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Use of flagellin and cholera toxin as adjuvants in intranasal vaccination of mice to enhance protective immune responses against uropathogenic *Escherichia coli* antigens

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ABSTRACT

Urinary tract infections (UTIs) caused by Uropathogenic *Escherichia coli* (UPEC) are among the most common infections in human. Antibiotics are common therapy for UTIs, but increase in antibiotic resistance will complicate future treatment of the infections, making the development of an efficacious UTI vaccine more urgent. In this study, we have evaluated intranasally the efficacy of FliC and FimH antigens of UPEC in different vaccine formulations with and without cholera toxin (CT) adjuvant. Immunization of mice with FliC in fusion form or admixed with FimH elicited higher levels of serum, mucosal and cell-mediated responses than FimH alone. Furthermore, the use of CT in synergism with FliC resulted in the stimulation of a mixed Th1 and Th2 responses against FimH and FliC as antigen and maintained the antibody responses for at least 24 weeks following the last vaccine dose. Of the vaccine preparations, Fusion, Fusion + CT, and FimH admixed with FliC and CT showed the best protection against UPEC. These data indicated that intranasal administration of a FliC and CT adjuvant-based vaccine has the potential to provide protective responses against UPEC strains.

1. Introduction

Urinary tract infections (UTIs) as one of the most common bacterial infections in human are predominantly caused by Uropathogenic *Escherichia coli* (UPEC) [1,2]. Episodes of UTI occur in about 40–50% of women that one in four of them will develop a recurrent infection [3]. Bladder is the common site of UTI, if left untreated, these infections can cause major complications including acute pyelonephritis, bacteremia, and sometimes death [4].

There is growing evidence that classical antibiotic treatment may not eradicate the causal organisms of acute UTI, particularly where intracellular reservoirs of UPEC are present [5,6]. Consequently, there is a need to develop an efficacious vaccine to prevent UTIs [6]. To date, efforts to develop a vaccine against UPEC have been limited success. The main reasons for the limited success are heterogeneous nature of UPEC strains and the use of monovalent vaccine candidates, regardless to intracellular reservoirs of UPEC, short longevity of protective immune responses, ineffectiveness in simultaneous stimulation of mucosal, systemic and cellular responses, and fails to simultaneous protection in bladder and kidney [5–7].

Fusion protein based vaccines that target multiple virulence factors can provide a solution to the problem of diversity in UPEC strains and also simultaneously protect the infection in bladder and kidneys [5,8]. Since the urogenital tract mucosa is the site of entry of UPEC, another alternative may be mucosal vaccination to generate a robust mucosal immune response in the urinary tract [5,9]. Furthermore, it has been shown that nasal vaccination with antigens combined with mucosal adjuvants stimulates mucosal, systemic and cellular immune responses against infectious diseases [10].

UPEC express a variety of virulence factors that play crucial roles in the development of UTI [7,11]. FimH as adhesin of type 1 pili demonstrated to be essential for the virulence of UPEC strains [5] and has the criteria of an ideal vaccine candidate including conservation among UPEC strains, highly immunogenicity, and expression at a high level during experimental UTIs [7,12]. For these reasons, we and others [13,14] selected the FimH of UPEC as a promising target for UTI vaccine development. The studies indicated that approximately 95% of UTIs caused by UPEC developed in

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an ascending manner, and flagella as a virulence determinant of UPEC are needed for the migration from the bladder into the kidneys [15]. Furthermore, the studies reported that flagellin (FliC) as the major protein of the flagellar filament is highly conserved among the motile bacteria [15,16] and could be another target for the vaccine development against UTI caused by UPEC.

The recent studies indicated that the mucosal surface of urinary tract contains receptors known as Toll-like receptors (TLRs), recognize the pathogen-associated molecular patterns (PAMPs) of UPEC, resulting in the activation of innate and adaptive immune responses [7,17]. In other studies, mucosal administration of flagellin as a PAMP was associated with significant mucosal and systemic immune responses [16,18,19] and the adjuvant properties were correlated with the capacity of flagellin to activate TLR5 on dendritic cells and other immune cells [16]. Because of the presence of TLR5 in the mucosal surfaces of the urinary tract, development of a mucosal UTI vaccine using a TLR ligand such as flagellin to induce significant immune responses could be of high importance [20].

We have recently evaluated the antigenic and adjuvant properties of flagellin (FliC) of UPEC strains subcutaneously (s.c.) with purified FimH protein admixed or genetically linked as fusion protein [13,21]. Here, we investigated the murine immune responses and protection to intranasal (i.n.) administration of different formulations of FimH and FliC with or without *Vibrio cholera* cholera toxin (CT) as a mucosal adjuvant.

2. Materials and methods

2.1. Protein expression and purification

Construction of fusion fimH.fliC, cloning and expression of *fimH*, *fliC* and fimH.fliC fusion genes and purification of the recombinant proteins has been previously described [21,22].

2.2. Mouse immunization

Female BALB/c mice 6–7 weeks were purchased from Pasteur Institute of Iran. Mice were housed in isolated and ventilated cages, and the animal works were approved by the Pasteur Institute of Iran ethical committee. Mice in groups of 18 were immunized intranasally on days 0, 14, and 28 with a 20 μl aliquot (10 μl per nostril) of phosphate-buffered saline (PBS) containing 25 μg of proteins FimH, FliC and fusion FimH.FliC alone or combined with CT (1 μg per dose) (Sigma, USA) as mucosal adjuvant. For immunization of mice with a mixture of FimH and FliC, 12.5 μg of each antigen was used. The control mice were inoculated with PBS or CT alone. Sera, nasal wash, vaginal wash and urine samples were collected from anesthetized mice as described previously [23,24] for further analysis.

2.3. Evaluation of humoral immune responses

FimH and FliC specific antibodies in serum and mucosal samples were analyzed by enzyme-linked immunosorbent assay (ELISA) as described previously [25]. Briefly, FimH and FliC proteins were coated onto 96-well microtiter plates (1 μg/well). Serial dilutions of sera (1:50–1:6400) and mucosal samples (undiluted to 1:50) in blocking buffer were applied as primary and HRP-conjugated anti-mouse IgG, IgG1, IgG2a and IgA (Sigma, USA) used as secondary antibodies.

2.4. Cell proliferation analysis

Spleens of immunized mice were removed 2 weeks after the last vaccine dose and single cell suspensions were made by forcing the organs through the sterile grinder. Red blood cells (RBCs) were lysed using RBC lysis buffer containing 8.02 mg/ml NH4Cl, 0.84 mg/ml NaHCO3, and 0.37 mg/ml EDTA in distilled water. Final suspensions were made in RPMI medium (supplemented with L-Glutamine, Gibco) with 1% sodium pyruvate, 1% penicillin/streptomycin, 1% non-essential amino acids, 10% fetal bovine serum (FBS) and 0.001% 50 mM β-mercaptoethanol. Finally, splenocytes (3×10^5 cells/well) were cultured with either medium alone or 10 μg/ml of purified proteins FimH and FliC. After incubation for 72 h at 37 °C, supernatants were harvested and cell proliferation assay was performed by cell proliferation ELISA kit, BrdU (colorimetric) according to the manufacturers (Roche Applied Science, Germany). Then, the stimulation index (ratio of mean OD 450 nm value from stimulated to non-stimulated cells) was calculated for each mice group.

2.5. Cytokine ELISAs

Levels of interferon-gamma (IFN-γ), interleukin-4 (IL-4), IL-2, IL-17 and IL-12 in supernatants from splenocytes cultured *in vitro* with FimH and FliC antigens were determined by ELISA kits (R&D Systems, USA) according to the manufacturer's recommendations.

2.6. Bacterial challenge

Finally, we determined whether the immune responses in vaccinated mice were correlated with the decrease of UPEC bacterial load in the bladder and kidney following transurethral challenge. In the previous study [13], we optimized the bladder challenge model with an UPEC isolated from a patient with acute UTI. Briefly, mice were anesthetized with a mixture of ketamine/xylazine and bacterial suspensions (50 μl/mouse) were delivered transurethrally using a sterile 0.28 mm inner diameter polyethylene catheter, with total inoculum of 1×10^8 CFU/mouse. For determination of CFUs, bladders and kidneys were harvested from euthanized animals at 48 h post-inoculation, homogenized in PBS and plated on Luria-Bertani agar to determine the CFU/ml of the organs.

2.7. Statistical analysis

Graphing and statistical analyses were performed using Prism version 6 (GraphPad Software, Inc.). The experimental groups were compared by one-way ANOVA, and Student's t-test. The results of challenge also were analyzed with the kruskal–wallis with the Dunn's multiple comparison. The level of significance was set at $P < 0.05$ for all experiments.

3. Results

3.1. Expression and purification

The recombinant proteins FimH, FliC and FimH.FliC were purified by Ni-NTA resin and confirmed by SDS-PAGE and Western blot [21,22]. The endotoxin level of the purified proteins was less than 0.01 EU/ml.

3.2. Serum IgG antibody responses

Two weeks after intranasal immunization with the vaccine formulations, anti-FimH and FliC IgG levels were significantly increased (Table 1). The antibody responses rose after second and third dose of vaccine in all mice groups compared to the control mice (Table 1). FimH + FliC and Fusion-immunized mice exhibited significantly enhanced levels of anti-FimH IgG antibody compared