MODELS OF INFECTION

Infection of BALB/c A mice by spiral and coccoid forms of *Helicobacter pylori*

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Helicobacter pylori exists in two different morphological forms, spiral and coccoid. This study demonstrated that both forms can infect BALB/c A mice. The animals were inoculated orally three times at 2-day intervals with 10^8 cfu of both spiral and coccoid forms of strain CCUG 17874 (NCTC 11637), strain 25 and strain 553/93. Infection was followed over a 30-week period by histological scoring of the grade of inflammation in gastric biopsies. At each time point sera were collected for analysis in ELISA and immunoblot analysis. Both spiral and coccoid forms of all *H. pylori* strains gave significantly higher inflammation scores than a control group of animals 1 week after inoculation. The histological evidence persisted throughout the entire 30 weeks. The inflammation was most severe in the pylorus and duodenum. Infection with strain 553/93 displayed the most severe gastritis. The spiral form of strain CCUG 17874 gave an immune response after only 4 weeks, whereas its coccoid form as well as strains 25 and 553/93 (spiral and coccoid forms) gave a significant increase in antibody response in ELISA and immunoblot after 16 weeks. It is concluded that both spiral and coccoid forms of *H. pylori* can cause acute gastritis in BALB/c A mice.

Introduction

Helicobacter pylori is a human pathogen associated with type B gastritis, peptic ulcer disease and gastric cancer [1–3]. In recent years an increasing number of animal models has been used to study the pathogenesis of *H. pylori* infection. Oral challenges with *H. pylori* resulted in infection in monkeys, gnotobiotic pigs and nude and euthymic mice [4–6]. Marchetti *et al.* [7] recently reported that vacuolating toxin (VAC)-producing strains of *H. pylori* can colonise the murine stomach and induce histopathological changes similar to type B gastritis in man.

H. pylori exists in two different morphological forms, spiral and coccoid. Coccoid forms of *H. pylori* have been described as ‘viable but non-culturable’ (VBNC). These cells may be viable and can revert to culturable forms in mice, but are no longer culturable on conventional media [8, 9]. The role of the coccoid form in the pathogenesis of *H. pylori*-associated gastritis has been disputed [10, 11]. A recent study

demonstrated chronic gastrointestinal colonisation of mice with *H. pylori* strains expressing high heparin-binding activity [12]. The present study describes the further development of this model in which BALB/c A mice were infected and the histopathological changes and systemic responses were followed at 1, 2, 4, 8, 16, 24 and 30 weeks.

Materials and methods

Bacterial strains and culture

H. pylori strain 17874 was obtained from the Culture Collection, University of Gothenburg (CCUG), Sweden (identical with strain NCTC 11637 from the National Collection of Type Cultures, 61 Colindale Avenue, London). *H. pylori* strain 25 [13] and strain 553/93 were freshly isolated from human gastric biopsies. The strains were grown on GAB-CAMP agar supplemented with horse serum 10% [14, 15] and were incubated for 48 h at 37°C in micro-aerophilic conditions to obtain a maximal yield of spiral-shaped *H. pylori* [12]. To obtain viable but non-culturable *H. pylori* cells, growth from 3–5-day-old agar cultures was harvested and resuspended in 20 ml of Ham’s F12 medium supplemented with calf serum (Flow Laboratories, Irvine) 10% and kept in a micro-aerobic environment for 3

days at 37°C and kept at 4°C. If no growth was observed after incubation for 5 days on GAB-CAMP agar at 37°C, harvested cells were defined as viable but non-culturable [12]. All bacteria were checked for the presence of spiral forms in the coccoid suspension and *vice versa*: the spiral form was not found in the coccoid suspension whereas only a few coccoid forms were observed among the spiral suspension (< 0.001%).

Animals

BALB/c A mice (6–8 weeks old) were used in this study [12]. Mice were housed with a 12-h light-dark schedule, fed a commercial rodent diet (B&K Universal AB, Sweden) and provided with water *ad libitum*.

Experimental design

Mice were inoculated orally through a feeding tube (OD, 0.1 mm) three times at 2-day intervals with 0.1 ml of either bacterial suspension (10^9 cfu/ml) or PBS. One hundred and fifty mice were divided into seven groups. Three groups of mice were inoculated with suspensions of strain CCUG 17874, strain 25 and strain 553/93, respectively. This experimental design was repeated with suspensions of the coccoid form of *H. pylori*; the control group received PBS. Three or four mice from each group were killed at 1, 2, 4, 8, 16, 24 and 30 weeks after inoculation. Mice were anaesthetised with ether and killed before collection of their stomachs.

The stomach was opened through the longer curvature with sterile surgical instruments. One half of the stomach and duodenum, covering all subtypes of mucosa, was sent for histopathological examination. The rest of the stomach was used for culture and PCR. Blood was drawn for measurement of the immune response.

Culture

Gastric mucosa samples were homogenised with PBS; then 100 µl of homogenate were placed on GAB-camp agar and incubated for 7 days in micro-aerobic conditions at 37°C. The presence of *H. pylori* on the culture plates was confirmed by the urease, catalase and oxidase tests, Gram's staining and PCR [16].

Histopathological examination

Each stomach was fixed in buffered formalin 10% (effective osmotic pressure 300 mosm/L) and embedded in paraffin. Sections (4 µm) were prepared and stained with haematoxylin and eosin, following standard procedures. Five areas of stomach were examined (Fig. 1); fundic mucosa with its stratified, keratinised, squamous epithelium; cardia in the transitive zone between fundus and corpus; body; antrum and canalis; and duodenum. The degree of inflammation was scored between 0 and 3 in the five different

Fig. 1. Diagram of the mouse stomach. A–E are the five areas examined by histology.

parts of the stomach and duodenum. The inflammatory infiltrate comprised mainly granular cells: 0, normal; 1, few inflammatory cells; 2, moderate inflammatory cells in several layers; 3, high level of inflammation with foci containing > 50 inflammatory cells, often more than three cell layers deep.

Transmission electron microscopy

The biopsy specimens were fixed immediately in glutaraldehyde 2% in 0.1 M sodium cacodylate buffer (pH 7.2), post-fixed in osmium tetroxide 2% in S-collidine buffer (pH 7.2), dehydrated in ethanol and embedded in agar resin 100. A semi-thin section was cut and examined by light microscopy. A representative area was chosen and ultra-thin sections, *c.* 50 nm, were cut on an LKB Ultratome III and contrasted with uranyl acetate and lead citrate. The grids were examined in a Zeiss CM 10 electron microscope at 60 kV.

ELISA and immunoblot

Sera were examined for total antibodies (mainly IgG) to *H. pylori* by ELISA as described by Guruge [15]. Serum samples (100 µl diluted 1 in 200) were added to each well, which had been coated with *H. pylori* antigen (acidic glycine extract) [17]. After incubation for 90 min at 37°C, wells were washed and 100 µl of HRP-labelled goat anti-mouse immunoglobulins (Dakopatts, Copenhagen, Denmark) diluted 1 in 2000 in the washing buffer were added. The plates were

incubated for 60 min at 37°C, followed by addition of substrate and stopping solution, and absorbance values at 450 nm were measured in a spectrophotometer.

Sera diluted 1 in 50 in washing buffer (10 mM Tris base, 30 mM NaCl, 1 mM NaN₃, pH 10.2 and Tween 20 0.1%) were incubated with strips from SDS-PAGE and blotted against a glycine extract of *H. pylori* overnight on a shaker at 4°C [15]. Anti-mouse immunoglobulins labelled with HRP (diluted 1 in 600) were added for 3 h on a shaker at 4°C and developed for 30–40 min with 50 mM sodium acetate containing carbazole 0.02% and H₂O₂ 0.3% at room temperature.

DNA extraction and PCR conditions

DNA was extracted from frozen homogenates of gastric biopsies from control and *H. pylori*-infected mice: 100 µl of homogenate were centrifuged at 12 000 g for 5 min and resuspended in 380 µl of TNE buffer with Triton X-100 1% and lysozyme 0.5 mg/ml. The samples were incubated at 37°C for 30 min, proteinase K was added to 1 mg/ml and incubated overnight at 37°C. DNA was isolated by extracting twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform. DNA was precipitated with 0.3 M sodium acetate and 2 volumes of absolute ethanol and centrifuged as described above, rinsed in alcohol 70% and dried in a speed vacuum [18]. The DNA pellet was dissolved in 50 µl of water plus Tween and stored at –20°C until the PCR was performed.

HPU primers, based on a urease A gene sequence of *H. pylori*, as originally described by Clayton *et al.* [19] were used. Primers were purchased from Scandinavian Gene Synthesis (Köping, Sweden). PCR conditions were according to Nilsson *et al.* [16]

Statistical analysis

The Mann-Whitney U test was used to compare the degree of inflammation and the changes in immune response. The level of significance selected was $p < 0.05$.

Results

In the infected groups, there was histological evidence of type B gastritis with infiltration of polymorphonuclear leucocytes (PMNL) or lymphocytes, or both, persisting for 30 weeks. In contrast, the uninfected control group and the group inoculated with formalin-killed *H. pylori* showed no evidence of gastritis. In several mice it was possible to demonstrate *H. pylori* in both spiral and coccoid form. Electron microscopy demonstrated well-preserved subcellular and membrane structures, giving the impression of viable bacteria. It was possible to demonstrate a clear attachment to the

mucosal cell surface membrane, especially around the coccoid form, and a tendency for pedestal formation was noticed (Fig. 2).

Both the spiral and the coccoid forms of *H. pylori* strains CCUG 17874 and 25 gave a significantly higher inflammation score in the gastric biopsies of mice 1 week after inoculation. For *H. pylori* strain 553/93, both spiral and coccoid forms gave more severe inflammation than the other strains tested (Fig. 3).

Pyloric and duodenal inflammation was most severe in infected groups compared to the control group. Finally, strain 553/93 caused a significantly higher inflammation score than the other types tested (Fig. 4).

The colonisation of mouse stomach by *H. pylori* was investigated by culture and PCR. The colonies on the GAB-camp agar and also DNA extraction from mouse stomach both showed a *H. pylori* urease-specific band (Fig. 5).

H. pylori strain CCUG 17874 produces vacuolating cytotoxin (VacA) and expresses cytotoxin-associated gene (CagA) protein. Strain 25, which was isolated from man and used for several years in our laboratory is VacA-positive and CagA-positive; and strain 553/93, which was freshly isolated from man is both VacA- and CagA-negative (data not shown).

Spiral forms of strain CCUG 17874 gave a significant increase in antibody response in ELISA 4 weeks after inoculation ($p < 0.05$). Eight weeks after inoculation the immune response showed a much stronger response than at 4 weeks ($p < 0.05$). Strain 553/93 spiral forms gave a small increase 8 weeks after inoculation and at 16 weeks gave significantly higher titres ($p < 0.05$). Strain 25, both spiral and coccoid forms, gave an increase 16 weeks after inoculation ($p < 0.05$). Coccoid forms of strains 17874 and 553/93 gave the same changes as strain 25 (Fig. 6).

Immunoblot analysis showed an increase in the number of bands and intensity, especially of proteins in the 14–20 and 60–80 kDa region (Fig. 7). the immunoblot results of the different groups showed similar changes to the ELISA titres.

Discussion

H. pylori infections occur in human populations throughout the world. Once acquired, the infection becomes chronic and probably persists for life if untreated [20]. Multiple lines of evidence demonstrate the casual role of *H. pylori* in the chronic inflammatory process. Ingestion of *H. pylori* by human volunteers resulted in gastritis, and eradication of *H. pylori* infection resolved this gastritis [21]. Oral challenges

Fig. 2. Histopathological analysis of mouse stomachs. (A) No inflammation at base, from a control mouse ($\times 280$); (B) severe inflammation at glandular base of 16 weeks after inoculation with *H. pylori* strain 553/93 (spiral form) ($\times 280$); (C) severe inflammation at base with formation of lymphoid follicle 16 weeks after inoculation with *H. pylori* strain 553/93 (coccoid form) ($\times 280$). (D) Electron micrograph: mouse stomach infected with *H. pylori* spiral form (upper right) and coccoid form (lower left); note the tendency for pedestal formation, especially around the coccoid form ($\times 24\,000$).

Fig. 3. Inflammation score in the stomach of BALB/c A mice infected by different *H. pylori* strains; $\square$ control, $\square$ 1 week, $\square$ 2 weeks, $\square$ 4 weeks, $\blacksquare$ 8 weeks, $\square$ 16 weeks, $\square$ 24 weeks, $\blacksquare$ 30 weeks. * $p < 0.05$ versus all infected groups through the whole 30 weeks.

Fig. 4. Inflammation score in different regions of stomach; + and * $p < 0.05$ versus the group infected by strain 553/93 (fresh isolate strain from human). Inflammation scores of jejunum, ileum, colon, liver and lung were all zero; $\square$ control, $\blacksquare$ all infected group, $\square$ infected by human isolates.

Fig. 5. PCR analysis. Lane 1, positive control; 2, negative control; 3, colony on GAB-camp agar from infected mouse stomach; 4, homogenate of stomach from control mice; 5, homogenate of stomach from infected mice.

Fig. 6. Antibody response in BALB/c A mice to different *H. pylori* strains. Strain 17874 spiral form (○) gave a significant increase 4 weeks after inoculation and much higher at 8 weeks after inoculation. Strain 553/93 spiral form (■) gave a significantly higher titre 16 weeks after inoculation and strain 25 spiral form (□) gave a significant change 16 weeks after inoculation. ** All coccoïd forms of these three strains showed the same results as strain 25 spiral forms in ELISA titre.

with *H. pylori* also resulted in gastritis in monkeys, piglets and rodents [4, 5, 12].

H. pylori can exist in two forms, spiral and coccoïd forms. Eaton *et al.* [22] reported that gnotobiotic piglets infected with spiral *H. pylori* developed lymphocytic gastritis and *H. pylori*-specific antibody but these changes were seen with coccoïd forms followed for 14 days after challenge. Cellini's study [9] showed that coccoïd *H. pylori* non-culturable *in*

Fig. 7. Immunoblot of mice sera from the group infected by strain 553/93 spiral form. Lane A, control mouse; B, 1 week after inoculation; C, 2 weeks; D, 4 weeks; E, 8 weeks; F, 16 weeks; G, 24 weeks; H, 30 weeks after inoculation. Similar patterns can be seen in the other infected groups, but the group infected with strain 17874 spiral form already showed an increase at 4 weeks after inoculation.

vitro had reverted in mice 2 weeks after inoculation and all colonised mice showed a systemic antibody response to *H. pylori*. Coccoïd forms of *H. pylori* can also exist in the human stomach and they were found more frequently and in larger numbers in cases of adenocarcinoma than in cases of benign ulcers [23]. In the present study, both spiral and coccoïd forms of *H. pylori* gave a significant increase in inflammatory cells in stomach biopsy samples from BALB/c A mice 1 week after inoculation and the inflammation continued throughout the 30 weeks of the study (Fig. 3). The inflammatory cells were mainly granular cells in this study. Inflammation was most severe in the pylorus and duodenum among infected animals compared to the control mice. Of the three strains of *H. pylori* used in this study, strain 553/93 caused a significantly higher inflammation score than the other two strains tested (Fig. 4). The distribution of *H. pylori* in the human stomach seems to have implications with respect to the disease process, and it has been suggested that the degree of gastritis can be used as an indicator of *H. pylori* density [24–26]. Danon *et al.* [27] observed that *H. felis* was mainly confined to the non-acid-producing regions, i.e., antrum and cardia, of the mouse stomach.

In spite of the advances in treatment and in the understanding of the epidemiology of type B gastritis, the mechanisms of *H. pylori* pathogenicity are still not well understood. Among many important virulence factors, the production of the vacuolating cytotoxin (VacA) and the expression of the cytotoxin-associated gene (CagA) protein were almost the first phenotypic characteristics described. However, these factors were not always present in all *H. pylori* strains. Thus, it is highly probable that they are associated with peptic ulcer disease, lymphoid proliferation and possibly gastric cancer. *H. pylori* strains producing the VacA toxin and CagA protein induced a more severe inflammation within the gastric mucosa in patients with peptic ulcer disease cases [7, 28]. The expression of CagA in the regulation of the inflammatory response to *H. pylori* infection is associated with increased secretion of interleukin-8 by gastric epithelial cells [29, 30]. Furthermore, CagA protein expression is also associated with an increased risk for development of gastric cancer [31]. *H. pylori* strain CCUG 17874 had commonly been recognised as a vacuolating cytotoxin (VacA)-producing and cytotoxin-associated gene (CagA) protein expressing strain [32]; strain 25 is a VacA-positive and CagA-positive strain; and strain 553/93 which was freshly isolated from man is both VacA- and CagA-negative. Gastrointestinal inflammation in the mice can be caused by both cytotoxic and non-cytotoxic strains of *H. pylori*.

Bacillary forms of strain 17874 gave a significant immune response 4 weeks after inoculation, while its coccoid forms gave a higher antibody response 16 weeks after inoculation. Strain 25 and 553/93 (both spiral and coccoid form) gave similar results to the coccoid form of strain 17874.

Finally, the study showed that both spiral and coccoid forms of *H. pylori* can induce gastritis in the gastric mucosa of BALB/c A mice and can produce an antibody response in the sera of infected mice. The finding that the coccoid form of *H. pylori* is active in the inflammatory process warrants further studies, which are now being done in our laboratory.

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