criterion-aggregative adherence suggests that adhesion has an important role in pathogenesis. Microscopy, genetic and phenotypic studies showed that enteroaggregative *Escherichia coli* (EAEC) adhesins are multiple and diverse. The aggregative adherence of EAEC is due to the presence of aggregative adherence fimbria (AAF), AAF/I, AAF/II and AAF/III [4, 5]. The first EAEC adhesin described at the molecular level was the aggregative adherence fimbriae I (AAF/I), expressed by EAEC strain 17–2. The cloned AAF/I fimbriae conferred the aggregative phenotype and agglutination of human erythrocytes on non-pathogenic *E. coli*. EAEC strain O42, which has been shown to be pathogenic in volunteers and does not express AAF/I fimbriae; instead, it expresses the antigenically distinct AAF/II fimbriae. The structural subunits for AAF/I and II fimbriae are 25% identical and 47% similar [6]. EAEC was described such that the bacteria appear as 'stacked brick' clumps which adhere to both the HEp-2 cells and the glass cover slip. The aggregative adherence of EAEC is due to the presence of

aggregative adherence fimbriae (AAF), AAF/I, AAF/II, and AAF/III [7, 8]. Adhesins are primarily what bacteria use to colonize the intestinal tract. Blocking the primary stages of infection, namely, bacterial attachment to the host cell receptors and colonization of the mucosal surface, may be the most effective strategy to prevent bacterial infections [9]. Bacterial adhesins are considered targets for vaccine development [9].

Therefore, in this investigation, the B subunit of Shiga toxin was fused to the structural genes encoding AAF/I and AAF/II subunits of the EAEC strain, i.e. 17–2 and 042, respectively.

The polypeptides hence obtained were examined in vivo in BALB/c mice for induction of antibody. The antibody obtained from immunized mice was evaluated for inhibition of adhesion and protection of the challenged immunized mice with a lethal dose of Shiga toxin.

Materials and methods

Construction of B-AAF/I and B-AAF/II hybrid peptide

E. coli strains O157, 17–2, 042 were used as bacterial strains for PCR amplification of the B subunit gene of Shiga toxin and structural genes of AAF/I and AAF/II, respectively [10–12]. Sequences of primers used for amplification and fusion of the peptides are shown in Table 1. The PCR condition for fusion of PCR fragments was as follows: denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min, repeated for five cycles; continued by denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, repeated for 20 cycles. The amplified fragments of AAFI/II (*aagA*, *aaf*A) and B subunit gene were fused as B-AAFI/II chimeric gene (Fig. 1). The amplified fused fragments were cloned in pTZ57R vector (MBI Fermentas). Each subunit was amplified separately by specific primers designed for amplification of subunits with and without N-terminus leader sequence. The amplified genes were cloned in a pBAD (Invitrogen Life Technologies) expression vector (Fig. 2). Different constructs have been made in order to express B-AAFI/II (in pBAD expression vector) by using gene III secretion signal in Top10 transformed bacteria (pBAD-B, pBAD-AAF/I, pBAD-AAF/II, pBAD B-AAF/I, pBAD B-AAF/II). The expression of the cloned genes was induced by different concentrations of L-arabinose (0.00002%–0.2%) under the control of ara promoter of the pBAD vector. Pellet of the induced clones were used for purification of the expressed protein. Expressed hybrid polypeptides were isolated by Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) and eluted from 12% SDS-PAGE gel by Electro-Eluter (Bio-Rad Laboratories).

The purified derivatives were applied to SDS-PAGE and electro transferred to a PVDF nylon membrane (Roche) after electrophoresis for further confirmation.

Protein bands were detected by the sera prepared from previous studies [13, 14]. The sera and the secondary antibody were diluted 100 and 1,000 times, respectively, before use.

Accession number

The B-AAFI/II constructed genes under study were sequenced, aligned with the Blast program for homology and the sequences obtained were deposited into GenBank under accession numbers EU368147 and EU368148.

Immunization of mice

Female BALB/c mice of age 6–7 weeks were purchased from the animal facility of Pasteur Institute of Iran. The animals were used in this study housed in standard Plexiglas cages with free access to food (standard laboratory rodent's chow) and water. The animal house temperature was maintained at 23±3°C with a 12 h light/dark cycle (light on from 6 AM). All animal experiments were carried out in accordance with the European Communities Council directive of 24 November 1986 (86/609/EEC) in such a way to minimize the number of animals and their suffering.

Mice were subcutaneously immunized on days 0 and 14 with 100-µl containing 100 µg of peptides. In groups of six, mice were injected with an equal amount of Complete Freund's Adjuvant (CFA) and a booster dose with the same dose of the antigens with an equal volume of Freund's incomplete adjuvant (Sigma) with each peptide alone or hybrid peptide (Table 2) on days 21 and 35. Sera were collected two weeks after the last immunization. The immunized mice were used for the challenge experiment. In another set of experiments, the immunization was performed with the same amount of the antigens without using adjuvant.

Evaluation of B and AAF specific antibody

Enzyme-linked immunosorbant assays (ELISA) were used for detection of immunoglobulin G (IgG) antibodies to pepides. Eluted peptides were dispensed to a 96-well plate (10 µg phosphate-buffered saline (PBS)/well) and incubated at 4°C for 16 h. After four washings with PBS/Tween20, wells were blocked for 1 h with 2% skim milk (Merck). Serial dilutions of serum samples were added and incubated at room temperature for 2 h. Peroxidase-conjugated goat anti-mouse IgG (Sigma) was added as the secondary antibody. For total IgG, 1:500

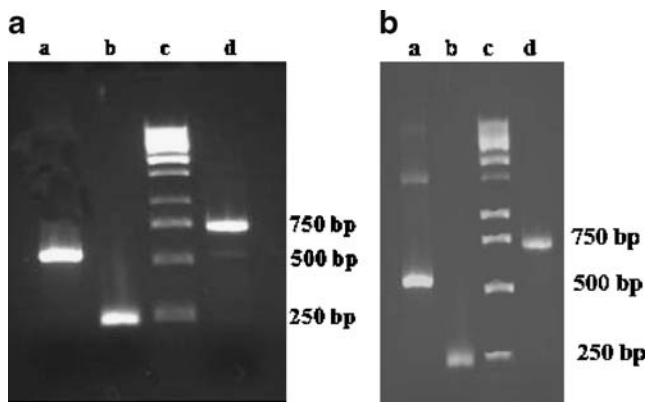**Table 1** Oligonucleotide primers used to amplify genes

Target gene	Oligonucleotide sequence (5′ 3′)	Predicted size (bp)
Bf	5′-CATGCCATGGCGGCGACGCCTGATTGTGTA-3	206 bp
Br/AAF/I	5′-TTTTAATGTTTTCAT.ACGAAAAATAACTTC-3′	
Br/AAF/II	5′-TCTGATTTTTTTCAT.ACGAAAAATAACTTC-3′	
AAF/If	5′-GAAGTTATTTTTCGT.ATGAAAACATTAAAA-3′	432 bp
AAF/Ir	5′-GCTCTAGAAAATTAATTCCGGCATGGAGT-3′	
AAF/IIf	5′-GAAGTTATTTTTCGT.ATGAAAAAAATCAGA-3′	396 bp
AAF/IIr	5′-GCTCTAGAATTTTAATTAAGGCCATACC-3′	

f forward primers, *r* reverse primers

dilutions were used (100 μl/well). After four more washings, development was done with single component TMB peroxidase substrate Kit (Bio-Rad Laboratories). An ELISA reader (Awareness Technology Inc.) was used to measure O.D. 450 nm. The sera from PBS and vector alone-injected mice were considered as control.

Determination of adherence to HeLa cells

HeLa cells from the National Cell Bank at the Pasteur Institute of Iran were grown in six-well microtiter plates (Nunc; 10^5 per well) for 24 h in RPMI medium (Biosera), pH 7.4, supplemented with 10% heat inactivated fetal bovine serum (Biosera) and 100 unit/ml penicillin-streptomycin (Biosera) at 37°C in a 5% CO2 atmosphere. Briefly, 50 μl of the overnight culture of the clone expressing hybrid peptides was incubated with 10^5 HeLa cells in 2 ml medium at 37°C. The monolayers were washed three times with PBS and 1 ml of fresh medium was added to each well. After adding 50 μl of overnight bacterial culture and a further 3 hr incubation period, the monolayers were washed thoroughly three times with PBS, fixed with methanol and acetic acid and stained with 10% (v/v) Giemsa.
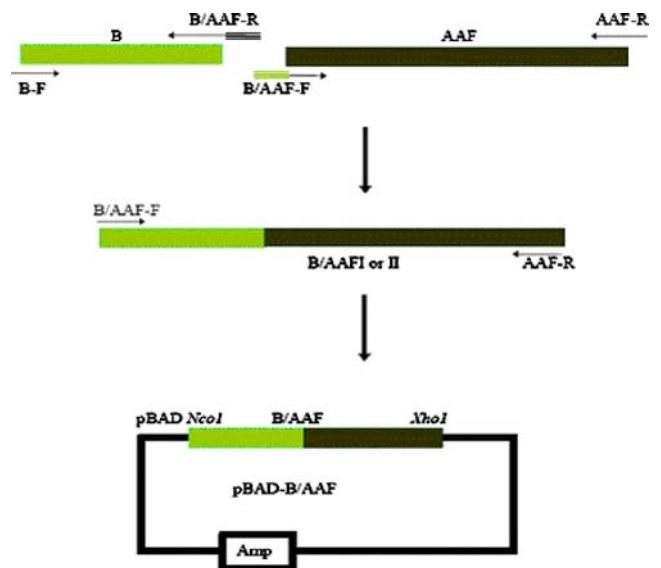
Inhibition of adherence to HeLa cells

Sera from immunized mice were added to 50 μl of the 'overnight' bacterial culture. It was further incubated at 37°C for a one hour period; the mixture was then added to the cell culture. The procedure continued as described for determination of adherence.

Challenge of mice

Two weeks after the last immunization, the immunized mice were challenged intraperitoneally with the toxic amount of recombinant Stx1 and observed for four days, and death, if any, was recorded [13].

Statistics

Data were subjected to ANOVA and Student′s *t*-test for statistical analysis, and a *p* value of <0.05 was considered to be significant.

Fig. 1 a Genes amplified by each primer are shown: *a* AAF/I gene (*aagA*), *b* B subunit, *c* molecular weight marker (MWM), *d* B-AAF/I. **b** Genes amplified by each primer are shown: *a* AAF/II gene(*aafA*), *b* B subunit, *c* molecular weight marker (MWM), *d* B-AAF/II

Fig. 2 Method used for fusion of two genes. The fused product is then cloned into a pBAD expression vector

Table 2 Mice immunized by peptides

| Group	Number of mice	Peptide injected
1	6	B
2	6	AAF/I
3	6	AAF/II
4	6	B-AAF/I
5	6	B-AAF/II
6	6	B, AAF/I separately
7	6	B, AAF/II separately
8	6	pBAD (control)

Results

Characterization of constructs

The eluted AAF/I, AAF/II, B, B-AAF/I and B-AAF/II peptides were characterized by SDS-PAGE electrophoresis and western blot analysis consequently (Fig. 3). The expression was different in each group shown by western blot. The purified eluted peptides were observed by SDS-PAGE and the amount of protein was calculated by Bradford protein assay used for mice injection.

Induction of Ag-specific Ab responses

Eight experimental groups of six inbred mice strains were injected by purified expressed peptides: AAF/I, AAF/II, B, B-AAF/I, B-AAF/II, B plus AAF/I (B+AAF/I) and recombinant B plus AAF/II (B+AAF/II), separately. The induction of antigen specific systemic antibody response was assessed by measuring total IgG response. Moreover, the ability of each peptide to modulate immune systems was compared with and without using CFA adjuvant. In this regard, serum pools of immunized mice were collected
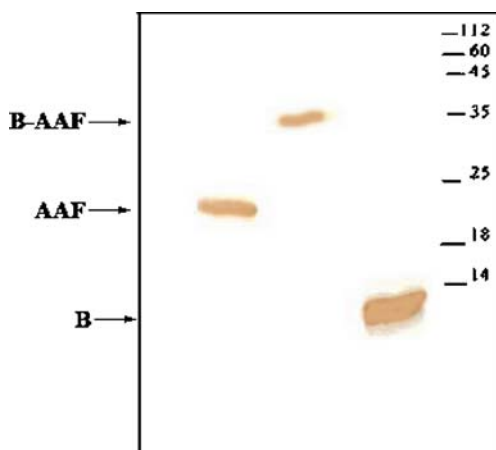
Fig. 3 Western blot analyses of the resulting peptides with each related antibody: *a* AAF peptide, *b* B-AAF peptide, *c* B peptide, *d* MWM

from each group. Antibodies against peptides were detected by ELISA in sera of all immunized mice. Specific IgG antibody response to each peptide after mice immunization was measured. It was shown that anti B-AAF response recognizes B and AAF antigen while the amount of raised antibody was similar when each peptide was used separately. Moreover, raising antibody to both antigens, B and AAF antigen (B+AAF), was similar to anti B-AAF response. Each antigen by itself raised more antibody than with Freund's adjuvant. The amount of anti B-AAF antibody production was significant ($p < 0.05$). The same result was observed with B-AAF/I and B-AAF/II, while the amount of antibody production in B-AAF/II was higher than B-AAF/I in mice.

Inhibition of adhesion

Characterization of AAF adhesin was examined in culture cells. As mentioned earlier, two standard strains were used for adhesion pattern of AAFI/II, 17–2 and 042, respectively. The result of challenging with strains that were already treated with B-AAF/I, B-AAF/II antibody separately has been shown (Fig. 4). It was observed that the antibody against two fusion peptides (B-AAF/I, B-AAF/II) drastically reduced the adherent bacteria to HeLa cells in each microscopic field, similar to the antibody against AAF/I and AAF/II peptides alone respectively.

Challenge

Mice immunized with AAF/I, AAF/II, B, B-AAF/I, B-AAF/II, B with AAF/I, B with AAF/II separately were challenged with a lethal dose of recombinant toxin two weeks after the last booster [13]. Twice the amount of LD_{50} of Stx was used for the challenge. All of the PBS immunized mice died by day 4, while B-AAF/I and B-AAF/II immunized mice survived within this period (Table 3). Protection was also observed for B+AAF/I and B+AAF/II immunized mice.

Discussion

Enteroaggregative *E. coli* (EACE) is emerging as a significant diarrheal pathogen in multiple population groups. Although most commonly associated with pediatric diarrhea in developing countries, EAEC is also linked to diarrhea in adults including HIV-positive patients and travelers and has been a cause of food-borne outbreaks in the industrialized world [15]. Pathogenesis is believed to be initiated with adherence to the terminal ileum and colon in an aggregative, stacked-brick type pattern by means of one of several different hydrophobic aggregative adherence

Fig. 4 The result of adherence to HeLa cells has been shown. Bacterial culture of standard strains 17–2 (**a**) and 042 (**b**) was added to the culture cells. Antibody against B-AAFI (**c**) and B-AAF/II (**d**) inhibited adherence of bacteria to cells
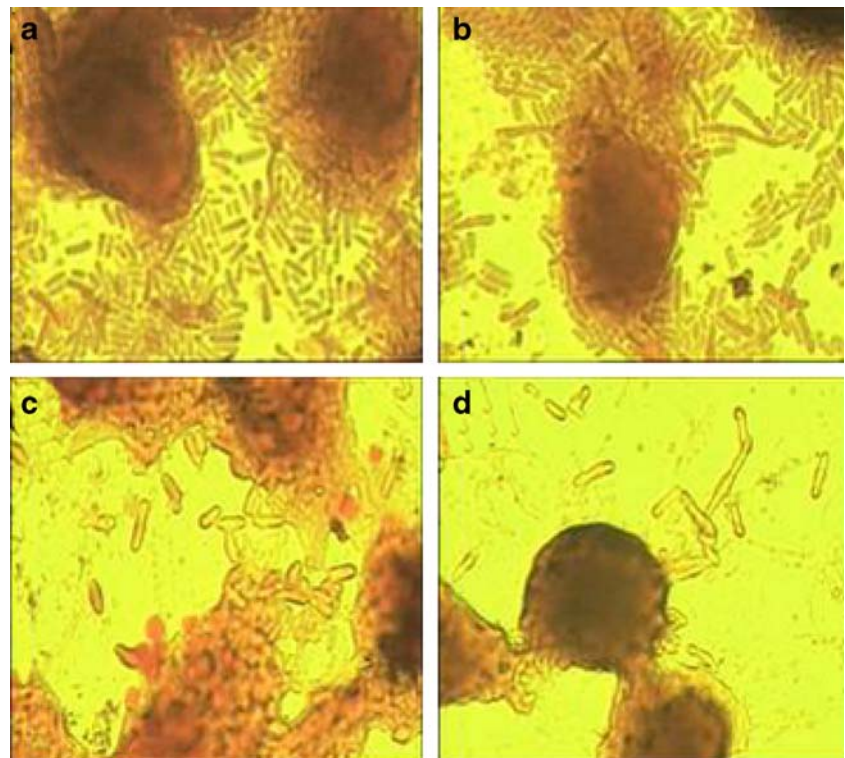
fimbriae [15]. Adhesins are the primary means that bacteria use to colonize the intestinal tract. Blocking the primary stages of infection, namely, bacterial attachment to the host cell receptors and colonization of the mucosal surface, may be the most effective strategy to prevent bacterial infection [16].

In 2001, the B subunit of Shiga toxin was used as a delivery system which transported exogenous DNA via vesicular traffic to the nucleus [17]. The B subunit of Shiga toxin was already fused to a tumor antigen and used as a vector to induce cytotoxic (CTL) immune response without the need for adjuvant [18]. Shiga toxin B subunit conjugation to keyhole limpet hemocyanin induced specific antibody response with cytotoxicity neutralizing activities in Ramos B cells [19].

Moreover, the Shiga toxin B subunit provided ligand mediated delivery of virus antigens to the gut-associated lymphoid tissues for enhanced stimulation of humoral and cellular responses [20]. In addition, the non-toxic B subunit of Shiga toxin (StxB) interacts with the glycolipid Gb3, which is coupled to OVA (Ovalbumin). Vaccination of mice with StxB-OVA inhibited tumor growth in prophylactic and therapeutic experiments [21].

Therefore, the B subunit of Shiga toxin has been used as a non-living and non-toxic attractive vaccine vector.

Currently, only vaccines against infection with rotavirus, *Vibrio cholerae* and *Salmonella* typhi, are commercially available. Inactivated whole cell *vibrio cholerae* O1 strains (WC) plus recombinant cholera toxin B subunit (WC-rBSCT), marked as Dukoral in Canada and some European countries, was shown to provide short-term protection [22].

There have been no vaccines yet licensed specifically to prevent diarrheal illness caused by *Shigella* spp. or ETEC [23]. Vaccines designed to protect against infection with ETEC that are in clinical trials did not demonstrate statistically significant protection [23].

Here we report for the first time this polypeptide strategy for promoting specificity of immune response against EAEC or dual protection. The dual responses (EAEC/STEC) not only induced specific antibody responses against each peptide alone, but also protection against Shiga toxin and inhibition of adhesion could be achieved simultaneously.

Although, we have to keep in mind the fact that the animal model does not completely reflect human infection. In addition, we have to point out that an analogy between inhibition of adhesion to HeLa cells and colonization of the human gut must be made with caution. While different small subunits (peptides) are the vaccine candidates, this

Table 3 Protection experiment of immunized mice challenged with recombinant peptides. Values given are number of surviving mice/total of immunized mice

Immunization				
AAFI/II	B	B-AAFI/II	B+AAFI/II	PBS
0/6	6/6	6/6	6/6	0/6

polypeptide strategy can be a helpful tool for any vaccine design against diarrheal diseases. In this study, the newly fused recombinant polypeptide can be considered as a potential new tool for vaccine development against EAEC and STEC infection.

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