Molecular Immunology 54 (2013) 32–39

Contents lists available at SciVerse ScienceDirect

Molecular Immunology

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

# Assessment of immune responses of the flagellin (FliC) fused to FimH adhesin of Uropathogenic *Escherichia coli*

Mohammad Reza Asadi Karam [a], Mana Oloomi [a], Mehdi Mahdavi [b], Mehri Habibi [a], Saeid Bouzari [a,*]

[a] Department of Molecular Biology, Pasteur Institute of Iran, Pasteur Ave., Tehran 13164, Iran
[b] Department of Virology, Pasteur Institute of Iran, Pasteur Ave., Tehran 13164, Iran

ARTICLE INFO

*Article history:*
Received 23 September 2012
Received in revised form 25 October 2012
Accepted 5 November 2012
Available online 3 December 2012

*Keywords:*
UTI
UPEC
FliC
FimH
Fusion
Montanide

ABSTRACT

Urinary tract infection (UTI) caused by Uropathogenic *Escherichia coli* (UPEC) is one of the most common infectious diseases in the world. Despite extensive efforts, a vaccine that protects humans against UTI is currently missing. In this study, the immunogenicity of flagellin (FliC) of UPEC strain in different vaccine combinations with FimH antigen of UPEC and conventional adjuvant Montanide ISA 206 was assessed. Finally, efficacy of the immune responses was evaluated for protection of the bladder and kidney of challenged immunized mice. Mice immunized with the fusion FimH·FliC induced significantly higher anti-FliC humoral (IgG1) and cellular (Th1 and Th2) immune responses than with FliC alone or FliC admixed with FimH. The Montanide enhanced the immune responses of FliC antigen and directed the anti-FliC responses preferentially toward Th1. The FliC vaccine combinations reduced bladder infection as compared to control mice. The fusion FimH·FliC and FliC admixed with FimH and Montanide combinations gave the best results in protection of kidney infection, compared to the control mice. The results of this study propose new promising vaccine combinations based on the FliC antigen and Montanide against UTI caused by UPEC.



## 1. Introduction

Urinary tract infections (UTIs) are among the most common infectious diseases that cause substantial medical costs and morbidity worldwide (Dhakal et al., 2008; Ragnarsdottir et al., 2008). It is estimated that approximately 50% of women will experience one UTI in their lifetime and many of them will suffer from recurrent infection (Nielubowicz and Mobley, 2010).

The studies suggest that UTIs often occur in an ascending manner, colonization of pathogen in periurethral area and then migration of the uropathogen from urethra into the bladder to establish bladder infection (cystitis), and if cystitis is left untreated, the pathogen may ascend the ureters to reach the kidneys to cause pyelonephritis. The uropathogen may migrate from the kidneys into the blood stream, causing bacteremia, sepsis and sometimes death (Walters and Mobley, 2009; Wright et al., 2005).

Uropathogenic *Escherichia coli* (UPEC) are the common cause of UTIs, accounting for more than 80% of all UTIs in humans (Bien et al., 2012; Wiles et al., 2008). The important virulence factors of UPEC consist of type1 pili, hemolysin, siderophores, flagella and capsule (Hagan and Mobley, 2007; Nesta et al., 2012). The flagella are important virulence factors of flagellated bacteria such as *Salmonella*, *Pseudomonas*, *Proteus* and *E. coli* species which are required for motility, colonization and invasiveness into the host tissues (Schwan, 2008; Smith et al., 2003). Bacterial flagella are composed of a basal body, hook, motor, and filament (Honko and Mizel, 2005). Flagellin is the major protein constituent of bacterial flagella encoded by *fliC* gene. Mutation of *fliC* in UPEC strain leads to loss of flagellation and motility (Lane et al., 2007a). Bacterial flagellin has long been studied as one of the major antigens of flagellated bacteria. Bacterial flagellins have been shown to bind extracellular Toll-like receptor 5 (TLR5) leading to activation of macrophages, dendritic cells and neutrophils to produce inflammatory mediators (McSorley et al., 2002; Smith et al., 2003). Although, due to possession of adhesin FimH, type1 pili is critical in causing UPEC bladder infections (Langermann and Ballou, 2001; Martinez et al., 2000), flagella have also shown to play important roles in ascension of UPEC from bladder into the kidneys (Lane et al., 2007b). In addition, it is suggested that flagella cause dissemination of UPEC to obtain new nutrients, contribution of UPEC in efficient colonization of the urinary tract, and escape from host immune system (Lane et al., 2005, 2007b).

Due to the increasing emergence of antibiotic-resistant UPEC strains, the development of an efficacious UTI vaccine would have a significant impact on public health and economic costs in societies (Billips et al., 2009; Song and Abraham, 2008). Some of the antigens of UPEC have been tested as vaccine candidates against UTI that have limited success. To date, no UTI vaccines have been approved

* Corresponding author. Tel.: +98 21 66953311; fax: +98 21 66492619.
*E-mail address:* saeidbouzari@yahoo.com (S. Bouzari).

0161-5890/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.molimm.2012.11.002

for human use and thus, we need to test new antigens or other strategies in order to develop an ideal vaccine against UTI (Billips et al., 2009; Hagan and Mobley, 2007).

One of the advantages of designing a vaccine candidate based on fusion protein technology is the incorporation of two or multiple target antigens in one molecule, which can prevent pathogenesis via several pathways (Cuadros et al., 2004). Construction of fusion FimH·FliC as a vaccine candidate against UTI can be an attractive idea, since FimH and FliC have critical roles in bladder (cystitis) and kidney (pyelonephritis) infection and the immune responses against the antigens can simultaneously prevent UTI in bladder and kidney. Furthermore, antibody response is likely an important component of the immune responses against UPEC and an ideal UTI vaccine target should be surface exposed and accessible to circulating antibodies. The targets also should be prevalent among clinical UPEC, possess conserved epitopes, and have an important role in pathogenesis (Hagan and Mobley, 2007; Russo et al., 2003). Based on the previous studies, virulence factors of FimH and flagellin (FliC) of UPEC strains have the criteria to present as an ideal vaccine candidate against UTI.

In the previous study, the immunogenicity of FimH was evaluated in mice immunized with different combinations of FimH and FliC with or without adjuvant Montanide ISA 206 (under consideration). However, in the present study, we investigated the humoral and cellular immune responses of FliC antigen in different vaccine combinations with and without adjuvant Montanide. Finally, efficacy of the immune responses was evaluated for inhibition of adhesion and protection of the bladder and kidney of challenged immunized mice.

## 2. Materials and methods

### 2.1. Expression and purification of recombinant proteins

In our previous study (Asadi et al., 2012), amplification of *fimH* and *fliC* genes of UPEC isolates was performed and fusion fimH·fliC was constructed by overlap PCR. Similarly, cloning and expression of the *fimH* (GenBank accession JX847135), *fliC* (GenBank accession JX847136) and fused *fimH·fliC* (GenBank accession JX624727) genes have also been previously described (Asadi et al., 2012). Recombinant proteins were purified using Ni-NTA resins (Qiagen) according to the manufacturer's instruction combined with the method of Reichelt et al. (2006) for simultaneous endotoxin removal by Triton X-114. Evaluation of the purified proteins was performed by SDS-PAGE and Western blot analysis with His-tag antibody.

In other study (under consideration), the immunogenicity of the fusion protein was evaluated by testing its TLR5 bioactivity on inducing IL-8 secretion. Endotoxin (LPS) level of the proteins was determined by the Chromogenic LAL test (Lonza, USA).

### 2.2. Immunizations

All animal experiments were carried out in accordance with the European Communities Council directive of 24 November 1986 (86/609/EEC). BALB/c mice were subcutaneously immunized on days 0, 14 and 28 with 100 μl PBS or Montanide (Seppic, France) only (control), PBS containing 50 μg FliC (FliC), 50 μg FliC admixed with Montanide (FliC + Montanide), 25 μg FliC admixed with 25 μg FimH (FliC + FimH), 50 μg fusion FimH·FliC (Fusion), 50 μg fusion admixed with Montanide (FimH·FliC + Montanide), 25 μg FliC admixed with 25 μg FimH and Montanide (FliC + FimH + Montanide). Sera were collected after each injection to determine the antibody response and the immunized mice were used for cytokines and challenge assays.

### 2.3. Analysis of antibody responses by ELISA

Antibody response to recombinant FliC protein was evaluated by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (Greiner, Germany) were coated with purified FliC diluted in PBS (10 μg/ml in PBS) and incubated overnight at 4 °C. Wells were then blocked with 3% bovine serum albumin (BSA; Sigma), washed and incubated with serial dilutions of immune sera starting from 1:10. Plates were then incubated with HRP-conjugated goat anti-mouse IgG (Sigma, USA) and developed with TMB substrate. The reaction was stopped with 2 N HCl and read on an ELISA plate reader at 450 nm. Detection of IgG isotypes and IgM was performed as described above, except that the secondary antibody was specific for mouse IgG1, IgG2a and IgM.

### 2.4. Cell proliferation analysis

Spleen of immunized mice (Six in each group) was removed two weeks after the last immunization and splenocytes were cultured in RPMI 1640 medium (Gibco, USA) ($3 \times 10^5$ cells/well; triplicate wells) supplemented with fetal bovine serum (FBS) and antibiotics in the presence or absence of FliC and fusion proteins (10 μg/ml) for 72 h at 37 °C. Cell proliferation was assessed by Cell Proliferation ELISA kit, BrdU (colorimetric) according to manufacturer's instruction (Roche Applied Science). Stimulation index (SI) was calculated by dividing the mean absorbance at 450 nm for the triplicate stimulated with the proteins by the mean absorbance for cells cultured with medium alone.

### 2.5. Cytokines measurement

To determine the antigen-specific cytokines secretion, interleukin-4 (IL-4), interferon-γ (IFN-γ) and interleukin-10 (IL-10) levels were measured in cultured splenocytes stimulated with FliC or fusion using DuoSet ELISA kits (R&D systems Inc.) in accordance with manufacturer's protocol. IL-10 production was measured by Mouse IL-10 kit (MABTECH AB) according to the manufacturer's instruction.

### 2.6. Urinary tract infection model

A murine urinary tract infection model was used to assess the efficacy of anti-FliC immune responses in prevention of bladder and kidney infection (Lane et al., 2005). We used a UPEC isolate that was obtained from a patient with acute urinary tract infection to challenge mice immunized with different vaccine combinations. The inoculum for challenge was prepared from a 48 h culture of the UPEC adjusted to deliver $1 \times 10^8$ colony-forming units (cfu). Two weeks after the last immunization, mice (six in each group) were anesthetized with ketamine/xylazine and inoculated transurethrally with a volume of 10 μl containing $1 \times 10^8$ cfu of bacterial suspension by means of a sterile polyethylene catheter. After 2 days post-inoculation, the bladder and kidney of mice were harvested and homogenized in 1 ml of sterile PBS. Serial dilutions of the homogenates were cultured on LB medium, and colonies were counted to determine the cfu/ml of the tissues.

### 2.7. Statistical analysis

The one-way ANOVA, Student's *t*-test and the Tukey HSD test were used to compare the differences between the mean values of the immunized groups using SPSS software. The results of challenge also were analyzed with the Kruskal–Wallis with the Dunn's multiple comparison, using Prism software (GraphPad). $p < 0.05$ of all results was considered as significant.