PREVENTION OF INFECTION

Protection of BALB/c mice against experimental *Helicobacter pylori* infection by oral immunisation with *H. pylori* heparan sulphate-binding proteins coupled to cholera toxin β -subunit

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The presence of *Helicobacter pylori* in the gastroduodenal mucosae is associated with chronic active gastritis, peptic ulcers and gastric cancers such as adenocarcinoma and low-grade gastric B-cell lymphoma. In response to the presence of antibiotic-resistant strains, the use of vaccines to combat this infection has become an attractive alternative. The present study used a murine model of infection by a mouse-adapted *H. pylori* strain to determine whether infection in BALB/c mice can be successfully eradicated by intragastric vaccination with *H. pylori* heparan sulphate-binding proteins (HSBP) covalently coupled to the β -subunit of cholera toxin (CTB). It was shown that vaccination confers protection against exposure of BALB/c mice to the pathogen, as revealed by microbiological, histopathological and molecular methods.

Introduction

The presence of *Helicobacter pylori* in human gastric mucosa is now established as the aetiological agent of chronic gastritis and most cases of peptic ulcer and gastric adenocarcinoma world-wide [1–5] and is considered as a class I carcinogen [6] by the World Health Organization.

Experimental infection of volunteers has shown that individuals develop severe polymorphonuclear leucocyte inflammation of the mucosa as a consequence of exposure to *H. pylori* [7], with the presence of immunoglobulin A (IgA) and IgG class antibodies. Nevertheless, this response fails to eradicate the bacteria and, although evidence exists of spontaneous eradication by the host, most untreated individuals remain infected with the organism, developing a chronic gastritis [5] characterised by gastric lymphoid tissue.

Current treatment of peptic ulcer disease consists of

acid suppression and antimicrobial therapy [8]. Although current treatment regimens are effective and the incidence of re-infection is low in industrialised countries, re-infection and recrudescence in the developing world are significant problems [9]. On the other hand, the emergence of antibiotic-resistant strains, poor patient compliance and the high cost of therapy and patient management represent additional problems for management of *H. pylori*-infected individuals, especially in the developing world, where antimicrobial treatment [6] has not been very effective.

In 1991, Karita and colleagues established transient *H. pylori* infections in immunodeficient BALB/c mice, thus demonstrating for the first time that it was possible to colonise a small laboratory animal with *H. pylori* [10]. More recently, there have been reports of the colonisation of immunocompetent mice with mouse-adapted *H. pylori* isolates [11]. Marchetti *et al.* used a mouse-adapted strain to study the immunoprotection of BALB/c mice against this gastrointestinal pathogen, so providing a suitable animal model for studying the pathology of *H. pylori* [12]. By screening various clinical isolates of *H. pylori* for their ability to colonise mice, Lee and colleagues [13] identified one *H. pylori* strain (SS1) [7] that, after adaptation to mice, was able to colonise the mouse gastric mucosae in high numbers for up to 8 months.

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In previous studies, significant protection against challenge with different species of *Helicobacter* has been demonstrated when various antigenic mixtures were co-administered by a mucosal route with a suitable adjuvant [14–19]. In those studies, vaccine antigens were found to induce protection against challenge. Although its role was not clearly defined, they suggested that antigen-specific secretory IgA (sIgA) can protect against infection. A role for sIgA in protection against *H. pylori* infection [11, 15] has been suggested with studies on passive immunity where human breast milk IgA titres were shown to correlate with a delay in the onset of *H. pylori* infection in infants.

Previous studies have shown that *H. pylori* expresses cell surface-associated proteins (outer-membrane proteins, OMP) and extracellular proteins (heat-shock bacterial proteins HSBP) which have affinity for the glucosaminoglycan heparan sulphate (Ruiz-Bustos *et al.*, unpublished data). It was also found that oral immunisation of BALB/c mice with HSBP elicited a specific mucosal and systemic immune response (Ruiz-Bustos *et al.*, unpublished data). Therefore, the present study is focused on the application of a HSBP-based vaccine to protect BALB/c mice against experimentally induced infection with a mouse-adapted *H. pylori* strain.

Materials and methods

Bacteria and growth conditions

The mouse-adapted *H. pylori* strain 25 was obtained from the gastric mucosa of BALB/c mice by repeated in-vivo passages through oral administration of 5×10^8 cfu (in 0.5 ml) of *H. pylori* [12]. After 10 days, mice were killed by cervical vertebral dislocation under light anaesthesia; stomach and small intestine were excised and homogenised in PBS. Then, 250 µl of this suspension was cultured in GAB-Camp medium supplemented with human blood 8.5% and horse serum 10% and the following antibiotics: vancomycin 100 µg/ml, amphotericin B 50 µg/ml and nalidixic acid 10.7 µg/ml. Plates were incubated for 48 h and *H. pylori* colonies were identified by morphology and confirmed by positive rapid urease reaction. The *H. pylori* strain had been passaged through mice up to eight times before its use in the present experiment.

Immunogen preparation

Briefly, HSBP and cholera toxin β -subunit (CTB) were each coupled to N-succinimidyl-(3-[2-pyridyl]-dithio) propionate (SPDP) at molar ratios of 1:30 and 1:10, respectively, according to the manufacturer's instructions (Pharmacia). The HSBP derivative was reduced with dithiothreitol in 0.1 M sodium acetate buffer–0.1 M NaCl, pH 4.5. The resulting HSBP preparation was incubated in equimolar proportions with the

adjuvant for 16 h at 22°C. The mixture was dialysed extensively against 0.01 M phosphate-buffer-0.15 M NaCl, pH 7.4.

Immunisation of mice

Eight-week old BALB/c mice were housed in polycarbonate cages in isolators and fed a commercial pellet diet with water *ad libitum*. Three groups of 15 mice each were immunised orally *via* feeding needles, with a priming dose on day 0 and booster doses on days 10 and 20, as follows: group I HSBP-CTB 20 µg; group II 0.2 M NaHCO₃ and group III saline only.

Experimental infections in mice with the mouse-adapted H. pylori strain

Ten days after the last immunisation, mice were exposed to an experimental infection with a mouse-adapted *H. pylori* suspension (5×10^8 cells) administered orally with feeding needles as described above with the exception of the group treated with saline, which remained uninfected. At 15-day intervals, five mice from each group were anaesthetised, blood was taken by heart puncture and gastric tissue was taken for further testing.

Isolation of H. pylori

Two biopsy specimens (body and antral regions of the gut) were collected from each animal for bacterial culture as described previously [20], plated on solid GAB-Camp medium and incubated for 48 h at 37°C in CO₂ 5% in air. *H. pylori* was identified as gram-negative, curved bacteria, catalase, oxidase and urease positive.

Identification of H. pylori cells in H. pylori-infected mice

H. pylori cells were identified by three different approaches.

Rapid urease test. Biopsy samples from the body and antral regions of each stomach were placed into tubes containing urea agar. The tubes were incubated at 37°C for up to 8 h; development of a pink colour, was indicative of a positive test.

Histopathology. Biopsy samples from the body and antral regions of the stomach were placed in buffered formalin 10%. These samples were embedded in paraffin, cut into 5-µm thick sections and stained with the Warthin-Starry silver stain to detect the presence of *H. pylori* cells. Similarly, 5-µm thick tissue sections were stained with haematoxylin and eosin to measure histopathological alterations in the stomach mucosa.

PCR. Biopsies taken from the body and antral region of each stomach were placed in a solution containing

brucella broth 70%, fetal bovine serum 20% and glycerol 10%. Samples were frozen at -70°C until required for DNA analysis. From these frozen samples (c. 15 mg of tissue), DNA was extracted as described previously [21]. Briefly, each biopsy sample was ground in 500 μl of Brucella broth for 10 s, transferred to a microcentrifuge tube and centrifuged for 5 min at 10 000 rpm. The supernate was discarded and the DNA was extracted by consecutive treatment with lysozyme 4 mg/ml, SDS 10% and proteinase K 10 mg/ml. DNA was purified by the phenol:chloroform:isoamyl alcohol (25:24:1) procedure and precipitated with ice-cold ethanol. PCR amplification of *H. pylori* DNA and selection of primers were based on previously published data [21–23]. The primers used were those coding for the urease enzyme of *H. pylori*.

Briefly, between 12 and 18 μl of the DNA preparation were added to 100 μl (final volume) of the reaction mixture containing $1 \times \text{Taq}$ polymerase supplemented with 1 M MgCl_2 (final concentration 2.25 mM), the two primers (each 0.5 μM) and deoxynucleotides (each 200 μM). The forward primer contained the sequence 5' (AAGCTTTTAGGGGTGTTAGGGGTTT)-3' and the reverse primer the sequence 5'(AAGCTTACTTTC TAACACTAACGC)3', derived from the *H. pylori ureC* gene sequence (accession numbers, EMBL X57132 and GenBank M60398), which amplify a 249-bp DNA fragment [21]. Samples were heated at 94°C for 4 min, centrifuged, and cooled to 65°C . Thirty cycles of amplification were performed in a DNA thermal cycler (GeneAmp system; Perkin-Elmer Cetus). Each cycle consisted of a 45-s denaturation step at 94°C , a 30-s annealing step at 59°C and a 90-s extension step at 72°C . The final cycle included an extension step (10 min at 72°C) to ensure full extension of the product. A DNA preparation from *H. pylori* was used as the positive control in each batch of PCR assays and distilled water in place of the DNA samples was used as a negative control.

Determination of serum, mucus and biliary antibody levels to H. pylori

Blood was obtained by cardiac puncture of anaesthetised mice prior to necropsy, sera were collected and stored at -20°C until assay. The small intestine and gall bladder were excised, extracting intestinal mucus and bile for antibody measurements. The determination was performed on 96-well ELISA plates coated with 10 μg of HSBP/well. Serum (100 μl diluted 1 in 100), bile and mucus (100 μl diluted 1 in 10) were added and incubated for 90 min at 37°C . After incubation, the wells were washed with PBS-Tween and the plates were incubated at 37°C for 90 min with 100 μl of a 1 in 1000 dilution (stock concentration of 0.5 mg/ml) of rabbit anti-mouse IgG- or IgA-horseradish peroxidase conjugates (Sigma). After washing with PBS-Tween, plates were developed with *o*-phenylene diamine as the substrate. The plates were then read at 492 nm.

Results

Mouse-adapted H. pylori strain

By means of consecutive passage through BALB/c mice intestinal tracts, *H. pylori* was able to adhere to the mouse gut-mucosal tissue, as revealed by histological examination. An interesting observation was that through subculture of the bacteria, the reaction in the urease test was slower than in the adapted strain, which gave a more rapid response in this test.

Isolation of H. pylori

After 48 h culture of tissue homogenates in GAB-Camp medium, individual, pinpoint, clear colonies were observed. Colonies were confirmed as *H. pylori* if they were urease, catalase and oxidase positive and if during microscopical examination the cells were gram-negative and had the characteristic curved-rod appearance. *H. pylori* was isolated from two (13%) of the 15 BALB/c mice orally immunised with the HSBP-CTB conjugate, while all 15 (100%) of the unvaccinated group were positive for *H. pylori*.

Identification of H. pylori cells in H. pylori-infected mice

Urease testing. The rapid urease tests were positive for the antrum from all animals of the non-immunised group after incubation for 8 h at 37°C in CO_2 5%. However, only one animal (6.6%) of the immunised group had evidence of the presence of *H. pylori*, as determined by this test.

Histopathology. Nineteen longitudinal biopsies were evaluated for the presence of *H. pylori* cells (Fig. 1). In the vaccinated group, no bacteria could be determined in 93.4% of animals orally immunised with the HSBP-CTB conjugate, whereas in the placebo group, stained tissue revealed numerous *H. pylori* organisms on the surface of the gastric epithelia and on the surface of the epithelial lining of the glandular crypts (Fig. 1). It was interesting that although the bacteria could be detected in the gastric pits and were associated with mucus-secreting cells, no evidence of gross lesions on the gastric mucosa could be observed in either the infected or the immunised mice.

PCR. The PCR assay successfully amplified a fragment of the expected 249 bp from a DNA preparation of the mouse-adapted *H. pylori* strain 25 with the UreC primers. When genomic DNAs prepared from the gastric tissue specimens were used as templates, the PCR assay yielded DNA fragments of the same mol. wt as that obtained from the *H. pylori* strain (Fig. 2). Of the immunised group, 14 animals were found to be free of the pathogen, whilst in the unvaccinated group PCR analysis showed that all the animals were infected.

Fig. 1. Haematoxylin and eosin-stained sections of BALB/c mice gastric mucosae from (A) control group, (B) mice orally immunised with HSBP-CTB, and (C) non-immunised mice. The presence of the bacteria (arrow) was determined by the detection of spiral rods within the mucus secretory cells (M) and in the mucosal crypts (MC).

Fig. 2. PCR detection of *H. pylori* UreC fragment in gastrointestinal tract of (a) unvaccinated and (b) vaccinated BALB/c mice.

Serum, biliary and local gastric immune response

High levels of IgG anti-HSBP of *H. pylori* were found in sera from immunised mice, while the serum IgA response was not as high, yet significantly greater than that found in the placebo group (Fig. 3). The mucosal IgA immune response against *H. pylori* HSBP was detected in the serum of immunised mice, as well as in intestinal mucus and biliary secretions (Fig. 3).

Protection against experimentally induced H. pylori infection

Mice orally immunised with HSBP covalently coupled to CTB were significantly protected against challenge with mouse-adapted *H. pylori* ($p < 0.005$), as revealed

by impairment of adhesion and colonisation of the murine stomach by mouse-adapted *H. pylori* cells, determined by rapid urease test, microbiological culture and PCR evaluations, in comparison with sodium bicarbonate-immunised controls. No significant differences were observed at any of the time points after infection, either in the presence of anti-HSBP antibodies (Fig. 4) or in the number of mice infected with the bacterium (Table 1).

Discussion

Although 15 years have elapsed since the discovery of *H. pylori*, an effective antimicrobial monotherapy or vaccine against *H. pylori* has not been fully developed.

Fig. 3. Antibody responses in sera (■), bile (▒), and gastric secretions (□) of BALB/c mice under different treatments. The bar shows the mean (SEM) OD₄₉₂ from a group of 15 mice.

Fig. 4. Mean (SEM) antibody titre at three time points after infection of BALB/c mice orally immunised with HSBP-CTB.

Table 1. *H. pylori* detection after challenge of orally immunised and untreated BALB/c mice

Treatment (number of mice)	Number (%) of mice positive for <i>H. pylori</i> by			
	Rapid urease test	Culture	Histopathology	PCR
HSBP-CTB (15)	1 (6.6)	2 (13)	1 (6.6)	1 (6.6)
Placebo (15)	15 (100)	14 (93)	15 (100)	15 (100)
Uninfected mice (15)	0 (0)	0 (0)	0 (0)	0 (0)

This is a most important goal to achieve, given the pathogenic potential of the bacteria in man. Besides man, domestic cats and non-human primates are the only animals in which *H. pylori* has been found to occur normally with subsequent histopathological damage [24]. Distinct animal models such as the rhesus monkey and Mongolian gerbils have been proposed. Marchetti and co-workers [12] used a murine model to evaluate a vaccine consisting of VacA and hLT (*Escherichia coli* thermolabile toxin), effectively preventing infection with virulent strains of *H. pylori*.

Different studies have shown that heparin, heparan sulphate (HS) and other glycosaminoglycans (GAGs) are involved in the binding of some specific viruses and pathogenic microbes to eukaryotic cells [25, 26]. The binding of heparan sulphate glucosaminoglycan to *H. pylori* cells has been proposed as a putative candidate for anti-adhesion therapy [27], and in this study, the protective role of the HSBP-CTB immuno-

gen was assessed in orally immunised BALB/c mice challenged with a mouse-adapted *H. pylori* strain.

Culture, considered specific for *H. pylori* although not sensitive, detected the bacterium in all the experimentally infected mice which had not received anti-*H. pylori* treatment. In this non-immunised group, *Helicobacter*-like colonies could be obtained consisting of gram-negative rods, that were catalase, oxidase and urease positive. In contrast, bacteria could be detected in only two (13%) of the immunised mice. As it is considered that studies relying on culture as the sole diagnostic method may underestimate the prevalence of *H. pylori*, other diagnostic methods were used.

Histological examination was positive for *H. pylori* in one of the 15 mice orally immunised with the HSBP-CTB immunogen, in contrast to the non-immunised group, where the bacteria could be recovered from 100% of the animals exposed to the pathogen.

Interestingly, these animals did not show signs of gastritis (Fig. 3), consistent with previous observations of the BALB/c mouse model [12], where although mice were positive for anti-*H. pylori* antibodies and the presence of the bacteria, no gastric inflammation was detected.

The absence of gastritis in *H. pylori*-infected mice might be due to the absence of certain pathways involved in the induction of inflammatory responses in the human gastric mucosa [7, 28]. For instance, mice do not produce a homologue of the human proinflammatory cytokine interleukin-8 (IL-8), identified as playing an integral role in the recruitment and activation of inflammatory cells in the gastric mucosae of *H. pylori*-infected individuals. On the basis of studies with murine *Helicobacter* models, it has been proposed that host factors (dietary requirements, cytokines), and not bacterial strain differences [29, 30], are the major factors contributing to the pathology associated with *H. pylori* infection.

The findings presented here suggest that the HSBP-CTB immunogen is able to prevent *H. pylori* infection in BALB/c mice, blocking the HSBP adhesins of *H. pylori*, thus preventing the adhesion of the bacteria to the gastric cells. With this mechanism of defence, the mouse is able to wash out any non-adherent bacteria by means of normal mucosal turnover.

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