Vaccine 28 (2010) 6923–6929

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Immunogenic properties of chimeric protein from *espA*, *eae* and *tir* genes of *Escherichia coli* O157:H7

Jafar Amani[a,b], Ali Hatef Salmanian[b,**], Sima Rafati[c], Seyed Latif Mousavi[d,*]

[a] *Applied Biotechnology Research Center, Baqiyatallah Medical Science University, Tehran, Iran*
[b] *National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran*
[c] *Molecular Immunology and Vaccine Research Laboratory, Pasteur Institute of Iran, Tehran, Iran*
[d] *Dept. of Biology, Faculty of Basic Sciences, Shahed University, Tehran, Iran*

ARTICLE INFO

Article history:
Received 23 February 2010
Received in revised form 16 July 2010
Accepted 19 July 2010
Available online 13 August 2010

Keywords:
Enterohemorrhagic *Escherichia coli*
Chimeric vaccine
Intimin
EspA
Tir

ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) comprise an important group of enteric pathogens causing hemorrhagic colitis and hemolytic uremic syndrome. These bacteria need EspA (E) as a conduct for Tir (T) delivery to the host cell and surface arrayed intimin (I) which docks the bacterium to the translocated Tir. This phenomenon leads to attaching and effacing (A/E) lesions. A trivalent recombinant protein called rEIT composed of immunologically important portions of EspA, Intimin and Tir was constructed as a candidate vaccine. For high-level expression, the EIT gene was synthesized with codon bias of *E. coli*. The immunization was conducted in mice with purified rEIT. The results showed that this chimeric protein induced strong humoral response as well as protection against live challenges using EHEC.

1. Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is significant zoonotic pathogen of human beings capable of causing severe gastrointestinal diseases, including hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) [1]. Outbreak of EHEC infection has been frequently reported worldwide [2,3]. Treatment of EHEC infection remains problematic since conventional use of antibiotics can enhance pathogenesis [4].

EHEC can form characteristic attaching and effacing (A/E) lesions on mammalian cells growing in vitro [5] and in vivo [6]. The first gene to be associated with A/E activity was *eae* gene encoding intimin; which is an outer membrane adhesion molecule essential for intimate bacterial attachment to eukaryotic host cells. It has been shown that intimin is required for colonization of *E. coli* O157:H7 in animal models [7]. The intimin encoded by *eae* gene is a part of a pathogenicity island found in enteropathogenic and enterohemorrhagic *E. coli* termed the locus of enterocyte effacement (LEE) [8,9]. The LEE encodes a type III secretion system (TTSS) which includes a translocated intimin receptor (Tir) [10] and three secreted proteins EspA, EspB, and EspD. These proteins are required for signal transduction in mammalian host cells and A/E lesion formation [11]. EspA is a structural protein and a major component of a large filamentous organelle that is transiently expressed on the bacterial surface and interacts with the host cell during the early stage of A/E lesion formation [12]. This filament may contribute to bacterial adhesion but greater significant role is of a component of translocation apparatus that is essential for the translocation of EspB, EspD [12] and Tir into host cells [10,13]. Secretion of Tir through the EspA filament leads to intimate attachment of the bacterium through intimin–Tir interaction [12,14].

The main components in EHEC A/E lesion formation are EspA which provides targets for disruption of bacterial–host cell interaction and therefore disease resistance strategies. EspA is immunogenic and its polyclonal antibody has been demonstrated to decrease adherence of EPEC and EHEC to host cells in vitro [15].

Intimin is a key colonization factor for EHEC O157:H7 in neonatal calves [7], young and weaned calves [16], adult cattle and sheep [17]. Studies on the different intimins from EPEC and EHEC have shown that receptor-binding activity is localized to the C-terminal 280 amino acids (Int280) which mediates the interaction with Tir [18]. Serological and phylogenetic analysis has identified at least six distinct intimin subtypes (designated Int- α, β, γ, δ, ε and θ) that vary in the sequence of the carboxy-terminal cell-binding domain [19].

Vaccination with the carboxy-terminal of intimin induced strong response of specific antibodies in serum and colostrums

* Corresponding author. Tel.: +98 21 51212600; fax: +98 21 51212601.
** Corresponding author. Tel.: +98 21 44580365; fax: +98 21 44580399.
E-mail addresses: salman@nigeb.ac.ir (A.H. Salmanian), slmousavi@shahed.ac.ir (S.L. Mousavi).

0264-410X/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2010.07.061

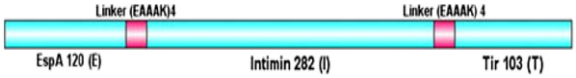

Fig. 1. Diagram of constructed recombinant proteins EIT containing EspA 120, Intimin 282, Tir 103.

of pregnant swines [16] and reduced the time of *E. coli* O157:H7 shedding in mice [20]. In the cattle, EHEC does not cause disease and localize on intestinal epithelium [21], mucosal antibodies against bacterial attachment can preclude colonization of bacteria in the intestinal cattle [22]. EHEC vaccines evaluated so far exhibited significant effect on subsequent EHEC colonization in cattle. EspA, Intimin and Tir proteins were individually proven to be immunogenic [23–26]. There is still a room to take measures in order to make the currently available vaccines more effective i.e. a combined triple EspA, Intimin, Tir antigens in a single recombinant protein would reduce the necessity of producing three separate antigens, which would make it more cost-effective and would ease the manufacturing. Thus, we designed a new construction containing EspA 120, lacking 36 amino acids from the N-terminal of the protein, Intimin 282 amino acids from the C-terminal of Intimin, and Tir 103, residues 258–361 which interact with Intimin [27,28]. In the present study immunogenicity of a trivalent recombinant protein (EIT) coded by a synthetic gene is reported.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

E. coli O157:H7 was obtained from reference laboratory, Booali Hospital (ATCC: 35218), Tehran. Plasmid pET-28a was from Novagen (USA). *E. coli* BL21 (DE3) and DH5α were procured from Pasteur Institute of Iran. All *E. coli* strains were grown in LB broth at 37 °C, media were supplemented, when required, with kanamycin (SIGMA, 40 μg/ml). *E. coli* isolates were stored at −70 °C in 20% glycerol.

2.2. Design and construction of chimeric EIT

GenBank was the main reference for retrieval of the sequences of the gene encoding EspA C120, Intimin C282 and Tir 103. The genes were back translated to nucleotide sequence. The codon optimization was performed by codon usage of *E. coli* and bioinformatic analysis was performed as reported in our previous work [28]. These genes were used to generate trivalent proteins by linkers $(EAAAK)^4$ in between (Fig. 1) and the restriction sites for enzymes *Bam*HI and *Hind*III at the 5′ and the 3′ sites respectively. The gene encoding target protein was verified by Gen-Script (NJ, USA) and synthesized by Shine Gene Molecular Biotech, Inc. (Shanghai, China) into pUC57 cloning vector. The final sequence is accessible under GenBank accession number FJ744505.

2.3. Expression of chimeric EIT

The synthetic gene was subcloned into pET28a with the 6X-His-tag at the N-terminal and expressed under the control of the T7 promoter. The pET-EIT recombinant plasmid was transformed into *E. coli* strain BL21 (DE3) and cells were stored at −70 °C. Glycerol stock cells (100 μl) were inoculated into 5 ml of LB medium and the culture was grown under agitation at 37 °C overnight. This culture was used to inoculate 10 ml of LB medium containing 20 μg/ml kanamycin. The culture was grown at 37 °C to an optical density (600 nm) of 0.5–0.7. Expression of the chimeric sequence was achieved by the addition of 1 mM IPTG (SIGMA). Cells were harvested by centrifugation at 5000 rpm/10 min and each pellet was resuspended in 0.3 ml of lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 0.2 mg/ml lysozyme). The samples were analyzed by SDS-PAGE.

2.4. Purification of recombinant fusion protein

The recombinant protein was purified using Nickel–nitrilotriacetic acid (Ni–NTA) (Qiagen) resin under native condition. 100 ml cell pellet was thawed and resuspended in 6 ml lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.2 mg/ml lysozyme). The suspension was kept at room temperature for 20 min for lysis. The lysate was then centrifuged at 14,000 rpm for 20 min. The supernatant was poured into the Ni–NTA affinity column and the flow-through of the soluble fraction was collected. The column was washed with washing buffer containing 40 mM imidazole and bound protein was eluted with 2 ml of 250 mM imidazole followed by 1 ml of 20 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer. The purification procedure was carried out at 4 °C and the purified chimeric protein (EIT) was monitored on 10% SDS-PAGE.

2.5. Western blot analysis

Purified protein from SDS-PAGE was transferred to nitrocellulose membrane using transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% SDS, and 20% methanol) by Bio-Rad Mini Protean II System. The membrane was incubated in the blocking buffer of 3% gelatin/phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, and 4.3 mM Na_2HPO_4, pH7.3) at 4 °C overnight. The membrane was then incubated in a 1:1000 dilution of mice anti-His-tag IgG in the PBS/T (PBS contain 0.05% Tween 20), with gentle shaking for 1 h at 37 °C. The membrane was washed with PBS/T three times and then incubated in 1:50,000 dilution of HRP-conjugated goat anti-mouse IgG antibody (SIGMA), with gentle shaking for 1 h at 37 °C. The membrane was washed three times with PBS/T and detection was carried out using HRP staining solution (DAB). Chromogenic reaction was stopped by rinsing the membrane twice with distilled water.

2.6. Animal immunization

Five-week-old female BALB/C mice (Pasteur Institute of Iran) were divided into test and control groups. Each mouse in the test group was injected subcutaneously in the back of neck with 20 μg recombinant EIT protein with complete Freund's adjuvant (SIGMA). Using incomplete Freund's adjuvant, 15 μg and 10 μg rEIT were injected after 20 and 35 days respectively as the booster doses. Fourteen days after the last booster, 5 μg rEIT was given intraperitoneally. As a negative control, PBS was injected with the same procedure. Blood samples were collected from the mice after the second, third and fourth injections. The sera were stored at −70 °C for further analyses [29,30].

2.7. Determination of serum IgG and fecal IgA antibody responses to recombinant EIT

Antibody specific responses were determined by an enzyme-linked immunosorbent assay (ELISA). Polystyrene 96-well plates (Nunc) were coated with 5 μg of rEIT protein in coating buffer (64 mM Na_2CO_3, 136 mM $NaHCO_3$, pH 9.8) at 4 °C overnight. The plates were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS/T) and the non-specific sites were blocked with 3% gelatin in PBS/T. Mouse serum samples were serially diluted to 1:500 in PBS/T and added to the ELISA plates and

incubated for 45 min at 37 °C. In order to recover IgA from feces, samples collected daily from each mouse were frozen at −80 °C. At the end of every week 1 g from the pooled fecal samples was mixed thoroughly with 500 μl of PBS and centrifuged (4 °C 6000 rpm 10 min). The supernatant was serially diluted in PBST and added to the ELISA plates which were incubated for 45 min at 37 °C. The plates were washed three times in PBS/T and HRP goat anti-mouse IgG (1/50,000 in PBST) (SIGMA) or HRP goat anti-mouse IgA antibodies (1/10,000 in PBST) (SIGMA) were added to the ELISA plates. Plates were incubated for 30 min at 37 °C and washed three times in PBST. The wells added with 100 μl of citrate buffer containing 0.06% (W/V) of O-phenylenediaminedihydrochloride (OPD) (SIGMA) and 0.06% (V/V) hydrogen peroxide were incubated at room temperature for 15 min. The reaction was stopped with 100 μl of 2 M H_2SO_4 and the OD_{492} was read on a microplate reader (Bio-Rad).

2.8. Determination of serum IgG and fecal IgA antibody responses to native E. coli O157:H7 proteins

Specificity of the antibodies was determined by probing Western blots and ELISA of heat-extracted whole-cell protein and precipitation of secreted protein from EHEC O157:H7. In brief the cultures were grown overnight in LB broth and subcultured (1:100) into M9 minimal media supplemented with 44 mmol/l $NaHCO_3$, 0.4% glucose, and 0.1% casamino acids (modified M9) and incubated at 37 °C, 5% CO_2, until the OD_{600} reached 0.7–0.8. For analysis of antibodies against crude whole-cell extract proteins, cells were harvested by centrifugation at 5000 rpm for 10 min, and resuspended in a 200 μl lysis buffer (Tris-base 10 mM pH:7). Secreted proteins were precipitated from the supernatants by TCA-PFE (10% trichloroacetic acid, 50 μg/ml, phenylmethylsulfonyl fluoride, 0.5 μmol/l EDTA, SIGMA) overnight at 4 °C. Precipitated proteins were centrifuged at 10,000 rpm for 30 min, washed with chilled 95% ethanol and recentrifuged and resuspended in lysis buffer.

For Western blotting the extracted proteins were resuspended in sample buffer, boiled for 5 min and electrophoresed on 10% SDS-PAGE and transferred onto nitrocellulose membranes. Also resolved proteins were coated in the plate for ELISA. The rEIT antisera were used as the primary antibody at various dilutions. HRP-conjugated goat anti-mouse IgG (1:50,000, SIGMA) antibody was used as the secondary antibody and the colorimetric reaction was visualized using substrate development buffer (Bio-Rad) [31]. Non-pathogenic *E. coli* and none immunized mice sera were used as control.

2.9. Challenging the immunized mice

The mice were challenged two weeks after last immunization. Prior to challenges, all groups were given drinking water containing streptomycin sulfate (5 mg/ml) to reduce the normal bacterial flora of the gut [32]. Following one day of treatment with streptomycin, mice were fasted overnight, and subsequently fed with 10^{10} colony forming units (CFU) of *E. coli* O157:H7 (ATCC: 35218) suspended in 100 μl of PBS. The animals were individually housed and permitted to access to food and water and libitum. The fecal samples from each mouse were collected at two days interval for two weeks. *E. coli* O157:H7 fecal shedding was monitored by adding approximately 0.1 g of feces to 1 ml of LB broth followed by incubation at room temperature for 2–4 h to allow the fecal pellets to soften. The mixture was then vortexed until the pellets were no longer visible. Serial dilutions of the supernatant were plated onto Sorbitol MacConkey agar plates containing cefiximine and tellurite. Plates were incubated overnight at 37 °C and *E. coli* O157:H7 colonies were enumerated. Bacterial colonies were tested for the O157 antigen by latex agglutination [23,33].

2.10. Binding inhibition assay and antibody treatment on bacterial growth

Caco-2 cells were grown to confluence in a culture flask, trypsinized and distributed onto a sterile round cover slip placed on the bottom of a 24-well cell culture plate. EHEC cells in the exponential growth phase were washed three times with PBS and adjusted to an optical density of 0.5 at 600 nm. The bacterial suspension (300 μl) was pretreated with 150 μl of immunized mice antisera for 20 min at room temperature. Non-immunized mice antisera and Caco-2 cell line were used as negative controls. The bacterial mixture was added to Caco-2 cells and incubated for 1 h at room temperature. After three times washing with PBS, the Caco-2 cells were fixed in 1 ml of 100% methanol for 5 min at room temperature. The fixed cells were stained with Giemsa staining solution (SIGMA) for 5 min and then destained twice with 1 ml of PBS. The cover slips containing the Caco-2 cells bound with EHEC O157:H7 were examined under the invert microscope at 1000× magnification. The number of EHEC O157:H7 cells that adhered to each Caco-2 cell was counted and the mean number of bacteria bound per cell was determined by examining 100 Caco-2 cells on each cover slip. The percentage of positive cells with at least one adhering bacterial cell was also noted [34].

To analyze the effect of antibody on bacterial growth the OD_{600} of an overnight culture of *E. coli* O157:H7 was measured and adjusted to 1 with LB broth. The immunized mice antisera were diluted in LB broth to a final concentration of 1:250. The serum from non-immunized mice served as negative control. The samples were incubated at 37 °C with gentle shaking. Aliquots of each sample (100 μl) was taken at 0, 1, 2, 4 and 6 h and spread onto Cefiximine tellurite Sorbitol MacConkey agar. Plates were incubated at 37 °C overnight and the colonies were enumerated to determine the total CFU/ml of each sample. Log 10 CFU/ml values were calculated from the mean of triplicate samples from experiments and subjected to statistical analyses [31].

2.11. Statistical analysis

The data in each figure was a representative of three independent experiments expressed as the mean ± standard deviation (SD). All statistical analyses were performed using a SPSS 12.0 statistical program. Student *t*-test was used to analyze the data for antibody responses between immunized and non-immunized groups, *t*-test was also applied to evaluate the significance of differences in inhibition of EHEC binding to Caco-2 cells generated by test sera. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Construction and subcloning of the synthetic gene

Sequence comparison of the synthetic construct by ClustalW showed high degree of conservation of the domains among different strains of *E. coli* O157:H7. The elicited structure was analyzed by bioinformatic software described previously [28] (Fig. 1). The synthetic gene was over expression in *E. coli*.

3.2. Expression and purification of recombinant protein

The synthetic *eit* gene was expressed in *E. coli* (BL21DE3), with the N-terminal 6X-His-tag, in different conditions (Fig. 2A). The expression of recombinant EIT protein was confirmed by reaction with the anti-His-tag antibodies with Western blotting (Fig. 2B). Purification of the recombinant EIT were carried

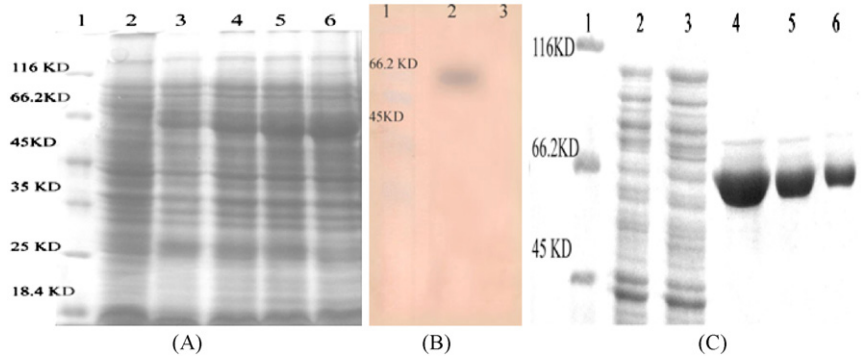

Fig. 2. Expression, Western blot analysis and purification of recombinant EIT. (A) Optimization of recombinant EIT at 4, 6, 8, 10 h (lanes 3, 4, 5, 6) with 1 mM IPTG. Lane 1, protein weight marker. Lane 2, uninduced *E. coli* BL21DE3/pET28a + *eit* gene as control. (B) Western blot analysis of rEIT using anti 6X-His-tag antibodies. Lane 1, protein weight marker. Lane 2, rEIT. Lane 3, total protein of *E. coli* BL21DE3/pET28a without *eit* gene after induction as control. (C) Purification of recombinant EIT protein with 6X-His-tagged from pET28a. Lane 1, protein weight marker. Lane 2, flow-through. Lane 3, wash column with 40 mM imidazole, Lanes 4–6, purified protein after elution with 250 mM imidazole.

out under native condition and SDS-PAGE analysis revealed the presence of 61.6 KD recombinant chimeric protein as a major band in all the eluted fractions as shown in Fig. 2C. Protein concentrations were estimated by the Bradford protein assay and the average yield for recombinant EIT was 100 mg/L culture.

3.3. Response to immunization with rEIT protein

Mice immunized subcutaneously with purified EIT protein showed significant EIT-specific IgG antibodies (Fig. 3A). Compared to control antisera the anti-EIT IgG antibody titer was clearly detectable even at 1:512,000 dilution. However, no EIT-specific IgA was detected in feces or serum following subcutaneous administration (Fig. 3B).

3.4. Recognition of the native protein antigens

The antibody specificity of mice antisera was confirmed against their native counterparts using whole bacterial cell and secreted cellular protein obtained from *E. coli* O157:H7 culture. Antibody against rEIT effectively targeted proteins of corresponding molecular weights to Intimin and EspA from crude cell extract or secreted Tir proteins from bacteria. There was no evidence of binding by control mice antisera to any proteins resembling the immunogens from *E. coli* O157:H7 (Fig. 4A–C).

3.5. Challenging of immunized mice with E. coli O157:H7

In order to determine that the EIT-specific antibodies in immunized mice serum could reduce or prevent the *E. coli* O157:H7 shedding in feces, subcutaneously immunized and non-immunized control mice were infected orally with 10^{10} CFU of *E. coli* O157:H7. Shedding of the organism was monitored in feces as described above. Non-immunized control mice shed high levels of *E. coli* O157:H7 (10^8 CFU) in their feces over the two-week sampling period whereas shedding of immunized mice reduced gradually and stopped completely after seven days (Fig. 5).

3.6. Binding inhibition of EHEC to Caco-2 cells and growth inhibition of EHEC by anti-EIT antibody

Examination of Caco-2 cell monolayers revealed that EHEC O157:H7 cells pretreated with serum from the non-immunized mice were densely distributed on the Caco-2 cells. Almost all the Caco-2 cells (97.2%) were observed to bind one or more bacteria with a mean number of 14.5 bacteria per cell. In contrast, pretreatment of the EHEC cells with immunized mice antisera remarkably blocked their binding to Caco-2 cells. 49% of the Caco-2 cells were found to attach the bacterial cells and mean number of 2.8 bacteria per cell. Comparison of pretreatment of the bacteria with non-immune antisera with that of serum obtained from an immunized mice, showed that immunized mice antibody greatly decreased their adhesion properties (Table 1).

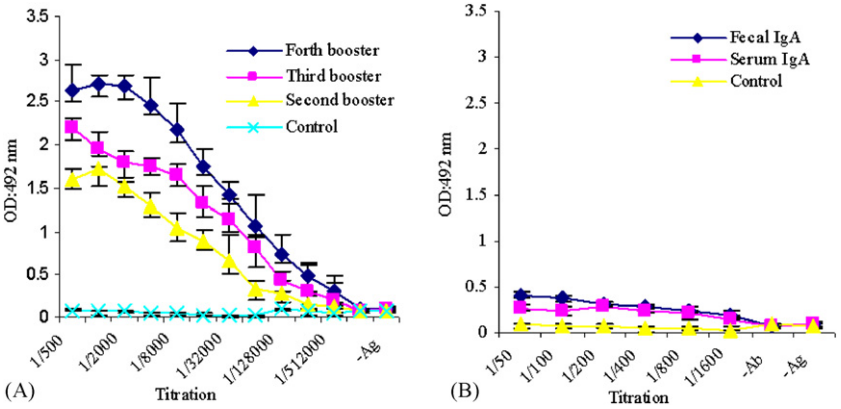

Fig. 3. EIT-specific serum IgG and fecal IgA following subcutaneous immunization. Mice were injected with recombinant rEIT using complete and incomplete Freund's adjuvants. Immunizations were performed four times within eight weeks. The sera were collected after second immunization and assessed for rEIT-specific IgG (Fig. 3A) and IgA (Fig. 3B) by ELISA method. Non-immunized mice sera were used as control ($p < 0.05$)

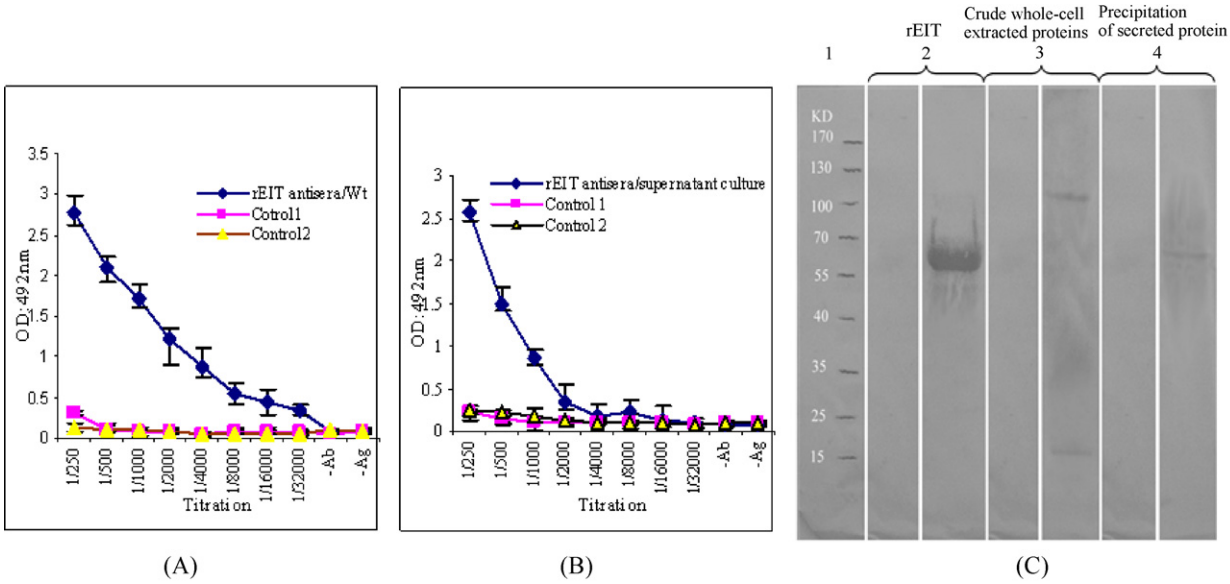

Fig. 4. Recognition of native proteins from whole cells of *E. coli* O157:H7. Serum specific EIT antibody was able to recognize native EspA, Intimin and Tir by ELISA and Western blotting. (A) Elisa results for bacterial whole-cell extract (B) Elisa results for precipitated proteins from EHEC supernatant culture. Non-pathogenic *E.coli* (control 1) and non-immunized mice sera (control 2) were used as control. (C) Western blot carried out with serum specific EIT antibody for identification of native EspA, Intimin in crude whole-cell extracted proteins and Tir in precipitated proteins from modified M9 supernatant culture of EHEC O157 H7. Lane 1, molecular weight marker. Lanes 2–4 was treated with serum specific EIT antibody and non-immunized mice sera as control.

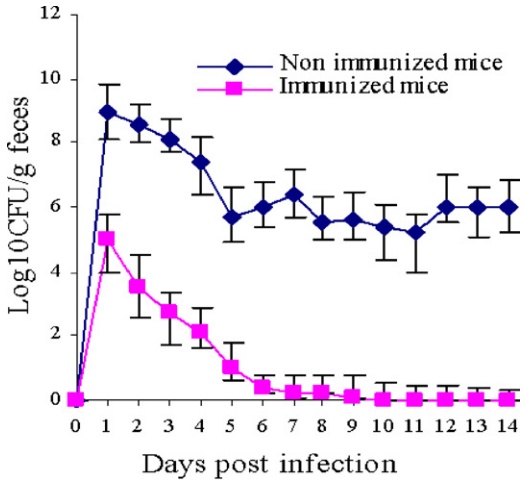

Fig. 5. *E. coli* O157:H7 shedding in feces following subcutaneous administration in mice. Immunized and non-immunized mice were orally fed 10^{10} *E. coli* O157:H7 and shedding was monitored in the feces for two weeks. The error bar is standard deviation and differences in outcome were assessed by *t*-test. Differences were considered significant whenever $p < 0.05$. The limit of detection for plating was 100 CFU/0.1 g feces. Data have been repeated in separate experiments ($p < 0.05$).

It is predicted that the growth of EHEC cells treated with antibodies having a bactericidal or bacteriostatic effect have to stop or to decline when compared with the effect of negative control antisera. Conversely, the growth curves of antibody-treated EHEC cells were similar with their controls ($p > 0.05$), indicating that these antibodies did not have any direct influence on the growth of the bacteria (data not shown).

4. Discussion

A number of EHEC vaccines have been evaluated in cattle and have primarily focused on immunization with bacterial proteins encoded by genes located within the locus of enterocyte effacement (LEE) that are known to play key roles in EHEC colonization in the bovine intestine [17,22,35]. Vaccination of cattle with secreted fraction of EHEC O157:H7 significantly reduced EHEC infection rate and considerably prevented its prevalence [23]. A vaccine derived from a mutant *tir* gene was described as less efficacious than a preparation from the wild type vaccine. These findings suggest that disruption of interaction between Intimin and Tir could be fruitful [23,26]. Finally immunization with a combination of recombinant EspA, Intimin and Tir has been previously shown to be protective against *E. coli* O157 challenge [26]. Based on a trivalent recombinant, EIT was constructed containing a truncated form of *espA* (lacking 36 amino acids from the N-terminal of the protein, designated EspA 120) which is exposed on the bacterial surface [15]. The 282 amino acids from the carboxy-terminal of Intimin have been reported to be involved in binding to its receptor Tir [17,27]. The region of Tir involved in the interaction with intimin has also been mapped (residues 258–361, designated Tir 103) [18].

So we combined three fragments with linkers $(EAAAK)^4$, reported in our previous study [28].

In this study we demonstrated that a combination of chimeric recombinant proteins designated EIT could protect the animal from EHEC O157:H7 colonization. In order to achieve an optimal induc-

Table 1
Inhibition of *E. coli* O157:H7 binding to Caco-2 cells.

Test sera	Positive Caco-2 cells (%)[a]	Mean number adhesion[b]
Non-immune mice sera	97.2 ± 0.62	14.5 ± 0.55
Immune mice sera	49.1 ± 0.9*	2.8 ± 0. 05*

[a] The percentage of Caco-2 cells with at least one adhering EHEC O157:H7 bacterial cell.
[b] The mean score of EHEC O157:H7 cells bound per Caco-2 cell.
* Statistical significance ($p < 0.001$) of difference in the binding inhibition was found in comparison with non-immune mice sera.

tion and powerful response, we applied booster doses at least three weeks after the first injection. The doses were gradually decreased to enhance affinity and to fully activate the B cell immune cascade. Finally fourth injection was intraperitoneal to activate clonal B cell effectively [29,30]. Accordingly a significant difference in the antibody titer was observed between sera of the immunize mouse and control groups (Fig. 3A and B). The reactivity of the same sera with the wild type and non-pathogenic *E. coli* strain, showed that antibodies against recombinant trivalent EIT are bound to native EspA filaments or Intimin. In a whole-cell ELISA and Western blot experiments, the low reactivity of immunized mice sera likely reflects the low amount of surface exposed EspA and Intimin antigens. The results from other studies have indicated that not all *E. coli* O157:H7 bacteria in a given population express EspA filaments at any one time under inducing conditions [36].

Roe et al. [36] demonstrated that Tir and EspA as type III secretion factors are inconsistently expressed among the population over time, implying that the TTSS is active at various points during the colonization process. As the TTSS is the only known mechanism for Tir secretion, hence we used Tir as an indicator of functionality of the TTSS apparatus. In addition previous studies showed that patients infected with EHEC exhibited high titers of anti-intimin and anti-EspA antibodies, indicating immunogenic properties of both proteins [37].

In this study we proposed that the agglutination or impairment of biological function mediated by antibody against specific components might have inhibited the growth of bacteria. The possibility was considered using direct interaction between the pathogenic factors and specific antibody. The present study showed that specific mouse chimeric antisera did not inhibit the growth of EHEC O157:H7 (data not shown). Our data showed that immunization with multicomponent EIT strongly protected mice from EHEC challenges and immunized mice did not shed *E. coli* O157:H7 following infection (Fig. 5). Moreover, attachment of EHEC on the epithelial cell culture was effectively reduced with immunized mice compared to the control groups (Table 1). Mucosal immunity, especially the production of IgA antibodies, is thought to be important for blocking the attachment of *E. coli* O157:H7 to epithelial cells [38]. However, it has been demonstrated with *Citrobacter rodentium* that secretory IgA is not necessary for preventing bacterial colonization in mice using intimin as a vaccine antigen [38].

The data presented here also indicate that secretory IgA is not absolutely necessary for the prevention of *E. coli* O157:H7 colonization in mice. In animals immunized subcutaneously with trivalent EIT vaccine, Tir, EspA, and Intimin-specific IgG were elicited without any detectable antigen-specific IgA. There was not bacteria colonization following experimental infection. However, it is likely that IgA antibodies are important in blocking the interaction of TTSS with host cells when the antigens are delivered to mucosal surfaces. The lack of TTSS-specific IgA in feces can be overcome by high Tir, EspA and Intimin-specific serum IgG or systemic priming of the immune system, resulting in a response following challenge. A recent report indicates that most IgG at mucosal surfaces is derived from the serum [39], although there is some evidence of active secretion via epithelial FcRn. Absorption of a bioactive Fc-fusion protein across the respiratory mouse epithelium has been demonstrated. Thus, IgG, like dimeric IgA, can cross-epithelial barriers by receptor-mediated transcytosis in adult animals [40,41].

Presumably, transudation of systemic IgG antibodies was responsible for this protection since adult mice were used in our study.

The results presented here are in accordance with those obtained previously in cattle and mice where the intramuscular and subcutaneous routes were used for immunization with TTSS [26,33].

Since the generation of Tir, EspA and Intimin-specific IgA in feces is not absolutely necessary to decrease colonization and shedding of *E. coli* O157:H7 in the mouse and cattle [26,33] models the current strategy for developing a cattle vaccine to reduce *E. coli* O157:H7 shedding appears to be feasible.

5. Conclusion

The present study is the first report showing that vaccination with a trivalent immunogen containing EspA, Intimin and Tir is an effective method to reduce shedding levels of *E. coli* O157:H7 following oral challenge. This level of reduction with combination of antigens can be used as a novel strategy for developing a vaccine against *E. coli* O157:H7 shedding. This could also be a new strategy applicable to further researches in this field.

Acknowledgements

Our grateful thanks are due to Dr. Rasooli for his critical evaluation of the manuscript. This work was supported by NIGEB Grant No. 368 and Basic Sciences Research Centre of Shahed University, Tehran-Iran, grant No. 57243.

References

[1] Girard MP, Steele D, Chaignat CL, Kieny MP. A review of vaccine research and development: human enteric infections. Vaccine 2006;24:2732–50.
[2] Khan A, Datta S, Das SC, Ramamurthy T, Khanam J, Takeda Y, et al. Shiga toxin producing Escherichia coli infection: current progress & future challenges. Indian J Med Res 2003;118:1–24.
[3] Aslani MM, Bouzari S. An epidemiological study on Verotoxin-producing Escherichia coli (VTEC) infection among population of northern region of Iran (Mazandaran and Golestan provinces). Eur J Epidemiol 2003;18:345–9.
[4] Wong CS, Jelacic S, Habeeb RL, Watkins SL, Tarr PI. The risk of the hemolytic-uremic syndrome after antibiotic treatment of Escherichia coli O157:H7 infections. N Engl J Med 2000;342:1930e–6.
[5] Knutton S, Baldwin T, Williams PH, McNeish AS. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic Escherichia coli. Infect Immun 1989;57:1290–8.
[6] Tzipori S, Wachsmuth IK, Chapman C, Birden R, Brittingham J, Jackson C, et al. The pathogenesis of hemorrhagic colitis caused by Escherichia coli O157:H7 in gnotobiotic piglets. J Infect Dis 1986;154:712–6.
[7] Dean-Nystrom EA, Bosworth BT, Moon HW, O'Brien AD. Escherichia coli O157:H7 requires intimin for enteropathogenicity in calves. Infect Immun 1998;66:4560–3.
[8] McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci USA 1995;92:4564–8.
[9] Perna NT, Mayhew GF, Pósfai G, Elliott S, Donnenberg MS, Kaper JB, et al. Molecular evolution of a pathogenicity island from enterohemorrhagic Escherichia coli O157:H7. Infect Immun 1998;66:3810–7.
[10] Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB. Enteropathogenic E coli (EPEC) transfer its receptor for intimate adherence into mammalian cells. Cell 1997;91:511–20.
[11] Elliott SJ, Wainwright LA, McDaniel TK, Jarvis KG, Deng YK, Lai LC, et al. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic Escherichia coli E2348/69. Mol Microbiol 1998;28:1–4.
[12] Knutton S, Rosenshine I, Pallen MJ, Nisan I, Neves BC, Bain C, et al. A novel EspA associated surface organelle of enteropathogenic Escherichia coli involved in protein translocation into epithelial cells. EMBO J 1998;17:2166–76.
[13] Fitzhenry RJ, Pickard DJ, Hartland EL, Reece S, Dougan G, Phillips AD, et al. Intimin type influences the site of human intestinal mucosal colonization by enterohaemorrhagic Escherichia coli O157:H7. Gut 2002;50:180–5.
[14] Caprioli A, Morabito S, Brugere H, Oswald E. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. Vet Res 2005;36:289–311.
[15] La Ragione RM, Patel S, Maddison B, Woodward MJ, Best A, Whitelam GC, et al. Recombinant anti-EspA antibodies block *Escherichia coli* O157:H7-induced attaching and effacing lesions in vitro. Microbes Infect 2006;8:426–33.
[16] Dean-Nystrom EA, Gansheroff LJ, Mills M, Moon HW, O'Brien AD. Vaccination of pregnant dams with intimin (O157) protects suckling piglets from Escherichia coli O157:H7 infection. Infect Immun 2002;70:2414–8.
[17] Cornick NA, Booher SL, Moon HW. Intimin facilitates colonization by Escherichia coli O157:H7 in adult ruminants. Infect Immun 2002;70:2704–7.
[18] DeVinney R, Stein M, Reinscheid D, Abe A, Ruschkowski S, Finlay BB. Enterohemorrhagic *Escherichia coli* O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated. Infect Immun 1999;67:2389–98.

[19] Oswald E, Schmidt H, Morabito S, Karch H, Marcheïs O, Caprioli A. Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic Escherichia coli: characterization of a new intimin variant. Infect Immun 2000;68:64–71.
[20] Judge NA, Mason HS, O'Brien AD. Plant cell-based intimin vaccine given orally to mice primed with intimin reduces time of Escherichia coli O157:H7 shedding in feces. Infect Immun 2004;72:168–75.
[21] Stevens MP, van Diemen PM, Dziva F, Jones PW, Wallis TS. Options for the control of enterohaemorrhagic Escherichia coli in ruminants. Microbiology 2002;148:3767–78.
[22] McNeilly TN, Naylor SW, Mahajan A, Mitchell MC, McAteer S, Deane D, et al. Escherichia coli O157:H7 colonization in cattle following systemic and mucosal immunization with purified H7 flagellin. Infect Immun 2008;76(6):2594–602.
[23] Potter AA, Klashinsky S, Li Y, Frey E, Townsend H, Rogan D, et al. Decreased shedding of Escherichia coli O157:H7 by cattle following vaccination with type III secreted proteins. Vaccine 2004;22:362e–9e.
[24] van Diemen PM, Dziva F, Abu-Median A, Wallis TS, van den Bosch H, Dougan G, et al. Subunit vaccines based on intimin and Efa-1 polypeptides induce humoral immunity in cattle but do not protect against intestinal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7 or O26:H-. Vet Immunol Immunopathol 2007;116:47–58.
[25] Van DJ, Hancock D, Rogan D, Potter AA. *Escherichia coli* O157:H7 vaccine field trial in 9 feedlots in Alberta and Saskatchewan. Can Vet J 2005;46:724–8.
[26] McNeilly TN, Mitchell MC, Rosser T, McAteer S, Low JC, Smith DGE, et al. Immunization of cattle with a combination of purified intimin-531 EspA and Tir significantly reduces shedding of Escherichia coli O157:H7 following oral challenge. Vaccine 2010;28(5):1422–8.
[27] Kühne SA, Hawes WS, La Ragione RM, Woodward MJ, Whitelam GC, Gough KC. Isolation of recombinant antibodies against EspA and intimin of Escherichia coli O157:H7. J Clin Microbiol 2004;42(7):2966–76.
[28] Amani J, Mousavi SL, Rafati S, Salmanian AH. *In silico* analysis of chimeric *espA*, *eae* and *tir* fragments of *Escherichia coli* O157:H7 for oral immunogenic applications. Theor Biol Med Model 2009;6:28.
[29] Schunk MK, Macallum GE. Applications and optimization of immunization procedures. ILAR J 2005;46(3):241–57.
[30] Cooper HM, Paterson Y. Production of antibodies. Curr Protoc Immunol 1995:2.4.1–9.
[31] Cook SR, Maiti PK, DeVinney R, Allen-Vercoe E, Bach SJ, McAllister TA. Avian- and mammalian-derived antibodies against adherence-associated proteins inhibit host cell colonization by Escherichia coli O157:H7. J Appl Microbiol 2007;103:1206–19.
[32] Wadolkowski EA, Burris JA, O'Brien AD. Mouse model for colonization and disease caused by enterohemorrhagic Escherichia coli O157:H7. Infect Immun 1990;58:2438–45.
[33] Babiuk S, Asper DJ, Rogan D, Mutwiri GK, Potter AA. Subcutaneous and intranasal immunization with type III secreted proteins can prevent colonization and shedding of Escherichia coli O157:H7 in mice. Microb Pathog 2008;45:7–11.
[34] Lee JY, Ye J, Henderson D, Langridge WHR. Plant- synthesized E.coli CFA I fimbrial protein protects Caco-2 cells from bacterial attachment. Vaccine 2004;23:222–31.
[35] Naylor SW, Roe AJ, Nart P, Spears K, Smith DG, Low JC, et al. *Escherichia coli* O157:H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the *LEE4* operon. Microbiology 2005;151:2773–81.
[36] Roe AJ, Yull H, Naylor SW, Woodward MJ, Smith DG, Gally DL. Heterogeneous surface expression of EspA translocon filaments by Escherichia coli O157:H7 is controlled at the post-transcriptional level. Infect Immun 2003;71:5900–9.
[37] Li Y, Frey E, Mackenzie AM, Finlay BB. Human response to Escherichia coli O157:H7 infection: antibodies to secreted virulence factors. Infect Immun 2000;68:5090–5.
[38] Ghaem-Maghami M, Simmons CP, Daniell S, Pizza M, Lewis D, Frankel G, et al. Intimin-specific immune responses prevent bacterial colonization by the attaching-effacing pathogen Citrobacter rodentium. Infect Immun 2001;69:5597–605.
[39] Meckelein B, Externest D, Schmidt MA, Frey A. Contribution of serum immunoglobulin transudate to the antibody immune status of murine intestinal secretions: influence of different sampling procedures. Clin Diagn Lab Immunol 2003;10(5):831–4.
[40] Spokesman GM, Finn PW, Ward ES, Dumont J, Dickinson BL, Blumberg RS, et al. Receptor-mediated immunoglobulin G transport across mucosal barriers in adult life: functional expression of FcRn in the mammalian lung. J Exp Med 2002;196:303–10.
[41] Yoshida M, Claypool SM, Wagner JS, Mizoguchi E, Mizoguchi A, Roopenian DC, et al. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. Immunity 2004;20:769–83.