A PLGA-encapsulated chimeric protein protects against adherence and toxicity of enterotoxigenic *Escherichia coli*

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**A B S T R A C T**

Enterotoxigenic *Escherichia coli* (ETEC) are the most common cause of diarrhea among children. Colonization factors and enterotoxins are the major ETEC candidate vaccines. Since protection against ETEC mostly occurs by induction of IgA antibodies, much effort is focused on the development of oral vaccines. In this study oral immunogenicity of a poly(lactic-co-glycolic acid) (PLGA) encapsulated chimeric protein containing CfaB, CsfH, CoA and LTB (Heat-labile B subunit) was investigated. The protein was encapsulated in PLGA by double emulsion method and nanoparticles were characterized physicochemically. Immunogenicity was assessed by evaluating IgG1, IgG2 and IgA titers after BALB/c mice vaccination. Non aggregated nanoparticles had a spherical shape with an average particle size of 252.7 ± 23 nm and 91.96 ± 4.4% of encapsulation efficiency. Western blotting showed maintenance of the molecular weight and antigenicity of the released protein. Oral immunization of mice induced serum IgG and fecal IgA antibody responses. Immunization induced protection against ETEC binding to Caco-2 cells. The effect of LT toxin on fluid accumulation in ileal loops was neutralized by inhibition of enterotoxin binding to GM1-gangliosides. Delivery of the chimeric protein in PLGA elicited both systemic and mucosal immune responses. The findings could be exploited to development of oral multi-component ETEC prophylactic measures.

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1. **Introduction**

Enterotoxigenic *Escherichia coli* (ETEC) are the most common causes of diarrheal disease associated with morbidity and mortality in children up to 5 years of age in developing countries and in travelers to high risk area (Tobias et al. 2011; Gupta et al. 2008; Qadri et al. 2005). Heat-labile (LT) and/or a heat-stable (ST) enterotoxin and colonization factors (CFs) are the major virulence factors in ETEC pathogenesis (Tobias and Vutukuru 2012; Svennerholm and Lundgren 2012; Qadri et al. 2005). ETEC must adhere to the epithelium of the small intestine by means of CFs to cause disease. Watery diarrhea is then produced due to the effects of the enterotoxins (Isidean et al. 2011). Several types of CFs and putative colonization factors have been identified (Isidean et al. 2011; Subekti et al. 2003). Of 25 CFs identified so far, seven, viz. CFA/I, CS1, CS2, CS3, CS4, CS5 and CS6 account for 50–70% of clinical ETEC isolates (Petri et al. 2008; Qadri et al. 2005). Vaccine development against ETEC has been identified as an important primary prevention strategy for children living in endemic regions and for adult travelers to those regions (Svennerholm and Tobias 2008; Sabiu et al. 2010).

Mucosal surfaces are the predominant entry site for most of the infectious pathogens including ETEC (Takahashi et al. 2009; Byrd et al. 2005). Mucosal immunization could provide the secretary IgA antibody (sIgA) responses in the intestine that prevents the attachment of bacteria to mucosa and are of particular importance for protection against ETEC infection (Tobias et al. 2011; Takahashi et al. 2009).

Adverse conditions or inadequate absorption renders native antigens as weak immunogens when administered to mucosal surfaces (Garino et al. 2007). Encapsulating antigens into nanoparticles such as poly(lactic-co-glycolic acid) (PLGA) can protect antigens from degradation in gastric conditions. PLGA enhances the immunogenicity of antigens by increasing antigen absorption by lymphoid tissue and controlling its release (Byrd et al. 2005; Eldridge et al. 1991). A multivalent ETEC vaccine containing the most prevalent colonization factors and LT may provide protection against a wide range of ETEC strains (Svennerholm and Tobias 2008). With this view, we constructed an ETEC candidate

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immunogen containing CFA/I, CS2, CS3 and LTB (named L2C3) encapsulated in PLGA particles. The anti-toxicity and anti-adherence properties of the immunogen was studied.

2. Materials and methods

2.1. Bacterial strains

The previously isolated ETEC strains from children with diarrhea, were characterized for CFS and enterotoxin profile. They were used as the challenge strains.

2.2. Design and construction of chimeric protein L2C3

Major subunit genes in CFA/I, CS2 and CS3 colonization factors are cfaB, cotA and cstH. These major subunits and B subunit from LT toxin were selected for construction of the chimeric gene. Related sequences were obtained from available sequence databases, from the National Centre for Biotechnology Information. The sequences were fused together by (EAAAK) 4 linker in order to find the best epitope exposing chimeric antigen (Fig. 1A). The bioinformatic analysis was performed as reported in our previous work (Nazarian et al. 2012). The gene was synthesized by Shine Gene Molecular Biotech, Inc. (Shanghai, China).

2.3. Expression and purification of chimeric protein

The synthetic gene was subcloned into pET28a and recombinant plasmids were introduced into the E. coli BL21(DE3) (Novagen). The transformants containing (pET28a-cfaB-cotA-cstH-ltb) were grown in Luria Bertani (LB) broth supplemented with 30 µg of kanamycin/ml. Expression of the chimeric protein was induced at OD600 of 0.5 by addition of 1 mM isopropyl-β-d-galactopyranoside (IPTG) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was purified by batch purification of His-tagged proteins from E. coli under denaturing conditions (Qiagen) and validated by SDS-PAGE. The denaturant (8 M urea) was removed by stepwise dialysis.

2.4. Western blot analysis

The proteins were blotted onto nitrocellulose membranes. The membrane strips were blocked with 5% nonfat dried milk and washed with PBS (137 mM NaCl, 2.7 mM KCl and 4.3 mM Na2HPO4). One strip was incubated with 1:5000 dilution of rabbit anti-cholera toxin antibody (CTX, Sigma) and the other one was incubated with rabbit anti-6X His tag antibody (Abcam). This was followed by incubation in 1:10,000 dilution of horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG antibody (Sigma). Detection was carried out using HRP staining solution.

2.5. Preparation of PLGA nanoparticles encapsulated with chimeric protein

L2C3 chimeric protein was encapsulated in PLGA (Sigma) with a copolymer ratio of lactide: glycolide 50:50 (Mw 40,000–75,000) using a water/oil/water double emulsion (w/o/w) method. 25 mg of PLGA was dissolved in 2.2 ml of dichloromethane to which 0.5 ml of an aqueous protein solution containing 1 mg L2C3. Emulsification was carried out by ultrasonicator at amplitude of 5 for 20 s in
an ice cooled bath. The w/o primary emulsion was added to 10 ml of 2% (w/v) aqueous polyvinyl alcohol (PVA) in a drop-wise manner and then emulsified with high speed homogenizer for 2 min at 12,000 × g. The w/o/w double emulsion was added to 0.5% PVA and was stirred to allow solvent evaporation. Nanoparticles were collected, washed with sterile deionized water and freeze-dried using 2.5% trehalose dihydrate as cryoprotectant.

2.6. Particle size measurement and zeta potential of PLGA nanoparticles

Particle size analysis and zeta-potential were determined by Zetasizer Nano ZS (Malvern Instruments, UK). Microscopic characteristics of PLGA nanoparticles were investigated by scanning electron microscopy (TESCAN Vega LMU, USA).

2.7. In vitro release of protein from the nanoparticles

Five milligrams of antigen loaded nanoparticles were dispersed in 2 ml PBS buffer (pH 7.4 or pH 2.2) and then was placed in a thermostatic shaker (37 °C, 300 rpm). The sample was centrifuged at 12,000 × g for 30 min and 300 μl of supernatant was removed and replaced with the same volume of fresh PBS at specified time intervals. The amount of the L2C3 release was determined using bicinchoninic acid (BCA) protein assay kit (Abnova).

2.8. Protein encapsulation efficiency

Encapsulation efficiency was determined by hydrolyzing the nanoparticles. 5 mg of prepared nanoparticle was dissolved in 0.5 ml of 1 M sodium hydroxide and incubated at 37 °C for 14 h. The solution was neutralized by adding 0.5 ml of 1 M hydrochloric acid. The solution was centrifuged and the supernatant was analyzed for protein content. The encapsulation efficiency (EE) was calculated by the following equation:

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\%EE = \frac{\text{total amount of L2C3} - \text{free L2C3}}{\text{total amount of L2C3}} \times 100
\]

2.9. Stability and antigenicity of the encapsulated protein

Stability and antigenicity of the protein released from PLGA nanoparticles were examined by western blotting as described above. In western blotting, mouse anti-CS3, -CS2, -CFA/I (developed in-house at our department) and -CTX IgG antibody were used to react with nitrocellulose bound chimeric protein.

2.10. Animals immunization

Female BALB/c mice (6–8 weeks old) were used for immunization experiments. For oral administration, a group of 10 mice received 50 μg dose of L2C3 encapsulated in PLGA and another group was immunized with free L2C3 in PBS. The primary immunization was followed by two boosts 2 and 4 weeks later. A control group received 25 μg dose of alum-L2C3 subcutaneously (s.c.) and a second control group received 50 μg dose of BSA-PLGA. Blood samples were taken from mice before and after immunization. IgA from feces was recovered as described previously (Amani et al. 2010).

2.11. Determination of anti-L2C3 IgG and IgA responses

Antibody titers were determined by enzyme linked immunosorbant assay (ELISA). Maxisorb plates (Nunc) were coated with 2 μg of LT, CfaB, CstH, CotA (expressed and purified at our department) and L2C3 proteins and blocked with 5% skim milk in PBST (PBS plus 0.05% Tween 20). Washed plates were incubated with serially diluted serum and fecal samples for 45 min at 37 °C. Other ELISA procedure steps were carried out as described previously (Amani et al. 2010). IgG1 and IgG2 isotypes were determined with ELISA using goat anti mouse IgG1, IgG2 antibodies (Sigma).

2.12. ETEC binding inhibition assay

ETEC strains possessing CFA/I, CS2 and CS3 were used for inhibition study. Caco-2 cells were grown in Dulbecco’s modified eagle media (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (PBS; Gibco). The cells were trypsinized and distributed onto a sterile round cover slip placed on the bottom of 6-well plates at a concentration of 5 × 10⁴ cells per well and grown to near confluence with 70% in a 5% CO₂ atmosphere. ETEC cells at 10⁷ CFU/ml concentration was pretreated with 40 μl of serum or fecal sample from immune and non immune mice for 30 min at room temperature. The mixture was added to Caco-2 cells and incubated for 1 h at room temperature. After washing with PBS, the cells were fixed in methanol, stained with Giemsa staining solution (Sigma) and then destained with PBS. The cover slips containing the Caco-2 cells bound with ETEC were examined under microscope. The number of ETEC cells that adhered to each Caco-2 cell was counted. The adhesion index was determined by examining 100 Caco-2 cells, corresponding to the mean number of bacteria per cell. The level of inhibition was determined by comparing the primary adherence index with and without the addition of antibody.

2.13. Toxin neutralization assay

To study the efficiency of serum and fecal samples from immunized mice to neutralize the toxicity of LT, rabbit ileal loop assay, GM1-ELISA and cyclic AMP were conducted. Serially diluted serum or fecal samples collected from immunized mice were incubated (37 °C, 1 h) with LT (2 μg/ml). The mixture was then added to 5 μg/ml of GM1 (Sigma)-coated well plates. Anti-CTX and HRP-conjugated goat anti-rabbit IgG were used as the primary and secondary antibodies. Optical density was measured at 492 nm after incubation in ortho phenylenediamine (OPD, Sigma) peroxidase substrate.

Rabbit ileal loop was assayed according to the method described by De and Chatterjee (1953). Briefly, New Zealand White rabbits (1.5 kg) were fasted for 24 h before surgery. The animals were anesthetized and 1 cm ligated ileal loops were constructed. ETEC bacteria at 1 × 10⁸ CFU/ml concentration were incubated with mouse serum or fecal sample, for 30 min. The bacterial mixture was inoculated into each ligated loop. The animals were sacrificed 18 h later, and the ratio of fluid accumulation against loop length (g/cm) was calculated as an index of enterotoxigenicity. Experimental infection of the rabbits was performed at the Faculty of Veterinary Medicine, University of Tehran. Research was conducted in compliance with the Animal Welfare Act and regulations related to experiments involving animals.

For cell rounding assay, Y-1 mouse adrenal cells (Pasteur. Institute of Iran) were grown in Dulbecco’s modified eagle media (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (PBS; Gibco). The Cells were seeded in cell culture plates at a concentration of 5 × 10⁴ cells per well and grown to near confluence at 37 °C in a 5% CO₂ atmosphere. Y-1 cells were mixed with LT pretreated with serum and fecal samples and incubated in 5% CO₂ incubator at 37 °C for 10 h. The morphological changes were observed under the microscope. In cAMP assay, CHO cells were seeded and grown in 24-well sterile culture plates as described above. 0.1 μg of LT was incubated with 50 μl mice serum or fecal
sample for 1 h. The mixture was added to each well for incubation at 37 °C in 5% CO₂ for 2 h. After a wash with PBS, the cells were lysed with 0.1 M HCl. The lysate was centrifuged and supernatant was tested for intracellular cAMP levels using a cAMP immunoassay kit (Abnova).

3. Results

3.1. Preparation and characterization of recombinant L2C3 protein

The synthetic l2c3 gene was expressed in E. coli BL21 (DE3). The recombinant protein was verified in insoluble pellets mainly by SDS-PAGE, and the molecular weight matched the theoretical prediction of 69.9 kDa. The expression of recombinant protein was confirmed by reaction with the anti-His-tag and anti-CTX antibodies with Western blotting (Fig. 1B). Purification of the protein were carried out under denaturation condition and SDS-PAGE analysis revealed the presence of the chimeric protein in the eluted fraction (Fig. 1C).

3.2. Characterization of L2C3–PLGA

PLGA nanoparticles were prepared by double emulsion method. Loading efficiency of L2C3–PLGA nanoparticles was 91.96 ± 4.4% with an average particle size of 252.7 ± 23 nm. Zeta potential of the nanoparticles was 11.2 ± 2.7. The external morphology of the nanoparticles studied by SEM revealed that nanoparticles were approximately spherical in shape having a smooth surface (Fig. 2).
In vitro release of chimeric protein from the PLGA in artificial gastric (pH 2.2) and in intestinal (pH 7.4) juice exhibited an initial burst release of 29 and 19 percent respectively within the first 24 h. A sustained release of protein was then observed thereafter under both acidic and neutral conditions. A sustained L2C3 release to a total of approximately 60.45% was recovered from the nanoparticles in the intestinal juice, whereas the release was only about 71.3% in artificial gastric juice. Antigenicity of PLGA encapsulated antigen determined by western blot analysis (Fig. 3) showed that the specific anti-CS3, -CS2, -CFA/I, -CTX and -L2C3 antibodies recognized the released chimeric antigen.

3.3. Serum and mucosal antibody responses

Animals remained healthy and showed no signs of abnormal behavior after vaccination. Following oral immunization, mice elicited significant IgG and IgA antibodies in serum and fecal samples compared to control mice (P < 0.05). Antibody responses were observed against all single antigens of chimeric protein (LTB, CfaB, CstH and CotA) (Fig. 4A–C). The IgG response from immunization with L2C3–PLGA was mainly dominated by the IgG1 subclass, followed by IgG2 (Fig. 4D). As expected, free L2C3 elicited a weak immune response and all the mice responded to L2C3 administered by s.c injection.

3.4. Binding inhibition of ETEC to Caco-2 cells

The ability of anti-L2C3 antibodies collected from immunized mice to block the binding of ETEC bacteria to Caco-2 cells was measured. Examination of Caco-2 cells revealed that ETEC CFA/I, CS2, CS3 positive strains were distributed on the Caco-2 cells. Almost all the Caco-2 cells were observed to bind one or more bacteria with an adhesion index of 22.2 ± 2.1, 17.5 ± 0.8 and 16.5 ± 0.9 bacteria per cell for CFA/I, CS2 and CS3 positive strains respectively. In contrast, pretreatment of the ETEC cells with anti-L2C3 antibody blocked their binding to Caco-2 cells (Fig. 5). Adhesion index was reduced to 3.4 ± 0.9, 2.9 ± 0.5 and 2.7 ± 0.8 bacteria per cell for CFA/I, CS2 and CS3 positive strains respectively. Anti-L2C3 antibody inhibited the adherence of CFA/I, CS2 and CS3-expressing bacteria to Caco-2 cells by approximately 84.68%, 83.42% and 83.63%.

3.5. Toxin neutralization assay

Binding of LT to the coated GM1 ganglioside was blocked as a result of subjecting antibodies from immunized mice to GM1-ELISA (Fig. 6A). The inhibitory effect of anti-L2C3 antibody on LT-induced fluid accumulation was confirmed in the rabbit ileal loop assay (Fig. 6B). The fluid accumulation was not observed in rabbit ileal loops 18 h post infection with ETEC treated with the immunized mice antibody. In contrast, infection with ETEC was accompanied by an increase of fluid secretion. No morphological changes...
were observed in Y1 cells incubated with LT pretreated with anti-L2C3 antibody, whereas normal serum had no inhibitory effects on rounding (Fig. 7). The cAMP immunoassay revealed that the antibody from immunized mice prevented the LT from stimulating intracellular cAMP in CHO cells (Fig. 8).

4. Discussion

The main strategy in the present study was to produce a PLGA encapsulated chimeric protein carrying epitopes from different ETEC serotypes and LT B as an adjuvant. This adjuvant plays a role...
not only in enhancing immunogenicity of the recombinant antigen, but also in elicitation of a broad mucosal immune response (Byrd and Cassels 2003; Kim et al. 2010; Verweij et al. 1998). In order to separate different domains of our chimeric protein, a well established linker (Amani et al. 2010; Arai et al. 2001; Chen et al. 2012) consisting of EAAAK repeats were used. For its nanoparticles being composed of biodegradable polymers that degrade to normal metabolic products (Kumari et al. 2010; Vert et al. 1994), PLGA was used for encapsulation of the chimeric protein. Other main advantages pertaining to PLGA include protection of protein from degradation, possibility of sustained release and enhancing both systemic and mucosal antibody responses (Danhier et al. 2012; Park et al. 2011; Shive and Anderson 1997). Oral administration of antigen used in this study is more advantageous than parenteral administration in the sense of its acceptability, simplicity of administration and elicitation of a stronger mucosal response (Pavot et al. 2012). Encapsulation of ETEC colonization factors was reported previously (Byrd and Cassels 2006; de Lorimier et al. 2003), de Lorimier et al. (2003) demonstrated that intranasal CS6–PLGA is safe and induces immunity. Byrd and Cassels (2006) showed that the CS3–loaded PLGA enhanced the immunogenicity of the CS3 protein. The present study is the first report of design, production and vaccination with a chimeric encapsulated immunogen inducing mucosal immune response against ETEC CFA/I, CS2 and CS3 positive strains. The chimeric protein was released from PLGA nanoparticles by polymer erosion.

An initial burst release of antigen was observed shortly after incubation of nanoparticles in neutral and acidic pH conditions. This release of antigen corresponds to a priming immunization dose. This initial burst effect is thought to be due to the rapid release of antigen located on or near the surface of the nanoparticles (McGee et al. 1994).
Slower release of the protein following initial burst release may be due to slow diffusion of the entrapped protein from nano-matrix into the medium (Coombes et al. 1998). Antigenicity of the released antigen detected by western blot analysis demonstrated that the chimeric protein remains intact retaining its antigenicity. The chimeric L2C3 protein described in this study could express LTB as a vaccine against ETEC enterotoxin and as a mucosal adjuvant for the recombinant protein. The development of efficient adjuvants for mucosal route is one of the more promising approaches (Borges et al. 2007). LTB has strong mucosal adjuvant capacity and can increase mucosal immune response against LTB conjugated protein due to its binding ability to GM1 on surface of M cells (Verweij et al. 1998; Wagner et al. 2004). The L2C3–PLGA administered orally induced an IgG1 subclass response followed by IgG2. This is in agreement with the response from previous reports on CFA1, CS6 and CS3 (Alves et al. 1998; Byrd and Cassels 2003; Byrd and Cassels 2006). These results indicate that antigens given by mucosal routes could be able to induce the humoral as well as cellular immunity. Although precautions were taken to add protease inhibitors to the extracts, the fecal IgA antibody titer was lower than that of the serum sample. This may be due to proteolytic degradation of antibodies prior to the addition of protease inhibitor (Qadri et al. 2000, 2003). LT neutralizing activities indicated that anti-LTB antibodies recognized native form of LTB in holotoxin and prevented LT binding to GM1-ganglioside. We also demonstrated that LT antibody was sufficient for the inhibition of LT induced cAMP accumulation and cell rounding. These results further support critical role of the antibody against LTB in protection against ETEC diarrhea (Fingerut et al. 2005; Kim et al. 2010; Sanchez and Holmgren 2005). Binding inhibition assay was performed to confirm production of anti-CFs antibodies in mice. A statistically significant (P < 0.005) reduction in ETEC adherence to Caco-2 cells was determined upon pretreatment of ETEC cells with antibodies. The results demonstrate that antibodies prepared against CFs efficiently blocked the binding of ETEC strain to Caco-2 cells. In conclusion; we demonstrated that the oral administration of the L2C3–PLGA nanoparticles induced systemic and mucosal responses. The results indicated that encapsulation of the recombinant chimeric protein containing different CFs and nontoxic enterotoxin antigens could help develop oral multivalent ETEC vaccine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.116/j.micres.2013.06.005

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