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Immunization with recombinant FliD confers protection against *Helicobacter pylori* infection in mice

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ABSTRACT

Nearly half of the world's population is infected with *Helicobacter pylori.* Clinical manifestations of this infection range from gastritis and peptic ulcers to gastric adenocarcinoma and lymphoma. Due to the emerging of antibiotic resistant strains and poor patient compliance of the antibiotic therapy, there is increasing interest in the development of a protective vaccine against *H. pylori* infection. The bacterial protein FliD forms a capping structure on the end of each flagellum which is critical to prevent depolymerization and structural degradation. In this study, the potential of FliD as a prospective *H. pylori* subunit vaccine was assessed. For this purpose, immunogenicity and protective efficacy of recombinant FliD (rFliD) from *H. pylori* was evaluated in C57BL/6 mice. Purified rFliD was formulated with different adjuvants and administered *via* subcutaneous or oral route. Subcutaneous immunization with rFliD elicited predominantly mixed Th1 and Th17 immune responses, with high titers of specific IgG_1 and IgG_{2a}. Splenocytes of immunized mice exhibited strong antigen-specific memory responses, resulting in the secretion of high amounts of IFN-γ and IL-17, and low levels of IL-4. Immunization with rFliD caused a significant reduction in *H. pylori* bacterial load relative to naïve control mice ($p < 0.001$), demonstrating a robust protective effect. Taken together, these results suggest that subcutaneous vaccination with rFliD formulated with CpG or Addavax could be considered as a potential candidate for the development of a subunit vaccine against *H. pylori* infection.

1. Introduction

H. pylori is a spiral-shaped, extracellular Gram-negative, and microaerophilic bacterium that has colonized the stomach of approximately 50% of the worldwide human population (Correa and Piazuelo, 2011). In 1994, *H. pylori* was classified as a class I carcinogen by the World Health Organization (Anderl and Gerhard, 2014). Infection is strongly associated with the development of several gastrointestinal diseases, including duodenal and gastric ulcers, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma (MALT) (Suzuki et al., 2009). Antibiotic therapy, although effective, is associated with several disadvantages including low patient compliance due to the drugs' side effects, treatment failure as a result of the emergence of drug-resistant strains, high costs, and the failure to prevent reinfection (Boyanova et al., 2002; Frenck and Clemens, 2003; Gisbert, 2005; Wheeldon et al., 2005). Therefore, alternative approaches to combat *H. pylori* infection are currently being pursued, including vaccination. Several *H. pylori* proteins have been identified as immunogenic in preclinical models, including Urease B (UreB) (Yang et al., 2005), Vacuolating toxin A (VacA) (Ghiara et al., 1997), *H. pylori* adhesion A (HpaA) (Sutton et al., 2007), neutrophil-activating protein A (NapA) (Satin et al., 2000), outer membrane protein (Omp) (Czinn and Blanchard, 2011), cytotoxin-associated antigen (CagA) (Ghiara et al., 1997), heat-shock proteins (Hsp) (Chionh et al., 2014a; Ferrero et al., 1995), OipA (Chen et al., 2012) catalase (Radcliff et al., 1997) and chimeric genes (Zhang et al., 2015; Li et al., 2012; Lv et al., 2014). Each of these antigens has the ability to reduce the bacterial load in animal models, but the elicited protection provided by them is less than ideal. Current strategies to enhance vaccine efficacy include the identification of most suitable immune targets and the combination of immunodominant antigens into multivalent formulations. Khalifeh Gholi et al. (Khalifeh Gholi et al., 2013) demonstrated that FliD, the flagellar

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Table 1
Vaccine formulations and route of application.

Route	Vaccine	Antigen dose	Adjuvant dose	Total volume	Number of mice
Subcutaneous	rFliD + CPG	30 μg	20 μg	100 μl	18
	rFliD + Addavax	30 μg	50 μl	100 μl	18
	rFliD + PBS	30 μg	0	100 μl	18
	CpG	0	20 μg	100 μl	18
	Addavax	0	50 μl	100 μl	18
	PBS	0	0	100 μl	18
Oral	rFliD + CTB	30 μg	5 μg	100 μl	18
	CTB	0	5 μg	100 μl	18

hook-associated protein 2, reacts with approximately 97 percent of sera obtained from patients infected with *H. pylori,* but not with sera from uninfected individuals, and reacts very weakly with sera from patients whose infections had been eradicated, suggesting that this is a common immune target in the infected human host (Khalifeh Gholi et al., 2013). FliD plays a crucial role in flagella assembly. Flagellin is important for bacterial motility and is essential for colonization and persistence of *H. pylori* in the stomach niche (Eaton et al., 1996).

The objective of the current study was to investigate the protective efficacy and underlying mechanisms of recombinant FliD (rFliD) antigen. Owing to inherent safety and the low risk of adverse reactions (Ghasemi et al., 2015), protein subunit immunization is an attractive vaccination approach. However, immune responses elicited by the administration of pure antigens are usually low. Therefore, we investigated whether formulations of FliD with different adjuvants, CpG, Cholera toxin subunit B (CTB), or Addavax, enhance protection of mice against *H. pylori* infection.

2. Materials and methods

2.1. Bacterial strains

E. coli strains BL21 and TOP10 were used for expression of FliD. Bacteria were routinely grown at 37 °C in LB broth or agar. The *H. pylori* strain SS1 was grown on *Brucella* agar supplemented with 5% sheep blood, 5 μg/mL trimethoprim, 161.5 μg/mL polymyxin B (Sigma), 10 μg/mL vancomycin (sigma) and 2.5 μg/mL amphotericin B (Sigma), in an anaerobic jar with microaerophilic gas generating kit (Merck, Germany) for 3 days at 37 °C. For infection experiments, bacteria were subcultured in brain heart infusion broth (BHI, Merck) containing 10 μg/mL vancomycin and supplemented with 10% Fetal Bovine Serum (FBS) (Sigma), and grown in microaerophilic conditions for 72 h at 37 °C.

2.2. Cloning, expression, and purification of recombinant FliD

Cloning and expression of FliD from *H. pylori* in BL21 (DE3) and its purification has been described previously (Khalifeh Gholi et al., 2013). Briefly, the *flid* gene was amplified from genomic DNA of *H. pylori* by PCR (forward primer: 5′-ATGGCAATAGGTTCATTAAGCT-3′, reverse primer: 5′- ATTCTTTTTAGCCGCCGCTT-3′). The DNA fragment was directly inserted into pTZ57R (InsTAclone PCR Cloning Kit, ThermoFisher, USA) and then subcloned into the pET28a + vector (Novagen, USA) to add a 6xHis tag. To express FliD, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used. Upon induction, rFliD was expressed in soluble form and purified under native conditions. Following SDS-PAGE separation, purity and identity of the recombinant protein was evaluated by Coomassie blue staining and Western blotting (Ghasemi et al., 2014a; Ghasemi et al., 2013). For immunodetection, size-separated proteins were transferred to a PVDF membrane and rFliD detected using anti-6xHis peroxidase (Roche, Germany) (1/40,000). Finally, the membrane was developed using 3, 3′-Diaminobenzidine tetrahydrochloride (Sigma). To preclude effects caused by residual endotoxin present in the protein preparation, only batches of purified protein with an endotoxin content of less than 0.05 endotoxin units per mg of protein (evaluated by Limulus amebocyte lysate analysis kit, Lonza, Basel, Switzerland) were used in further studies. The Bradford method was used to determine the concentration of the recombinant protein (Stoscheck, 1990).

2.3. Mice

Six- to eight-week-old female C57BL/6 mice were purchased from the Pasteur Institute of Iran and maintained under specific pathogen-free conditions. Mice were handled under optimal conditions of temperature, hygiene, humidity and light (cycles of 12 h dark/light). All experimental animal procedures were approved by the ethical committee of the Kashan University of Medical Science.

2.4. Immunization and H. pylori inoculation of mice

One hundred and forty-four mice were randomly divided into eight groups (n = 18 each). Five groups were immunized subcutaneously (s.c.) three times at 2-week intervals with either 30 μg rFliD formulated with 20 μg/mouse CpG (CpG, ODN1826 5′-TCCATGACGTTCCTGAC GTT- 3′, synthesized by TAG Copenhagen, Denmark), 30 μg rFliD formulated with (1:1) Addavax (Invivogen, USA), or PBS, or 20 μg/mouse CpG, Addavax, and PBS alone. The two remaining groups were orally immunized with rFliD and 20 μg/mouse CTB (Sigma, Germany) or CTB alone (Table 1). To enable immunization in a small volume, the recombinant antigen was lyophilized and then reconstituted in a volume of 100 μl PBS containing the respective adjuvant.

Two weeks after the final immunization, eight mice from each group were challenged orally thrice in 2-day intervals with 5×10^8 CFU mouse-adapted *H. pylori* strain SS1 in 100 μl brain heart infusion broth. Five mice were bled to obtain sera at days 0, 15, 30, 45, and 75 after the first immunization. The remaining five mice were sacrificed to evaluate immune responses including cytokine production, sIgA secretion, and IgG immune responses on the day of the challenge. For measuring sIgA secretion, gastric fluid was collected as described previously (Chionh et al., 2014b).

2.5. Humoral and mucosal immune responses

To measure rFliD-specific serum IgG_1, IgG_{2a} and gastric fluid sIgA titers in immunized mice, an enzyme-linked immunosorbent assay (ELISA) was used. 96-well polystyrene plates (Greiner Bio-One, Frickenhausen, Germany) were coated with the purified rFliD (1 μg/mL). After overnight incubation, TBST buffer (Tris-buffered saline, pH 7.4, and containing 0.05% Tween 20) were used to wash the plates three times followed by blocking with 300 μl 10% FBS in PBS for 2 h at 37 °C. After that, plates were incubated with serial dilutions of mouse sera or gastric and intestinal fluid for 2 h at room temperature and then washed. HRP-conjugated goat-anti-mouse IgG_1, IgG_{2a} or sIgA antibodies (BD Pharmingen, USA) were added to the wells for another 90 min at 37 °C. After the last washing step, specific reactivity was