Experimental Infection of Mice with Tightly Coiled Spiral Bacteria ("Candidatus Helicobacter suis") Originating from the Pig Stomach

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Summary

Mice (n = 34) were inoculated orally with a gastric homogenate from a pig infected with tightly coiled spiral bacteria (TCSB). In mice killed in pairs at 16 intervals up to 108 weeks post-inoculation (pi), TCSB were invariably found, mainly in the mucosal surface, gastric pits, intercellular spaces, cytoplasm of surface epithelial cells, and lumina of gastric glands. Histopathologically, infiltration of lymphocytes and plasma cells was seen from 8 weeks pi onwards, gradually increasing as infection progressed. From 64 weeks pi onwards, the formation of large follicles was observed in the lamina propria and submucosa, together with severe necrosis of surface epithelial cells. Glandular epithelial cells in the fundic mucosa were markedly dysplastic and intruded through the basement membrane into the submucosal layer. Common antigenicity between TCSB and Helicobacter pylori was demonstrated by Western blotting, ELISA, and immunohistochemistry. The sequence of the 16S rDNA fragment of 374 bp showed 100% homology with the 16S rRNA gene of "Candidatus Helicobacter suis". Experimental infection of the gastric mucosa of mice with TCSB was closely associated with chronic gastritis and dysplastic lesions.

Keywords: bacterial infection; Candidatus Helicobacter suis; Helicobacter pylori; mouse; pig; stomach.

Introduction

Tightly coiled bacteria were first described in pigs by Queiroz et al. (1990). These bacteria, which were named Gastrospirillum suis (Mendes et al., 1990), were morphologically similar to Helicobacter helimannii, and the 16S rRNA gene sequence of the two organisms showed 99.5% homology (Queiroz et al., 1995). Recently, based on 16S rRNA gene sequence data, the organism was renamed "Candidatus Helicobacter suis" (De Groote et al., 1999).

Association of the spiral organism with gastric disease in pigs is still unclear. Queiroz et al. (1996) suggested that infection was closely related to gastric ulceration in the pars oesophagea, but Park et al. (2000) found that the organism was restricted to the pyloric mucosa and was associated with chronic pyloric gastritis. Earlier, Mendes et al. (1991) had reported that infection was closely related to chronic pyloric gastritis, accompanied by the formation of lymphoid follicles.

This bacterium has not so far been cultured in vitro, but experimental infection has been reported in CFW mice and Wistar rats (Moura et al., 1993; Mendes et al., 1996). The aims of the present study were (1) to produce a murine model for the infection, and (2) to clarify the association between infection and gastritis in laboratory mice.

Materials and Methods

Gastric Homogenate Preparation

Gastric samples from 6-month-old pigs (>100 kg body-weight) were obtained from a slaughterhouse in Suwon, Korea. Smears on glass slides were
stained with carbol fuchsin and examined for tightly coiled spiral bacteria (TCSB), as described by Rocha et al. (1989). From a positive sample and a negative sample, two 10% (w/v) homogenates in phosphate-buffered saline (PBS) were prepared with a homogenizer (Ultra-turrax T25; Ika, Staufen, Germany) and used as infective and control inocula, respectively.

**Mouse Infection and Sampling**

Male, specific pathogen-free (SPF) ICR mice aged 4 weeks were purchased from Korea-Biolink, Suwon, Korea and used in accordance with the laboratory animal guidelines of Seoul National University. The animals were dosed orally (0.5 ml per mouse) with either the infective inoculum (34 mice) or the control inoculum (14 mice). Two mice from the infected group were killed by exsanguination under deep ether anaesthesia at each of the following times post-inoculation (pi): 3, 7, 10, 17, 21, 28 days and 8, 12, 16, 20, 24, 32, 40, 64, 72 and 108 weeks. Two control mice were similarly killed at 7, 17, 24 days and 8, 16, 24 and 72 weeks pi. Samples of blood and stomach were collected immediately, the stomachs being removed and opened along the greater curvature. Some gastric tissue samples were frozen at $-20\,^\circ\text{C}$ for polymerase chain reaction (PCR) assay; others were fixed in 10% neutral formalin for at least 24 h for histopathological, immunohistochemical or ultrastructural examination.

**Histopathological Examination**

Fixed tissues were dehydrated in an alcohol-xylene series, and embedded in paraffin wax. From each block, sections ($2\,\mu m$) were prepared and stained with haematoxylin and eosin (HE) and modified Steiner’s silver stain (Sigma, St Louis, MO, USA) for histopathological examination and for the detection of TCSB, respectively.

**Rapid Urease Test**

To examine urease production, 2 ml of test solution (containing urea 2%, phenol red 0.05%, 157 mg Na$_2$HPO$_4$$\cdot$12H$_2$O, 80 mg NaH$_2$PO$_4$$\cdot$2H$_2$O, and 20 mg NaN$_3$, pH 6.2) were added to each well of a 24-well cell culture plate. Mouse gastric mucosa samples (50 mg) were then added individually to the wells and the plate was incubated at 37 $^\circ\text{C}$ for 3 h. A positive result was indicated by a colour change of the test solution from yellow to orange.

**Bacterial Culture**

A portion of gastric tissue from each mouse examined was washed in PBS and cut into small pieces with a sterile razor blade. These were streaked on (1) GC modified chocolate agar (Difco, Detroit, MI, USA) supplemented with horse serum 10%, amphotericin B, trimethoprim, polymyxin B and vancomycin, and (2) brain heart infusion agar (BBL, Cockeysville, MD, USA). The plates were incubated anaerobically in a sealed jar containing a Gaspak (GasPakPlus™; BBL) at 37 $^\circ\text{C}$ for 4–7 days.

**Western Blot**

*Helicobacter pylori* (ATCC 43504) grown on GC modified chocolate agar plates, was harvested, washed, ultrasonically disintegrated, suspended in 2% sodium dodecysulphate (SDS) and boiled for 10 min to extract protein. The extract was then mixed with sample buffer (0.1 M Tris-HCl, pH 6.8, glycerol 10%, SDS 2%, 2-mercaptoethanol 5%, bromophenol blue 0.05%) and boiled for 5 min. It was separated on 12.5% polyacrylamide gel at 200 V for 45 min, transferred to a nitrocellulose membrane and subjected to 100 V for 1 h. The membrane was then blocked with 5% skimmed milk at 4 $^\circ\text{C}$ for 2 h and incubated with sera (1 in 100 dilution) of infected or control mice and with a rabbit anti-*H. pylori* antibody (Biodesign, Kennebunk, ME, USA) at 4 $^\circ\text{C}$ overnight; after washing, the membrane was treated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Zymed, South San Francisco, CA, USA) and anti-rabbit IgG antibody (Zymed), as appropriate, at room temperature (RT) for 1 h, and the reaction was “visualized” with 3,3$'$-diaminobenzidine (Vector, Burlingame, CA, USA).

**Immunohistochemistry**

Sections (3 $\mu m$) of formalin-fixed, paraffin wax-embedded tissues were placed on poly-L-lysine-coated slides and dried overnight at 55 $^\circ\text{C}$. After dewaxing and rehydration with a xylene-alcohol series, the sections were incubated with H$_2$O$_2$ 10% in methanol for 30 min to block endogenous peroxidase activity, and subsequently with normal goat serum for 1 h at 4 $^\circ\text{C}$ to block non-specific reactions. They were then incubated overnight at 4 $^\circ\text{C}$ with a rabbit polyclonal antibody against *H. pylori* (Biodesign), diluted 1 in 100 in 0.05% Tween 20-PBS (PBS-T). The sections were washed three times for 5 min and incubated with biotinylated mouse anti-rabbit IgG antibodies (Zymed) at
RT for 30 min. They were then treated with peroxidase-conjugated avidin-biotin complex (ABC) (Vector). Peroxidase activity was evaluated with 3,3’-diaminobenzidine (Vector) as the chromogen. Finally, the sections were counterstained with Mayer’s haematoxylin for 30 sec and mounted.

**Enzyme-linked Immunosorbent Assay (ELISA)**

Each well of an ELISA plate was coated with 2 µg of *H. pylori* total protein, prepared as described above, and incubated at 4 °C overnight. After washing with PBS (pH 7.4), the plate was blocked by treatment with 1% bovine serum albumin at 4 °C for 2 h, incubated with the various mouse sera (1 in 100 dilution) at RT for 2 h, and washed three times with PBS-T. Plates were then incubated with 1 in 15 000 dilutions of HRP-conjugated anti-mouse IgG antibodies (Zymed) at RT for 1 h. After washing, plates were visualized with o-phenylenediamine dihydrochloride (Sigma). The absorbance was measured at 450 nm.

**DNA Extraction and PCR Amplification**

For isolation of total genomic DNA, gastric samples were wrapped with foil, quickly frozen in liquid nitrogen, and ground by striking with a hammer. The samples were then suspended in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, SDS 0.5% w/v, pH 8.0) with 100 µg of proteinase K. After incubation of the lysates at 56 °C for 3 h, the DNA was extracted from each sample with phenol–chloroform and precipitated in isopropyl alcohol with sodium acetate. Each DNA sample was washed twice in 70% alcohol and resuspended in Tris-EDTA (TE; 10 mM Tris, 15 mM EDTA, pH 8.0) buffer. For PCR assay, primers (F, 5’-CTATGACGGGTATCCGGC-3’; R, 5’-TGGGAGAGGTAGGTGGAAT-3’) were designed from the 16S rRNA gene of *H. pylori*. The PCR mixture (100 µl) contained 2 µg of DNA, 100 pmol of each primer, 1 × PCR buffer with MgCl₂, 200 µM dNTP, and 2.5 units of Taq polymerase (PCR core Kit; Boehringer Mannheim, Mannheim, Germany). PCR conditions were as follows: 3 min of denaturation at 95 °C; followed by 35 cycles consisting of 30 sec of denaturation at 94 °C, 30 sec of annealing at 55 °C, and 1 min of extension at 72 °C; and 7 min of extension at 72 °C after amplification. The PCR product was detected by electrophoresis of 12 µl of reaction solution in 1.5% agarose gel containing ethidium bromide 1 µg/ml.

**16S rDNA Gene Sequencing**

Sequencing of the PCR product was performed to determine the *Helicobacter* species present with Big Dye terminator cycle sequencing kit, sequence analysis being resolved with the ABI PRISM 377 DNA sequencer (Perkin Elmer, Shelton, Connecticut, USA).

**Transmission Electron Microscopy (TEM)**

Formalin-fixed gastric tissue containing large numbers of TCSB was selected for electron microscopic evaluation. Gastric tissue was cut into cubes (1 mm³), post-fixed in 1% OsO₄ and rinsed with 0.1 M sodium cacodylate buffer (pH 7.2) for 30 min. After dehydration in an alcohol–propylene oxide series, the sections were embedded in Epon mixture at 60 °C. Ultrathin sections were stained with 1% uranyl acetate and 1% lead citrate, and examined with an electron microscope (JEM 100CX II, JEOL, Japan) at an accelerating voltage of 80 kV.

**Results**

**Urease Activity, Bacterial Culture, and Histopathology**

A positive urease reaction was observed in all infected mice except for one of the two mice killed at 3 days pi. The control mice, however, were invariably negative. The TCSB could not be cultured on artificial media, but with silver staining they were observed in both the fundic and pyloric (antral) mucosa, but not in the cardiac mucosa. The bacteria, which were tightly coiled, were found in all infected mice, mainly in the mucosal surface.

*Fig. 1. Tightly coiled spiral bacteria (TCSB) in the cytoplasm of surface epithelial cells and in intercellular spaces of the pyloric mucosa, 8 weeks post-infection (pi). Steiner’s silver stain. × 400.*
gastric pits, intercellular spaces, cytoplasm of surface epithelial cells, and lumen of gastric glands (Fig. 1), in numbers that remained large throughout the experimental period. No such bacteria were seen in the control mice. During the first 3 weeks pi, inflammatory changes in the gastric mucosa of infected mice were mild or absent. However, from 21 days pi onwards, moderate infiltration of the lamina propria with mononuclear cells was observed. From 8 weeks pi onwards, the inflammatory response gradually increased, and gastric lesions (formation of lymphoid follicles, cystic dilatation and hyperplasia, and focal degeneration of glandular epithelia in the fundic and antral mucosa) were observed (Fig. 2A–C). From 64 to 108 weeks pi, glandular epithelial cells in the fundic mucosa were severely hyperplastic and intruded through the basement membrane into the submucosal layer. The lumina of glands were expanded

Fig. 2. (A) Glandular hyperplasia and cystic dilatation, (B) focal necrosis of glandular epithelial cells, and (C) lymphoid follicles in the fundic mucosa at 20 weeks pi. (D) Severely hyperplastic and dysplastic glandular epithelial cells intruded through the basement membrane into the submucosal layer, 64 weeks pi. HE. (A) × 33; (B) × 500; (C) × 80; (D) × 66.

Porcine Spiral Bacteria in Mice

Fig. 3. Immunolabelling with *H. pylori* antiserum in the lumina of gastric glands of an infected mouse, 20 weeks pi. ABC × 200.
to a variable degree and the epithelial cells were flattened and dysplastic (Fig. 2D). In the pyloric mucosa, atrophic changes with erosion and focal necrosis of the surface epithelial cells were observed. Mild to moderate infiltration of mononuclear cells was observed in the gastric mucosa of all control mice, but degeneration and necrosis of epithelial cells were absent.

Immunohistochemistry

Positive signals were mainly observed in the mucus, mucosal surface, gastric pits, the cytoplasm of parietal cells, and the lumina of gastric glands (Fig. 3), but not in lymphoid aggregates. No immunolabelling was observed in the gastric mucosa of the control mice. The immunohistochemical results indicated shared antigenicity between the TCSB and H. pylori.

Western Blot

Total protein of H. pylori was allowed to react with a rabbit polyclonal anti-H. pylori antibody and with the sera of infected and control mice, to identify cross-reactivity between H. pylori and the TCSB. Reaction of the protein with H. pylori antibody produced several major and minor bands. Moreover, more than eight major bands and a few minor bands between 21 and 75 kD were observed as a result of reaction with infected mouse serum. On the other hand, no positive bands were produced by control mouse serum (Fig. 4).

ELISA

The antibody titres against H. pylori increased progressively in TCSB-infected mice. From 7 to 24 days pi, the antibody titres of the infected mice were over 240ld higher than those in control mice, and by the end of the experiment were 440ld to 8-fold higher (Fig. 5).

PCR Amplification and Sequencing of 16S rDNA Gene

By means of the PCR, with genus Helicobacter specific primers, a 16S rRNA fragment of 374 bp was successfully amplified in the gastric samples of infected mice but not control mice (Fig. 6). When the sequence was compared with those present in BLAST software databases (Pubmed, NCBI, Bethesda, MD, USA), 100% homology was observed with the 16S rRNA gene of “Candidatus H. suis” (Genbank accession no. AF127028) (De Groote et al., 1999).
Ultramicroscopically, the organism had a tightly coiled appearance with two to six spiral turns, and was approximately 2–8 μm long and 0.5–0.8 μm wide. Two to five flagella, about 20 nm in diameter, were found on each pole (Fig. 7). However, the TCSB had no periplasmic fibrils such as those found on Helicobacter felis by Paster et al. (1991). At higher magnification, a three-layered cell membrane and a cell wall, which are characteristically observed in gram-negative bacteria, were clearly seen.

**Discussion**

*H. heilmannii* has been identified in nonhuman primates, dogs, cats, cheetahs, pigs and human beings. In man, it colonizes the antrum only rarely, and induces a much milder gastritis than that caused by *H. pylori* (Dent et al., 1987; Heilmann and Borchard, 1991). On the other hand, cats and dogs are infected at relatively high rates (61–100%), regardless of the presence of chronic gastritis (Weber et al., 1958; Henry et al., 1987; Eaton et al., 1996; Yamasaki et al., 1988), the bacteria colonizing both the fundic and pyloric mucosa. Infection in pigs is associated with chronic pyloric gastritis or ulceration of the pars oesophagea (Mendes et al., 1991; Queiroz et al., 1996; Park et al., 2000), and the organism has been reported in both the pyloric and fundic mucosa at infection rates of 10.8% in Brazil (Mendes et al., 1991), 9.4% in Italy (Grasso et al., 1996) and 8.0% in Korea (Park et al., 2000). When CFW mice and Wistar rats were inoculated with homogenate of a porcine stomach infected with *G. suis*, the bacteria colonized only the antral and oxyntic mucosa (Moura et al., 1993; Mendes et al., 1996); at an early stage of infection, mononuclear cell and polymorphonuclear cell infiltrates and lymphoid follicles were observed in the subglandular region of the antral mucosa. In the present study in mice, the TCSB were observed in the fundic and pyloric (antral) mucosa, the inflammatory response being similar to that described by Moura et al. (1993) and Mendes et al. (1996). However, from 64 weeks pi onwards, hyperplasia of the glandular epithelial cells was observed in the gastric mucosa, and the newly proliferating cells were dysplastic and intruded through the basement membrane into the submucosal layer. Western blotting and immunohistochemistry with a rabbit polyclonal antibody against *H. pylori* and serum of a mouse infected with TCSB revealed a common antigenicity between the two organisms. The antibody titre against *H. pylori* gradually increased in TCSB-infected mice as infection progressed. On the basis of 16S rDNA analysis, the TCSB showed 100% homology with "Candidatus Helicobacter suis". In addition, electron microscopy showed close similarity between TCSB and "G. suis" reported by Mendes et al. (1991). TCSB infection in the gastric mucosa of mice was closely associated with chronic gastritis and dysplastic lesions. This animal model should prove useful for preventive and therapeutic studies on infection with TCSB.
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References


