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Preclinical immunogenicity and protective efficacy of an oral *Helicobacter pylori* inactivated whole cell vaccine and multiple mutant cholera toxin: A novel and non-toxic mucosal adjuvant

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ABSTRACT

Mucosal vaccines against *Helicobacter pylori* consisting of either whole cell bacteria or recombinant antigens can induce immune protection against challenge in mice only when co-administrated with a strong mucosal adjuvant such as cholera toxin (CT) or *Escherichia coli* heat labile enterotoxin (LT). The strong enterotoxicity of these adjuvants however preclude their use in human vaccines. The recently developed multiple mutant CT (mmCT) is a strong, yet practically non-toxic novel mucosal adjuvant which here was admixed with a formalin-inactivated *H. pylori* whole cell vaccine (WCV) as a potential vaccine candidate against *H. pylori* infection. We report that intragastric immunizations with *H. pylori* WCV together with mmCT, similar to immunization with WCV together with CT, resulted in 50–125-fold reduction in colonization of *H. pylori* in the stomach of mice associated with rises in both serum IgG and intestinal-mucosal IgA anti-*H. pylori* antibody responses and strong T cell and IFNγ and IL-17A cytokine responses. Data presented in this study also supports that the proposed vaccine can be grown in a bioreactor and would be effective against infection caused by a multitude of pathogenic *H. pylori* strains isolated from patients from various continents. The results warrant immunization studies in humans to evaluate the safety, immunogenicity and efficacy of the proposed *H. pylori* WCV and mmCT.

1. Introduction

Approximately half of the world's population is infected with *Helicobacter pylori*. The infection, which in most cases is chronic, is usually acquired during childhood and commonly under crowded living conditions where access to clean water and good food hygiene is limited [1]. Similar to other gastrointestinal infections the transmission of *H. pylori* can occur through contaminated food and water and via infected vomitus of patients with diarrhea caused by *Escherichia coli* or *Vibrio cholerae* [2]. For these reasons, the highest incidence and prevalence of *H. pylori* infection are found in low- and middle-income countries where up to 90% of the adult population are chronically infected with *H. pylori* [3].

At variance from most other gastrointestinal infections, infection with *H. pylori* does not elicit a protective immune response that can lead to the eradication of the infection, and therefore in most infected individuals remains chronic. This may also be explained by the fact that in *H. pylori* infected individuals $CD4^+$ regulatory T cells are recruited to the gastric mucosa that dampen the anti-bacterial response and promote persistence of the infection [4,5]. Chronic infection with *H. pylori* is associated with inflammation of the stomach mucosa (gastritis) in all infected individuals, which is often asymptomatic. In 15–20% of the infected individuals, however, chronic infection will lead to peptic ulcers and in 1–2% to gastric cancer [6]. Eradication of *H. pylori* infection with antibiotics has the potential to cure peptic ulcer disease and reduce the risk of gastric cancer development. However, limited compliance with the treatment and emerging antibiotic resistance present significant challenges to successful eradication of the infection.

The importance of *H. pylori* infection and disease as a global health problem has stimulated much interest in the development

Abbreviations: CT, cholera toxin; FCS, fetal calf serum; mmCT, multiple mutant cholera toxin; IG, intragastric; SL, sublingual.

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of an effective *H. pylori* vaccine that could prevent the acquisition of infection and if given to infected individuals, could promote eradication, prevent reinfection and block the progression towards cancer development. Extensive studies in mice and other small animals have yielded substantial knowledge about the immune response against *H. pylori* after vaccination or infection. Besides identifying several protective *H. pylori* antigens and the importance of $CD4^+$ T cells producing interferon-gamma and/or IL-17 for immune protection, these studies have demonstrated the importance of a mucosal route of immunization as well as the critical need for a mucosal adjuvant for inducing an effective immune response associated with protection against infection [7–10]. Mucosal immunization by intragastric, sublingual or nasal routes with killed *H. pylori* bacteria, whole-cell lysates or purified protective *H. pylori* antigens given together with an effective mucosal adjuvant (often cholera toxin [CT]) has been found to induce significant but not sterilizing immune protection against challenge with live, virulent *H. pylori* bacteria [11].

Our strategy for designing a safe and effective *H. pylori* vaccine has been to use our experience from developing oral-mucosal vaccines for human use against other gastrointestinal mucosal infections, specifically cholera and enterotoxigenic *E. coli* (ETEC) diarrhea [12]. We have shown that immunization with either an inactivated whole-cell *H. pylori* vaccine given orally/intragastrically or a cocktail of *H. pylori* antigens administered sublingually, both together with CT as an adjuvant, induce upon infection with *H. pylori* bacteria a significant, approximately 100-fold reduction in bacterial load compared to unvaccinated infected mice [10,13]. Since the presence of a strong mucosal adjuvant in the vaccine is necessary for inducing protection against *H. pylori* infection [8] and CT cannot be used as an adjuvant in humans due to its toxicity, it is noteworthy that also oral or sublingual immunization with *H. pylori* lysate antigen together with dmLT, a practically nontoxic E. coli heat-labile enterotoxin-derived adjuvant, could induce comparable mucosal immune responses and protection against *H. pylori* challenge as immunization using CT as adjuvant [9,14,15].

In the present study, we have evaluated the feasibility of an oral vaccine containing formalin inactivated *H. pylori* bacteria together with a recently developed non-toxic adjuvant, multiple- mutated CT (mmCT) [14], with similar adjuvant activity and safety profile as dmLT but easier to produce and purify in large quantities. We report that intragastric immunization with this adjuvanted *H. pylori* WCV induces levels of mucosal immune responses and protection against *H. pylori* challenge that are fully comparable to those achieved using CT as an adjuvant. The promising preclinical findings presented provide a sound basis for proposed safety and immunogenicity studies in humans with the described, easily produced, low-cost inactivated mmCT-adjuvanted oral *H. pylori* WCV.

2. Materials and methods

2.1. Animals

Six- to eight-week old specific-pathogen-free female C57BL/6 mice were purchased from Taconic (Denmark) and housed at the Laboratory for Experimental Biomedicine (EBM), University of Gothenburg for the duration of the study. All experiments were approved by the ethics committee for animal experiments (Gothenburg, Sweden).

2.2. Formalin inactivated H. pylori whole cell vaccine

The *H. pylori* Hel-305 strain, a clinical isolate from a patient with peptic ulcers was used as the primary vaccine strain. The strain was characterized with respect to its expression of previously identified or proposed protective antigens using commercial and in-house produced monoclonal antibodies to CagA (Austral Biologics, San Ramon, CA), Flagellin, HpaA and Urease in a semi-quantitative dot blot immunoassay and was found to produce consistent, high levels of all of the above mentioned antigens (Supplementary Fig. 1).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.07.073.

Additionally, *H. pylori* strains from the University of Gothenburg culture collection (17874) and from our own clinical strains collection (Cancer strain 1 and 2) were grown and characterized in the same way as described for the Hel-305 strain.

2.2.1. Preparation of formalin-inactivated H. pylori Hel-305 WCV (Grown in flasks)

Bacteria from −70 °C stock cultures were grown on Columbia-iso agar plates for 5–6 days at 37 °C under microaerophilic conditions until a confluent growth was reached. Bacteria from each plate was then harvested in sterile PBS, and the bacterial suspension was vortexed vigorously to resuspend bacterial aggregates where after the optical density at 600 nm (OD) of the suspension was measured. The bacterial suspension was diluted with sterile PBS to an OD of 1.5, and formaldehyde was then added to 50 ml bacterial suspensions in 250 ml flasks to a final concentration of 0.01 M. The flasks were tightly closed and incubated at 37 °C for 2 h with agitation followed by overnight shaking at room temperature. The bacterial suspension was then washed three times with sterile PBS and the final bacterial pellet was re suspended in sterile PBS to OD 1.5. From each flask one hundred (1 0 0) microliter of the washed bacterial suspension was plated onto Columbia-Iso agar plates and incubated as described above to check inactivation of the bacteria. Strict aseptic handling procedure was followed during the process of preparing the inactivated *H. pylori* WCV from strain Hel 305 (hereafter referred to as Hel-305 WCV) and then stored at 4 °C until use.

2.2.2. Preparation of formalin-inactivated H. pylori Hel-305 WCV (Grown in bioreactor)

Batches of *H. pylori* Hel-305 WCV were produced in small-scale bioreactors (Multifors 250 ml, Infors HT, Switzerland). *H. pylori* strain Hel305 from −70 °C stock cultures were first grown on Columbia-iso agar plates for 3 days in microaerophilic conditions. Bacteria were scraped off and suspended in PBS and used as an inoculum. To a starting volume of 150 ml of sterile brucella Broth (BD, USA), 8% fetal calf serum (Corning, USA) and 2.5% Dent (Thermo Fisher Scientific, USA) was added. The temperature in the vessel was set to 36 °C with a stirrer rate at 600 rpm and a gas of 5% oxygen, 10% carbon dioxide, and 85% nitrogen (AGA, Sweden) was introduced at a flow rate of 0.15–0.6 lpm (litre/minute). Rapeseed oil (Axfood, Sweden) was used as antifoam agent. The pH was continuously measured together with pO_2 and OD_{600} (Supplementary Fig. 2A). Formalin inactivation of the bioreactor grown *H. pylori* strain Hel305 was carried out as described above.

Using the dot blot assay and antigen-specific monoclonal antibodies the bioreactor grown Hel-305 WCV was shown to contain each of the tested antigens CagA, Flagellin, HpaA and Urease (Supplementary Fig. 2B).

2.3. Immunizations

Mice were immunized by three intragastric rounds of immunization on two consecutive days at biweekly intervals (Fig. 1A), with laboratory or bioreactor grown Hel-305 WCV using either mmCT or CT as an adjuvant. Each administration provided approximately 1×10^9 inactivated bacteria with either 7.5 μgCT (List Biological Laboratories, Inc., Campbell, CA) or 7.5, 15 or 30 μg mmCT in

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