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Intranasal immunization with fusion protein MrpH·FimH and MPL adjuvant confers protection against urinary tract infections caused by uropathogenic *Escherichia coli* and *Proteus mirabilis*

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ABSTRACT

Urinary tract infections (UTIs) caused by Uropathogenic *Escherichia coli* (UPEC) and *Proteus mirabilis* are among the most common infections in the world. Currently there are no vaccines available to confer protection against UTI in humans. In this study, the immune responses and protection of FimH of UPEC with MrpH antigen of *P. mirabilis* in different vaccine formulations with and without MPL adjuvant were assessed. Mice intranasally immunized with the novel fusion protein MrpH·FimH induced a significant increase in IgG and IgA in serum, nasal wash, vaginal wash, and urine samples. Mice immunized with fusion MrpH·FimH also showed a significant boost in cellular immunity. Addition of MPL as the adjuvant enhanced FimH and MrpH specific humoral and cellular responses in both systemic and mucosal samples. Vaccination with MrpH·FimH alone or in combination with MPL showed the highest efficiency in clearing bladder and kidney infections in mice challenged with UPEC and *P. mirabilis*. These findings may indicate that the protection observed correlates with the systemic, mucosal and cellular immune responses induced by vaccination with these preparations. Our data suggest MrpH·FimH fusion protein with or without MPL as adjuvant could be potential vaccine candidates for elimination of UPEC and *P. mirabilis*. These data altogether are promising and these formulations are good candidates for elimination of UPEC and *P. mirabilis*.

1. Introduction

Urinary tract infections (UTIs), including cystitis and pyelonephritis, are among the most common infectious diseases with significant morbidity and mortality in humans (Dhakal et al., 2008; Ragnarsdottir et al., 2008). Approximately 50–80% of women and 14% of men will suffer at least one episode of UTI in their lifetime and 20–50% of women will experience recurrent episodes (Ejrnaes, 2011; Luo et al., 2012). UTI is caused by several different pathogens, but in 80–95% of uncomplicated UTIs the etiological agent is uropathogenic *Escherichia coli* (UPEC), while *Proteus mirabilis* is associated with complicated infections, long term catheterization and stone formation (Armbruster and Mobley, 2012; Qin et al., 2013; Scavone et al., 2011). Although in majority of cases antibiotic therapy is effective, but the continual increase in antibiotic resistance among pathogens causing UTIs is severely limiting the effectiveness of this treatment, making development of a preventative vaccine a global target (Billips et al., 2009; Scavone et al., 2004). Ideally a UTI vaccine should be multivalent with broad coverage, but the pathogens involved produce an array of virulence factors which has made designing an effective vaccine challenging (Brumbaugh and Mobley, 2012; Wieser et al., 2010). Several UTI associated virulence factors expressed by UPEC and *P. mirabilis* have been tested as candidate vaccines of which those involved in colonization of uroepithelia and establishment of infection have attracted more attention (Hagan and Mobley, 2007; Scavone et al., 2004). It has been shown that FimH, the minor subunit of type 1 fimbriae of UPEC is responsible for bacterial adhesion and its use in various forms and in combination with FimC or fused to FliC the major subunit of flagellin as vaccine candidate has resulted in significant protection and prevention of UTI in a mouse model (Asadi Karam et al., 2013; Connell et al., 1996; Langermann et al., 2000, 1997).

Similarly immunization with MrpH, the tip adhesin of mannose-resistant *Proteus*-like (MR/P) fimbriae, which plays an important

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role in bacterial colonization of host mucosal surfaces, protects mice against experimental infection (Li et al., 2004a).

However, although vaccination with these proteins induces a significant immune response, but administration of a mucosal adjuvant can augment both mucosal and systemic responses to vaccine antigens (Baldridge et al., 2000). It is now evident that mobilization of adaptive immunity in response to vaccines is directed by innate immune response through Toll-like receptors (TLRs) resulting in antigen-specific acquired immunity (Huleatt et al., 2007; Nielubowicz and Mobley, 2010). Monophosphoryl lipid A (MPL) a non-toxic, immunoactive derivative of the lipopolysaccharide obtained through modification of *Salmonella minnesota* LPS by Edgar Ribi and colleagues enhances adaptive immunity via binding to TLR4 complex (Casella and Mitchell, 2008). Furthermore, since the mucosal immune system is the first line of defense against the invading pathogens, mounting a strong response to prevent bacterial colonization and establishment of the UTI infection is essential (Alteri et al., 2009; Brumbaugh and Mobley, 2012) and it has been shown that administration of MPL-adjuvanted vaccines results in enhanced production of protective IgA antibodies both locally and at distal sites (Baldridge et al., 2000).

Fusion protein technology provides a platform for incorporation of target antigens from one or more pathogen in one molecule to simultaneously obstruct different virulence mechanisms or prevent infection caused by different pathogens (Cuadros et al., 2004; Skeiky et al., 2002; Weinrich Olsen et al., 2001).

In this study, the immunogenicity and the ability of a fusion protein composed of FimH from UPEC and MrpH of *P. mirabilis* with and without MPL adjuvant by intranasal route to protect against UTI caused by UPEC and *P. mirabilis* were evaluated.

2. Materials and methods

2.1. Expression and purification of recombinant proteins

In this study *fimH* of UPEC and *mrpH* gene of *P. mirabilis* were amplified from clinical isolates and the sequence of the PCR products was verified by a commercial facility (Eurofins MWG, Germany). Alignment of the sequences obtained showed ≥98% homology with the data deposited in the GenBank therefore, the genes amplified from locally isolated bacteria (*fimH*: GenBank accession JX847135.1, *mrpH*: GenBank accession KJ130024.1) were used for construction of *mrpH·fimH* fusion gene (GenBank accession KJ182940.1) by overlap PCR (Asadi et al., 2012) and subsequent cloning and expression. The sequence-verified genes were cloned in pET28a (Novagen, USA) and expressed in *E. coli* BL21 (DE3) (New England Biolabs, USA) as 6×his-tagged proteins using IPTG (Sigma, USA). Recombinant proteins were purified by Ni-NTA resin (Qiagen, Germany) according to the manufacturer's instructions combined with the method of Reichelt et al. (2006) for simultaneous endotoxin removal by Triton X-114. After dialysis, the purified proteins were characterized by SDS–PAGE and electro-transferred to nitrocellulose membrane (Schleider and Schuell, Germany) for Western blotting. Membranes were blocked with 0.5% skim milk (Merck, Germany) in TPBS (PBS containing 0.05% Tween 20, pH 7.2) for 1 h at 37 °C. The blocked membranes were incubated for 90 min with HRP-conjugated anti-His monoclonal antibody (Invitrogen, USA) at a concentration of 1:5000 in TPBS containing 0.5% skim milk and after 3 min × 5 min washes with TPBS were developed with diaminobenzidine (DAB; Sigma, USA).

The purified proteins were quantified using Bradford method and endotoxin (LPS) contamination was determined by chromogenic Limulus amebocyte lysate test (Lonza, USA).

2.2. Mucosal immunization

Six to 8 weeks old female BALB/c mice were obtained from Pasteur Institute of Iran and all animal experiments were carried out in accordance with the European Communities Council directive of 24 November 1986 (86/609/EEC). Mice intraperitoneally anesthetized with a mixture of ketamine and xylazine in groups of 18 were intranasally immunized on days 0, 7 and 14 with a total of 20 μl (10 μl in each nostril) containing 25 μg proteins of each vaccine formulation (FimH, MrpH or fusion MrpH·FimH) in sterile PBS or emulsified in MPL adjuvant (Sigma, USA). For immunization of mice with a mixture of FimH and MrpH, 12.5 μg of each antigen was used. The control groups were vaccinated with PBS or MPL alone. MPL adjuvant was prepared in accordance with the manufacturers' instructions and diluted to 1 mg/ml and used at the final concentration of 10 μg in each preparation. On day 21, blood and urine samples, nasal and vaginal wash were obtained from anesthetized mice (Arakawa et al., 2005; Li et al., 2004b). Briefly, nasal cavities were gently flushed from the posterior opening of the nose with PBS and for vaginal washes, sterile PBS was flushed gently in the vaginal tract through the opening of the vulva of an anesthetized mouse and the fluid was collected by a micropipette. Gentle abdominal massage was used to induce bladder voiding for urine sample collection. The samples were centrifuged at 10,000 × *g* for 15 min to remove cellular debris, and the supernatants were stored at −20 °C until used. Blood samples collected from a tail vein, were incubated at 4 °C overnight and centrifuged at 3000 rpm for 10 min for serum collection. Sera were also collected after each injection and stored at −20 °C for further analysis.

2.3. Evaluation of antibody responses

FimH and MrpH-specific antibody levels in sera and mucosal secretions were measured by ELISA. Briefly, 1 μg/well of FimH or MrpH proteins were coated onto 96-well microtiter plates and incubated overnight at 4 °C. The plates were washed with washing buffer (PBS + 0.05% Tween 20) and were blocked with 3% BSA in PBS for 2 h at room temperature. Serial dilutions of serum (1:50–1:6400) or mucosal samples (undiluted-1:50) were added to wells, and incubated for 2 h at room temperature. Plates were then washed and horseradish peroxidase conjugated goat anti-mouse IgG (Sigma, USA) was added to the wells and incubated for 1 h at 37 °C. After washing, TMB (3,3,5,5-tetramethylbenzidine; Sigma, USA) was used and the absorbance was measured at 450 nm by a plate reader. Detection of antibody isotypes (IgG1, IgG2a) and IgA was performed as described using a specific secondary antibody for each isotype and IgA.

2.4. Lymphocyte proliferation assay

After the last immunization, spleens were aseptically removed from the mice (six/group) and cell suspensions were prepared after mechanical homogenization. The cells (3×10^5 cells/well; triplicate wells) were cultured in RPMI 1640 medium (Gibco, USA), supplemented with 10% FBS and antibiotics in the presence of 10 μg/ml purified FimH, MrpH or medium alone at 37 °C and 5% CO_2 in a humidified incubator for 72 h. Cell proliferation was assessed by colorimetric BrdU cell proliferation ELISA kit according to the manufacturer's protocol (Roche, USA). The stimulation index (SI) was calculated as the ratio of the average OD_{450} value of wells containing antigen-stimulated cells to the average OD_{450} value of wells containing un-stimulated cells.

2.5. Analysis of cytokine responses

Spleen cells (3×10^5 cells/well) were cultured in triplicate in flat-bottom 24-well microtitre plates. Supernatants from the