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Immunization with a recombinant fusion protein protects mice against *Helicobacter pylori* infection

Amir Ghasemi [a,b,*], Nazanin Mohammad [a], Josef Mautner [c], Mehrnaz Taghipour Karsabet [a], Jafar Amani [d], Abolfazl Ardjmand [e], Zarichehr Vakili [f]

[a] Department of Microbiology and Immunology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran
[b] Department of Infectious Disease and Immunology, College of Veterinary Medicine, University of Florida, FL, USA
[c] Technische Universität München & Helmholtz Zentrum München, Munich, Germany
[d] Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[e] Physiology Research Center, Kashan University of Medical Sciences, Kashan, Iran
[f] Department of Pathology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran

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ABSTRACT

More than 50% of the world's population is infected with the bacterium *Helicobacter pylori.* If left untreated, infection with *H. pylori* can cause chronic gastritis and peptic ulcer disease, which may progress into gastric cancer. Owing to the limited efficacy of anti-*H. pylori* antibiotic therapy in clinical practice, the development of a protective vaccine to combat this pathogen has been a tempting goal for several years. In this study, a chimeric gene coding for the antigenic parts of *H. pylori* FliD, UreB, VacA, and CagL was generated and expressed in bacteria and the potential of the resulting fusion protein (rFUVL) to induce humoral and cellular immune responses and to provide protection against *H. pylori* infection was evaluated in mice. Three different immunization adjuvants were tested along with rFUVL: CpG oligodeoxynucleotides (CpG ODN), Addavax, and Cholera toxin subunit B. Compared to the control group that had received PBS, vaccinated mice showed significantly higher cellular recall responses and antigen-specific IgG2a, IgG1, and gastric IgA antibody titers. Importantly, rFUVL immunized mice exhibited a reduction of about three orders of magnitude in their stomach bacterial loads. Thus, adjuvanted rFUVL might be considered as a promising vaccine candidate for the control of *H. pylori* infection.

1. Introduction

H. pylori is a spiral-shaped, microaerophilic, Gram-negative, flagellated bacterium that colonizes the stomach in more than 50% of the world's human population [1,2]. Infection with this bacterium may cause chronic gastritis and peptic ulcers and is also associated with gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma (MALT) [3]. The strong epidemiological relation between *H. pylori* and distal (non-cardia) cancer led the World Health Organization Agency to classify *H. pylori* as a definite (type I) carcinogen in 1994 [4]. Current therapies used to eradicate *H. pylori* include the use of three or four antibiotics in combination with a proton-pump inhibitor, an approach that has only been slightly changed over the three decades since *H. pylori* was identified [5,6]. Patient compliance, increasing antibiotic resistance (especially to clarithromycin and metronidazole), recurrence, and the high cost of the treatment have led to renewed interest in developing a vaccine to prevent against *H. pylori* infection [7–10].

Several *H. pylori* antigens have been investigated as vaccine candidates, but the protection provided by each antigen is less than ideal [11–19]. To improve efficacy, combinations of multiple protective antigens have been tested. For example, immunization with three different antigens derived from the vacuolating cytotoxin (VacA), cytotoxin-associated gene A (CagA), and *H. pylori* urease subunit B (UreB), was able to decrease *H. pylori* colonization in mice with pre-existing infection [20]. Because of the time-consuming and difficult process of producing three separate recombinant proteins, the simultaneous synthesis of antigenic polypeptides would be highly desirable. However, most of the reported *H. pylori* protective antigens are large and expressing them combined as a fusion protein is hardly feasible. Thus, a

* Corresponding author at: Department of Microbiology and Immunology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran.
E-mail address: amghasemi@ufl.edu (A. Ghasemi).

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chimeric antigen comprising various antigenic epitopes that have the potential to provoke robust cellular and humoral immune responses may be more effective and convenient than a single recombinant antigen [21–23].

We have recently reported an *in silico* study in which a chimeric antigen was designed using four antigenic parts of *H. pylori* flagellar hook-associated protein (FliD), UreB, VacA, and cytotoxin-associated gene L (CagL) proteins [24]. Also, we showed that vaccination of C57BL/6 mice with recombinant FliD combined with an adjuvant could stimulate protective immune responses against *H. pylori* infection [25]. Therefore, we sought to employ FliD as the main component of a polyvalent antigen by adding additional epitopes based on published findings. Since the $UreB_{327-334}$ B-cell epitope from urease prompts antibody responses that nullify urease activity and $UreB_{373-385}$ is considered a novel immunodominant CD4+ T-cell epitope [26,27], the region from UreB covering both epitopes ($UreB_{327-385}$) was fused to FliD. In addition, amino acids 744–805 from VacA, which is deemed a major virulence factor of *H. pylori* type I strains [28], contain an MHC class-II binding peptide that consistently stimulates CD4+ T-cell responses [20]. Thus, this region was also incorporated into the chimeric antigen. Finally, we have previously shown through a bioinformatics approach that amino acids 51–100 of the CagL protein ($CagL_{51-100}$), a versatile type IV secretion system (T4SS) surface protein that facilitates the translocation of CagA into host cells [29], contains numerous domains with antigenic activity [24] and was therefore also included into the potential vaccine candidate.

Following recombinant expression and purification, the chimeric polypeptide was used to immunize mice in combination with three different adjuvants: CpG ODN, Addavax, and Cholera toxin subunit B (CTB). The potential of the recombinant fusion protein to elicit immune responses in C57BL/6 mice and the prophylactic effect of the vaccine against subsequent challenge with *H. pylori* was investigated.

2. Materials and methods

2.1. Animals

Six- to eight-week-old female C57BL/6 mice were purchased from the Pasteur Institute of Iran (IPI). All animal procedures were approved by the Experimental Animal Ethics Committee of the Kashan University of Medical Science. Mice were acclimated for one week after arrival before starting the experiments.

2.2. Bacterial strains and growth conditions

The *H. pylori* strain SS1 (a kind gift from Prof. James G. Fox, Massachusetts Institute of Technology) was grown on Brucella agar supplemented with 5% sheep blood, 5 μg/mL trimethoprim, 161.5 μg/mL polymyxin B (Sigma-Aldrich, St. Louis, MO, USA), 10 μg/mL vancomycin (Sigma-Aldrich, St. Louis, MO, USA) and 2.5 μg/mL amphotericin B (Sigma-Aldrich, St. Louis, MO, USA), in an anaerobic jar with microaerophilic gas generating kit (Merck Group, Darmstadt, Germany) for 3 days at 37 °C. For infection experiments, bacteria were subcultured in Brucella broth (Merck Group, Darmstadt, Germany) containing 10 μg/mL vancomycin and supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), and grown in microaerophilic conditions for 72 h at 37 °C.

2.3. Construction and expression of recombinant protein

The *fuvl* chimeric gene design was described previously [24]. Briefly, sequences encoding FliD (1–600), UreB (327–385), VacA (744–805) and CagL (51–100) polypeptides were obtained from GenBank. To facilitate epitope exposure, flexible glycine-serine (GS) linkers were inserted between the gene segments. In addition, the chimeric gene sequence was optimized for expression in *Escherichia coli* and a tag comprising six histidine amino acids was added at the C terminal of the gene to facilitate detection and purification of the recombinant protein. The *fuvl* gene synthesis was performed by Biomatik (Cambridge, Ontario, Canada) and subcloned into the pET26a vector using HindIII and EcoRI restriction enzymes. Subsequently, *E. coli* BL21 DE3 cells were transformed with the pET-*fuvl* plasmid and expression of the recombinant fusion protein induced by 1 mM isopropyl-d-1-thioga lactopyranoside (IPTG) for 4 h at 37 °C in the presence of 50 μg/ml kanamycin. rFUVL was expressed in soluble form and purified under native conditions. Identity and purity of the recombinant protein were evaluated by SDS-PAGE and visualized by Coomassie blue staining and Western blotting [30,31]. Briefly, purified rFUVL was size-separated by SDS-PAGE and transferred to a PVDF membrane. Next, the membrane was incubated with anti-6xHis peroxidase (Roche, Basel, Switzerland) (1/40,000) for 2 h. Finally, the membrane was developed using 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA). Contamination of the recombinant protein with endotoxin was prevented by the inclusion of 0.1% Triton X-114 in all wash buffers used during purification. Only purified recombinant protein containing an endotoxin content of less than 0.05 endotoxin units per mg of protein (evaluated by Limulus amebocyte lysate analysis kit, Lonza, Basel, Switzerland) was used for further studies [30,31]. The Bradford method was used to determine the concentration of recombinant protein [32].

2.4. Immunization and infection with H. pylori

One hundred and sixty mice were randomly divided into eight groups (n = 18 each). Five groups were immunized subcutaneously (SC) three times at fifteen-day intervals with 30 μg rFUVL formulated with 20 μg/mouse CpG ODN (CpG, ODN1826 5′-TCCAT GACGTTCCTGACGTT-3′, synthesized by TAG Copenhagen, Frederiksberg, Denmark) or Addavax (Invivogen, San Diego, CA, USA), 30 μg rFUVL alone, 20 μg/mouse CpG ODN and Addavax alone, or only PBS. In addition, to evaluate the protection can be provided by the killed bacteria, eight mice were received 100 μg formalin-fixed *H. pylori* subcutaneously to evaluate the protection can be provided by the whole lysate of bacteria. To avoid immunizing with excessive volume, only 100 μl PBS comprising the antigen and the respective adjuvant was used for each vaccination. The two remaining groups were orally immunized with rFUVL and 5 μg/mouse CTB (Sigma-Aldrich, St. Louis, MO, USA) or CTB alone (Table 1). Distilled water was used to dissolve adjuvants.

Forty-five days post initial immunization, five mice from each group were challenged orally thrice in two-day intervals with 5×10^8 colony-forming units (CFUs) of mouse-adapted *H. pylori* strain SS1 in 100 μl brain heart infusion broth. In each group, five mice were bled to obtain sera at days 0, 15, 30, 45, and 75 after the initial immunization. Another five mice were sacrificed to obtain tissue and evaluate immune responses including cytokine production, IgA secretion, and humoral immune responses on the day of the challenge. To ensure the observed findings resulted from immunization treatment, the challenge experiment was repeated once with the same numbers of mice.

2.5. Protection experiment

To assess whether the vaccination of mice with adjuvanted rFUVL was able to reduce bacterial load in the stomachs of infected mice, *H. pylori* CFUs were quantified four weeks post-infection. For

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