Oral Immunization with Recombinant *Mycobacterium smegmatis* Expressing the Outer Membrane Protein 26-Kilodalton Antigen Confers Prophylactic Protection against *Helicobacter pylori* Infection†‡

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*Helicobacter pylori* infection is prevalent worldwide and results in chronic gastritis, which may lead to gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer. We have previously reported that oral immunization with recombinant *Mycobacterium smegmatis* expressing the *H. pylori* outer membrane protein 26-kilodalton (Omp26) antigen affords therapeutic protection against *H. pylori* infection in mice. In the present study, we investigated the prophylactic effects of this vaccine candidate on *H. pylori* challenge in mice. We found that oral immunization with recombinant *Mycobacterium Omp26* significantly reduced *H. pylori* colonization in the stomach compared to inoculation with wild-type *M. smegmatis* in control mice. Six of the recombinant *Mycobacterium*-immunized mice (60%) were completely protected from *H. pylori* infection. The severity of *H. pylori*-associated chronic gastritis assessed histologically was significantly milder in mice vaccinated with recombinant *Mycobacterium* than in control animals. Mice immunized with recombinant *Mycobacterium* showed enhanced antigen-specific lymphocyte proliferation and antibody responses. Moreover, immunization with recombinant *Mycobacterium* resulted in an increased expression of interleukin-2 and gamma interferon in the stomach and spleen, as determined by reverse transcription-PCR analysis. Our results collectively suggest that vaccination with recombinant *Mycobacterium Omp26* confers prophylactic protection against *H. pylori* infection. The inhibition of *H. pylori* colonization is associated with the induction of antigen-specific humoral and cell-mediated immune responses.

*Helicobacter pylori* is a spiral-shaped Gram-negative bacterium that inhabits various areas of the stomach, particularly the antrum. It causes a chronic low-level inflammation of the stomach lining and is strongly linked to the development of gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer (23, 25, 28, 31). Approximately 50% of the global population has been estimated to be infected by this bacterium, with a higher prevalence in developing countries (2). Current regimens for treatment of *H. pylori* infection consist of a proton pump inhibitor (PPI) with any two antibiotics of amoxicillin, clarithromycin, and metronidazole. Despite a high eradication rate of greater than 80%, there are some limitations associated with the PPI-based triple therapy, such as poor patient compliance, emerging antibiotic resistance, frequent reinfection, and high cost (4). Vaccination against *H. pylori* would represent an attractive alternative or complement to standard antibiotic therapy.

Identification of protective antigens that can induce effective immune responses is a crucial step for vaccine development. To date, many protein molecules expressed by *H. pylori* have been identified to possess immunogenicity, including urease, cytotoxin-associated antigen (CagA), neutrophil-activating protein A (NapA), *H. pylori* adhesin A (HpaA), vacuolating toxin A (VacA), catalse, and outer membrane protein (Omp) (4, 6). Numerous vaccination studies performed in animal models have demonstrated that immunization with various *H. pylori* antigens or combinations confers protective immunity against this bacterium, leading to a significant reduction in bacterial load (11, 13, 20). However, sterilizing immunity, which completely prevents or eradicates infection, is rarely achieved (7). Most importantly, no effective and safe vaccine against *H. pylori* is currently available for humans.

The development of effective vaccines requires an efficient antigen delivery system. *Mycobacterium smegmatis* is a species of rapidly growing mycobacteria and generally regarded as nonpathogenic. These properties make this bacterium an ideal vaccine vector (3, 9, 10, 34). It has been documented that recombinant *M. smegmatis* engineered to express human immunodeficiency virus type 1 (HIV-1) Env elicits HIV-1 envelope-specific CD8+ T-cell responses (3). Falcone and colleagues (9) reported that immunization with recombinant *Mycobacterium* bearing *Mycobacterium bovis* BCG genes limits the growth of virulent *Mycobacterium tuberculosis* within the lung and spleen in mice. Unlike other mycobacterial species, such as BCG, that survive in host cells by inhibiting phagosome...
Maturation, M. smegmatis is rapidly destroyed by phagolysosomal proteases in the phagosomes of infected cells (14, 17), thus facilitating rapid uptake of expressed antigens in M. smegmatis and cross-presentation of antigen.

Our previous work has revealed the therapeutic benefits of recombinant Mycobacterium expressing the H. pylori Omp26-kilodalton (Omp26) antigen in the clearance of H. pylori infection (16). In this study, we sought to check whether immunization with recombinant Mycobacterium Omp26 would provide protective effects against H. pylori challenge in mice. We also evaluated the immune responses induced by this vaccine candidate.

MATERIALS AND METHODS

Generation of recombinant Mycobacterium expressing H. pylori Omp26. Recombinant Mycobacterium expressing H. pylori Omp26 was generated as described previously (16). Briefly, M. smegmatis MC\(^{\text{I-155}}\) was grown in Middlebrook 7H9 medium supplemented with an albumin-dextrose-catalase enrichment (ADC; Difco, Detroit, MI). A 594-bp fragment containing H. pylori Omp26 was amplified from the pET32a+(+) Omp26 plasmid (kindly provided by Z. Jiang, Chongqing Medical University, Chongqing, China) and cloned into the PLA73 Escherichia coli-mycobacterium shuttle vector. The Omp26 expression vector was transformed into the M. smegmatis MC\(^{\text{I-155}}\) strain by electroporation (22). Transformed mycobacterial clones were selected for kanamycin resistance on Middlebrook 7H10 agar plates (Difco) supplemented with oleic acid-ADC enrichment containing 30 \(\mu\)g/ml of kanamycin. Expression of the Omp26 protein was assessed by Western blotting of mycobacterial lysates. Recombinant bacteria in 10% glycerol were stored at -80°C until use.

Animal experiments. Forty-five specific-pathogen-free (SPF), 7-week-old female BALB/c mice (weighing 17 to 19 g) were obtained from the Chongqing Medical University Laboratory Animal Center (Chongqing, China) and housed in a pathogen-free environment. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the Chongqing Medical University. Animals were randomly divided into 3 groups (\(n = 15\) for each) and were orally immunized with 2 \(\times\) 10\(^7\) CFU of wild-type M. smegmatis or a recombinant Mycobacterium strain expressing Omp26 per mouse or given phosphate-buffered saline (PBS) as a control. No boosting immunization was done.

Four weeks after immunization, 5 mice per group were killed, and their stomachs, spleens, and sera were harvested for evaluation. The remainder of mice were orally infected with 12,000 \(\times\) 5 \(\times\) 10\(^7\) CFU of wild-type H. pylori (Sydney strain 1) or a recombinant H. pylori expressing the Omp26 antigen in the clearance of H. pylori infection (16). Six of the recombinant Mycobacterium expressing H. pylori Omp26 were selected for kanamycin resistance on Middlebrook 7H10 agar plates (Difco) supplemented with oleic acid-ADC enrichment containing 30 \(\mu\)g/ml of kanamycin. Expression of the Omp26 protein was assessed by Western blotting of mycobacterial lysates. Recombinant bacteria in 10% glycerol were stored at -80°C until use.

Evaluation of H. pylori infection. The intact stomachs from vaccinated and control mice were divided longitudinally into four parts: one for microbiological culture, one for real-time PCR analysis, one for histology, and one (snap-frozen in liquid nitrogen) for total RNA extraction and reverse transcription-PCR (RT-PCR). For bacterial culture, the stomach sample was homogenized in 2 ml physiological saline with a tissue homogenizer. The homogenate was serial diluted and plated on bruccella broth agar plates supplemented with 7% goat blood, polymyxin B (2,500 \(\mu\)g/liter), vancomycine (3,000 \(\mu\)g/liter), amphotericin B (2 mg/liter), and vitamin K1 (0.1 mg/liter) (24). For bacterial counts, samples were plated onto MacConkey agar plates supplemented with 5% sheep blood, and incubated at 37°C for 24 h. The number of colonies was counted after 24 h.

RESULTS

Immunization with recombinant Mycobacterium confers protection against H. pylori infection. We first evaluated the efficacy of recombinant Mycobacterium immunization on experimental H. pylori infection in mice. Bacterial culture revealed that immunization with recombinant Mycobacterium expressing H. pylori Omp26 significantly reduced the H. pylori load in the stomach (\(P < 0.05\) compared to control group) (Fig. 1A). Six of the recombinant Mycobacterium-immunized mice (60%) were completely protected from H. pylori challenge. However, inoculation with wild-type M. smegmatis offered no detectable protection against H. pylori infection, except in one mouse in which no H. pylori was detected in the stomach. The rapid urease test confirmed a significantly lower rate of H. pylori infection in the recombinant Mycobacterium group (50%) than in the normal control (100%) and M. smegmatis (90%) groups (\(P < 0.05\)) (Fig. 1B).
Recombinant Mycobacterium immunization decreases H. pylori-associated gastric lesions. Next, we sought to determine whether vaccination with recombinant Mycobacterium had a protective effect on the gastric lesions associated with H. pylori challenge. Mice in the nonimmunized group (Fig. 2A) and the wild-type M. smegmatis group (Fig. 2B) showed marked gastric mucosal degeneration and inflammatory cell infiltration induced following H. pylori inoculation. The severity of H. pylori-associated chronic gastritis was significantly (*P < 0.001) milder in mice vaccinated with recombinant Mycobacterium, and no neutrophil infiltration into or degeneration of the gastric mucosa was detected (Fig. 2C and D). These data, combined with the results of the biological and histological assessment of the H. pylori load, indicate that the recombinant Mycobacterium vaccine candidate prevents H. pylori infection and associated gastric damage in mice.

Immune responses elicited by recombinant Mycobacterium. In vitro lymphocyte proliferation assay revealed that splenic lymphocytes from recombinant Mycobacterium-immunized mice had a significantly (*P < 0.05) higher stimulation index than their counterparts from immunized controls, either in the presence of ConA or H. pylori extracts (Fig. 3A). This finding suggests an induction of lymphocyte proliferation responses to H. pylori after vaccination with recombinant Mycobacterium.

We also evaluated the humoral immune response induced by recombinant Mycobacterium in immunized mice. Four weeks after immunization, there were significantly (*P < 0.001) higher titers of serum IgA and IgG specific to H. pylori in recombinant Mycobacterium-immunized mice than in immunized controls for which no detectable IgA or IgG was found in the serum (Fig. 3B). Similar results were observed for the serum levels of IgG1 and IgG2a (*P < 0.001 between recombinant Mycobacterium-immunized mice and controls). Moreover, IgG2a titers were greater than IgG1 titers, reflecting a predominant Th1 response.

Local cytokine expression profile in response to recombinant Mycobacterium. To characterize the local cytokine expression profile in response to recombinant Mycobacterium vaccination, we examined the mRNA levels of IFN-γ, IL-2, IL-4, IL-6, IL-10, and IL-12 in the stomach and spleen by RT-PCR. Both stomachs and spleens from mice vaccinated with recombinant Mycobacterium expressed significantly (*P < 0.05) more...
Our data demonstrates that vaccination with recombinant 'Mycobacterium' Omp26 provides protection against 'Helicobacter pylori' infection in mice, reducing 'H. pylori' Omp26 provides protection against 'H. pylori' colonization in the stomach. The outer membrane is a continuous structure on the surface of Gram-negative bacteria and consists of an asymmetric bilayer with phospholipids in the inner monolayer and the bulky glycolipid lipopolysaccharide in the outer monolayer. 'H. pylori' strains express a large set of Omps (21), whose role is still not completely understood. Several lines of evidence suggest that some Omps are essential for 'H. pylori' colonization in the stomach (5, 32). Thus, these cell surface-expressed proteins are logical candidates for vaccine development. Talebkhan and colleagues (27) have reported that an 'H. pylori' bacterial ghost containing recombinant Omp18 is capable of stimulating specific antibodies and reducing gastric colonization by 'H. pylori' in mice. Our present data reveal the strong immunogenicity of native 'H. pylori' Omp26. Omp26-loaded 'M. smegmatis' was found to induce humoral and cell-mediated adaptive immune responses. These findings encourage a thorough evaluation of the immunogenicity of other Omps. 'H. pylori' is known to cause chronic active gastritis, which is typically characterized by considerable infiltration of inflammatory cells into the gastric mucosa. Neutrophils are thought to mediate gastritis activity and play a critical role in 'H. pylori'-induced mucosal injuries (8, 33). In response to 'H. pylori' infection, neutrophils are recruited to the site of inflammation and generate reactive oxygen and nitrogen species and proteases. They not only protect the host from the bacterium but also cause mucosal damage during inflammation. Tanko and colleagues (29) reported that there is a statistically significant relationship between 'H. pylori' colonization intensity and the degrees of neutrophil activation, chronic gastritis, and intestinal metaplasia. In support of this finding, we showed that recombinant 'Mycobacterium'-immunized mice had no detectable neutrophil infiltration into the mucosa after 'H. pylori' challenge, coupled with reduced bacterial load and gastric inflammation. In contrast, control animals with high 'H. pylori' burden exhibited marked neutrophil infiltration into the mucosa and gastric damage.

The induced immune responses during 'H. pylori' infection play a complex role in gastroduodenal diseases. Several earlier studies have documented that a CD4+ T cell-mediated IFN-γ response is not only essential for vaccine-induced protection, but also involved in the induction of gastric inflammation caused by 'H. pylori' infection (19, 26). It has been proposed that Th1 immune responses are crucial for 'H. pylori'-specific protective immunity (1, 30). Oral inoculation of the present vaccine candidate was found to induce a prominent Th1 response characterized by increased expression of Th1 cytokines (IFN-γ and IL-2) and high IgG2a serum titers. However, after 'H. pylori' challenge, the antigen-specific response appeared to be polarized toward a Th2-type response, producing predominantly IL-4. The acceleration of the Th2 immune response may inhibit the Th1 response and minimize the undesired tissue-damaging effects of inflammatory cytokines. In support of this view, the gastric IFN-γ and IL-2 levels were found to be slightly lower in recombinant 'Mycobacterium'-immunized mice after 'H. pylori' challenge than in control animals. These results suggest that the recombinant 'Mycobacterium' Omp26 vaccine candidate...
elicits a balanced Th1 and Th2 response, restraining *H. pylori* colonization in the stomach without exacerbating tissue inflammation.

In summary, our data highlight a prophylactic role against *H. pylori* infection for recombinant *Mycobacterium* expressing Omp26. Immunization with this vaccine candidate induces antigen-specific humoral and cell-mediated immune responses that may thus interfere with *H. pylori* colonization and reduce associated gastric lesions.

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