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Sjunnesson, Håkan; Sturegård, Erik; Hynes, Sean; Willén, R; Feinstein, R; Wadström, Torkel

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PO Box 117  
221 00 Lund  
+46 46-222 00 00

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## Five month persistence of *Helicobacter pylori* infection in guinea pigs

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HÅKAN SJUNNESSON,<sup>1</sup> ERIK STUREGÅRD,<sup>1</sup> SEAN HYNES,<sup>1</sup> ROGER WILLÉN,<sup>2</sup>  
RICARDO FEINSTEIN<sup>3</sup> and TORDEL WADSTRÖM<sup>1</sup>

<sup>1</sup>Department of Medical Microbiology, Dermatology and Infection, Lund University, Lund,

<sup>2</sup>Department of Pathology, Sahlgrenska University Hospital, Göteborg, and

<sup>3</sup>National Veterinary Institute, Uppsala, Sweden

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Seven Dunkin-Hartley guinea pigs were infected with the Sydney strain of *H. pylori* (SS1). Gastric histopathology was evaluated and serum antibody response to *H. pylori* cell-surface proteins was analysed by enzyme immunoassay (EIA) and immunoblot. Tissue and faecal samples from five control animals were analysed for the presence of naturally occurring *Helicobacter* spp. infection by culture and *Helicobacter* genus-specific PCR. The *H. pylori* infection persisted for 5 months, in most animals accompanied by a histologically severe antral gastritis, exhibiting focal degeneration and necrosis of gastric crypt epithelium. Increased numbers of mitotic figures were observed in the gastric epithelium, indicating a regenerative process. Infected animals displayed specific antibodies towards *H. pylori* cell-surface proteins in immunoblot, whereas EIA was of dubious value creating false-positive results. Serum complement C3 and cholesterol levels appeared to be elevated in infected animals. *Helicobacter* spp. infection was not detected in the control animals. The persistent infection, accompanied by severe gastritis and a prominent serum antibody response, and the apparent absence of a natural *Helicobacter* spp. infection makes the guinea pig model useful in *H. pylori* research.

Key words: *Helicobacter pylori*; animal model; guinea pig; gastritis.

Torkel Wadström, Dept MMDI, Lund University, Sölvegatan 23, S-22362 Lund, Sweden. e-mail: torkel.wadstrom@mmb.lu.se

*Helicobacter pylori* infection causes acute and chronic gastric inflammation and peptic ulcer in man. In addition, previous studies linked the infection with gastric cancer and led to *H. pylori* being classified as a class one carcinogen by the WHO International Agency for Research on Cancer (IARC) in 1994 (1). Natural hosts for *H. pylori* include humans, nonhuman primates, and possibly cats and sheep (2–4). Important laboratory animals for experimental infection include mice, Mongolian gerbils and guinea pigs (5–8). Immunocompetent mice do not develop

severe inflammation such as is seen in humans, but promising data from studies on IL-10 cytokine-deficient (IL-10<sup>-/-</sup>) mice showed induction of a more severe inflammation in these animals following *H. pylori* infection (9, 10). Mongolian gerbils were shown to develop intestinal metaplasia in the gastric mucosa following 26 weeks of *H. pylori* infection and gastric adenocarcinoma after 62 weeks of infection (11, 12). The level of gastric inflammation in *H. pylori*-infected guinea pigs has been similar to that in the gerbil during short-term experiments; however, there have not been any long-term infection studies (7, 8). This animal has a stomach anatomy resembling that of the human, and is probably the

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only small laboratory animal with a stomach entirely lined by glandular epithelium (13). The composition of the stomach glands is similar to what is seen in humans (13). Guinea pigs express the cytokine IL-8 (14), an important factor in the local inflammatory response, and requiring dietary vitamin C (15), which is most useful in studies of how dietary factors affect the *H. pylori* infection.

The *Helicobacter* genus has expanded to comprise more than 20 gastric and enterohepatic species (16). These non-*pylori Helicobacter* spp. have been detected in a wide range of hosts, and some colonizing the enterohepatic niche seem to be associated with enteritis and hepatitis in these hosts (3). Recently, interest in this area has been directed towards the potential association of *Helicobacter* infection and the development of inflammatory bowel disease and hepatitis in humans (17). Indigenous *Helicobacter* infections in laboratory animals can be confounding factors in *H. pylori* experimental infections, and should be excluded in order to standardise these models.

In the present study we followed an experimental *H. pylori* infection in guinea pigs over a 5-month course. Gastric histopathology and serological immune response to *H. pylori* cell surface antigens was analysed, and serum C3 and cholesterol levels, previously suggested to correlate with *H. pylori* infection and increased risk of developing cardiovascular disease, were measured (9). We also investigated the presence of potential natural *Helicobacter* infections in this animal.

## MATERIALS AND METHODS

### *Animals and experimental protocol*

Fourteen male Dunkin-Hartley guinea pigs were used in the present study. The animals were divided into five groups: Group 1: Two control animals maintained for 5 months; Group 2: Five *H. pylori*-inoculated animals maintained for 5 months. Animals were inoculated three times within one week using 3 ml of a suspension of *H. pylori* strain SS1 (18); Group 3: Two animals inoculated with 3 ml of a suspension containing four strains from different *Helicobacter* species: *Helicobacter bilis* (CCUG 38995), *Helicobacter hepaticus* (CCUG 33637), *Helicobacter pullorum* (CCUG 33840) and one canine isolate of "*Flexispira rappini*" (strain no. KT0201, M-L Hänni-

nen, University of Helsinki, Finland). Following inoculation, animals were maintained for 3½ months; Group 4: Two animals inoculated twice with a suspension of 3 ml of *H. pylori* strain SS1 and maintained for 3½ months; Group 5: Three control animals maintained for 3½ months. Animals in groups 1 and 2 were 11 weeks old at time of bacterial inoculation and animals in groups 3 to 5 were 7 weeks old.

### *Bacterial strains and culture conditions*

*H. pylori* was cultured in tryptone soya broth (Oxoid, Basingstoke, England) at 37°C in a 5% CO<sub>2</sub> incubator under microaerobic conditions prior to inoculation. *H. pylori* inocula contained between 5×10<sup>5</sup> and 8×10<sup>6</sup> colony-forming units (cfu)/ml, measured by dilution series. *H. bilis*, *H. hepaticus*, *H. pullorum* and "*F. rappini*" were cultured on selective (vancomycin 10 mg/l, polymyxin 2500 IU/l and trimethoprim 5 mg/l) Brucella agar (BBL, Cockeysville, MD, USA) supplemented with defibrinated horse blood (5% v/v), IsoVitalex (BBL) (1% v/v), haemin (0.002 g/l) and activated charcoal (1 g/l) (Brucella blood agar), at 37°C in an environment containing 10% CO<sub>2</sub>, 10% H<sub>2</sub>, 75% N<sub>2</sub> and 5% O<sub>2</sub> (MART microbiology, Lichtenvoorde, Holland, microaerobic cycle), prior to inoculation (19). Bacteria were suspended in phosphate-buffered saline (PBS) and the inoculum contained 2×10<sup>5</sup> cfu/ml of *H. bilis*, 2.5×10<sup>4</sup> cfu/ml of *H. pullorum* and 2×10<sup>6</sup> cfu/ml of "*F. rappini*". Concentration of *H. hepaticus* could not be determined due to culture failure from the serial dilutions of this inoculum.

### *Processing of faecal samples*

Fresh faecal samples from each animal were collected prior to inoculation and at several time-points during the experiment. All samples were analysed using a *Helicobacter* genus-specific PCR, an *H. pylori*-specific PCR, a faeces antigen test and culturing methods. Faecal culture samples were suspended in PBS and filtered in two steps first using an 8-µm pore-size filter and subsequently through an 0.8- or an 0.45-µm pore-size filter (Millipore Corporation, Bedford, USA). Filtered as well as unfiltered samples were inoculated onto selective and nonselective Brucella blood agar and incubated at 37°C under microaerobic conditions as described above, and under anaerobic conditions consisting of 10% CO<sub>2</sub>, 10% H<sub>2</sub> and 80% N<sub>2</sub> in a MART microbiology unit. Isolated colonies were tested for catalase, urease and oxidase, and examined by Gram's stain for morphology and by *Helicobacter* genus-specific PCR. *H. pylori*-specific and *Helicobacter* genus-specific PCR methods are described below. The amplified IDEIA HpSTAR (DAKO, Glostrup, Denmark) faeces antigen test was performed according to the manufacturer's instructions (20).

### *Tissue collection and processing*

Animals were sacrificed using CO<sub>2</sub> and blood was drawn from the heart. Stomachs collected from all

animals were divided into three parts and were, respectively, frozen for PCR analysis, put in formalin for histopathology, and used for culture. Samples were also taken from liver/gallbladder, pancreas, jejunum, caecum/terminal ileum and colon of animals in groups 3 to 5. Stomach tissues were cultured by scraping directly onto selective Gab-Camp agar (7) and selective Brucella blood agar, at 37°C in a 5% CO<sub>2</sub> incubator under microaerobic conditions. Both solid and homogenized liver-, gallbladder- and caecum tissue samples were inoculated onto selective and nonselective Brucella blood agar under microaerobic and anaerobic conditions (MART) as described above. Caecum homogenates were also cultured following filtration through a 0.8-µm pore-size filter (Millipore Corporation, Bedford, USA). Isolated colonies were tested as described above for isolates from faecal samples.

#### *Helicobacter* genus-specific PCR

A two-step *Helicobacter* genus-specific PCR was performed on the tissue samples, faecal samples and selected colonies recovered from culture. Bacterial DNA was extracted from faecal samples using the QIAamp® DNA Stool Mini Kit (QIAGEN®, Hilden, Germany) and from tissue samples using the QIAamp® DNA Mini Kit (QIAGEN®) according to the manufacturer's instructions with the modification that faecal samples were lightly mechanically homogenised prior to extraction. PCR amplification was carried out using a GeneAmp 2400 thermocycler. Detection of *Helicobacter* spp. was carried out by semi-nested *Helicobacter* genus-specific PCR assay, using primers previously described by Goto *et al.* (Table 1) (21). The reaction mixture of the first step (25 µl) contained 0.5 µM amounts of each primer (1F and 1R), 0.2 mM concentrations of each deoxyribonucleotidetriphosphate (dNTP) (Amersham Pharmacia Biotech, Uppsala, Sweden), 1× chelating buffer, 2.5 mM MgCl<sub>2</sub>, 0.4% (wt/vol) BSA, 1.25 U of rTth dna polymerase (Applied Biosystems, Foster City, CA), and 5 µl of extracted DNA. The amplification program for the first step was 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and finally 72°C for 5 min. The reaction mixture of the second step (25 µl) con-

tained 0.5 µM amounts of each primer (1F with GC-clamp and 2R), 0.2 mM concentrations of each dNTP, 1× buffer II, 2.5 mM MgCl<sub>2</sub>, 1.0 U of AmpliTaq Gold™ DNA polymerase (Applied Biosystems), and 2 µl of 10× diluted PCR product from the first step. The amplification program for the second step was 95°C for 10 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and finally 72°C for 5 min.

For both the *Helicobacter* genus-specific and the *H. pylori* PCR, 10<sup>-8</sup> g/ml of *H. pylori* (CCUG 17874) DNA was added to the reaction mixture as a positive control, and 5 µl of sterile Millipore-filtered deionized water was added as negative control. PCR products were visualized by electrophoresis in an agarose gel (1.5%) containing ethidium bromide (22). The gel was analysed in a GelFotoStation (Tehtum Lab., Klippan, Sweden).

#### *H. pylori* PCR

An *H. pylori*-specific semi-nested PCR assay was used to detect the *cagA* gene in faecal samples. DNA extraction was performed as described above for the *Helicobacter* genus-specific PCR. Amplifications were carried out using a GeneAmp 2400 thermocycler (Applied Biosystems). The CagF1 and CagB1 primers were described by Tummuru *et al.* (23), and the CagIF primer was designed by W. Abu Al-Soud & HO. Nilsson (personal communication) (Table 1). The reaction mixture of the first step was the same as described for the *Helicobacter* genus-specific PCR except for the primers used (CagF1 and CagB1). The amplification program for the first step was 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and finally 72°C for 7 min. A 25 µl reaction mixture of the second step contained the same contents as described above for the second step of the *H. pylori*-specific PCR, except primers (CagIF and CagB1) and template (5 µl of 50× diluted PCR product from the first step). The amplification program for the second step was the same as for the first step.

#### Serology

Enzyme immunoassay (EIA) and immunoblot to detect *H. pylori*-specific immune responses were per-

TABLE 1. Primers used in the PCR assays

Amplification	Primers	Primer sequence	Size bp	Ref.
<i>H. pylori</i> <i>cagA</i>				
Step one	CagF1	GAT AAC AGG CAA GCT TTT GAG G	~350	(23)
	CagB1	CTG CAA AAG ATT GTT TGG CAG A		(23)
Step two	CagIF	TTG TCG TGG AAT CTT CCA CAA A	~210	
N16s <i>Helicobacter</i> genus PCR				
Step one	1F	CTA TGA CGG GTA TCC GGC	~780	(21)
	1R	CTC ACG ACA CGA GCT GAC		(21)
Step two	2R	TCG CCT TCG CAA TGA GTT T	~470	(21)

formed on serum samples collected before bacterial inoculation and after sacrifice of the animals.

The *H. pylori* EIA was performed as described previously (7). In brief, Maxisorp immunoplates (NUNC, Roskilde, Denmark) were coated with 100 µl per well of 5-µg/ml acid glycine-extracted *H. pylori* cell-surface proteins (24). Serum diluted 1:200 was added and the plates were incubated for 90 min at 37°C. Horseradish peroxidase-conjugated rabbit anti-guinea pig immunoglobulins (Dako, Glostrup, Denmark) diluted 1:1000 were used as the second antibody. The substrate solution was 10 mg of 1,2-phenylenediamine diluted in 25 ml of 0.1 M sodium citric acid phosphate with 5 µg of H<sub>2</sub>O<sub>2</sub> (30%). To correct for inter-assay variation, relative antibody activity (RAA) was calculated using the formula: RAA = mean absorbance × 100 / mean absorbance of reference wells. Human gammaglobulin was used in the reference wells (gammaglobulin 165 mg/ml, Pharmacia & Upjohn, Stockholm, Sweden).

Immunoblot assays with acid glycine-extracted cell-surface proteins of *H. pylori*, *H. bilis*, *H. hepaticus*, *H. pullorum* and "*F. rappini*", respectively, were performed as described elsewhere with minor modifications (25). In brief, SDS-PAGE was performed in Criterion™ cell electrophoresis equipment (Bio-Rad, Hercules, CA, USA). Cell-surface protein extracts and molecular weight standards ranging from 14.4 to 97 kDa (Amersham Pharmacia Biotech) were separated in a 10 to 20% gradient gel with a 5% stacking gel (Criterion™ Precast Gel, Bio-Rad) for 10 min at 50 V and then at 200 V for approximately 1.5 h, until the dye front reached the end of the gel. Separated *Helicobacter* proteins were electrophoretically transferred to a PVDF membrane (Osmonics, Westborough, MA, USA) in semidry electroblotter equipment (Amersham Pharmacia Biotech) for 1.5 h with current of 0.8 mA/cm<sup>2</sup>. Membranes were saturated for 2 × 15 min in blocking buffers and rinsed. Strips were cut and incubated with guinea pig serum diluted 1:50 in washing buffer for 16 h at 8°C. Human pooled sera were used as positive control in the *H. pylori* assay and sera from immunized rabbits were used as positive control in the *H. bilis*, *H. hepaticus*, *H. pullorum* and "*F. rappini*" assays (26). Horseradish peroxidase-conjugated rabbit anti-guinea pig immunoglobulins (Dako, Glostrup, Denmark) diluted 1:600 were used as the second antibody and bound antibodies were detected by reaction in 50 mM sodium acetate buffer containing 0.04% 3-amino 9-ethylcarbazole and 0.015% H<sub>2</sub>O<sub>2</sub>.

Serum levels of C3 were determined as described previously and serum cholesterol levels were analysed using the CHOD-PAP enzymatic test (Roche Diagnostics, Mannheim, Germany) (9, 27).

#### Histopathology

Samples for histopathology were fixed in 10% buffered formaldehyde, processed routinely, embedded in paraffin, cut, and stained in a haematoxylin &

eosin and Alcian blue, pH 2.5. Subsequently, two examiners blindly graded gastritis in samples from the antrum and corpus regions on a scale ranging from 0 to 3, where 0 represents no gastritis and 3 severe gastritis (7).

## RESULTS

Stomachs from four of the five guinea pigs of group 2 (infected for 5 months with *H. pylori*) and one of the two animals of group 4 (infected for 3½ months) were positive for *H. pylori* either by culture or PCR. Four of these five animals were PCR positive, whereas three of the five were positive by culture. No non-*pylori Helicobacter* species could be detected in tissue or faeces of any animal, including the two animals challenged with a mixture of non-*pylori Helicobacter* species. Neither the HpSTAR faeces antigen test, nor the semi-nested PCR for the *cagA* gene, could detect *H. pylori* in faecal samples from challenged animals, including samples collected 24 h postinoculation.

*H. pylori*-infected animals showed high antibody titres to *H. pylori* cell-surface proteins in EIA. Animals infected with a mixture of other *Helicobacter* species also exhibited an elevation of anti-*H. pylori* titres, as did two of the five control animals. The remaining three control animals and the *H. pylori*-challenged animals that had not been colonized displayed low titres.

Immunoblot showed specific antibodies to *H. pylori* cell-surface antigens in sera from all *H. pylori*-infected animals at sacrifice. Antibodies were directed to proteins with relative molecular masses ( $M_r$ ) of 16-, 18-, 24-, 28- and 33-kDa (Fig. 1). Serum from these animals displayed no bands before bacterial challenge. Some control sera exhibited weak bands towards 24- and 28-kDa proteins (Fig. 1). Animals challenged with a mixture of *H. bilis*, *H. hepaticus*, *H. pullorum* and "*F. rappini*" displayed a weak cross-reactivity in immunoblot with *H. pylori* cell-surface proteins ( $M_r$  16-, 24- and 27-kDa) (Fig. 1). Immunoblot to cell-surface proteins of *H. bilis*, *H. pullorum* and "*F. rappini*" displayed no specific antibody reactivity in the sera from *Helicobacter* mixture-challenged animals, whereas *H. hepaticus* immunoblot recognized antibodies towards 18- and 22-kDa proteins.

Serum C3 and cholesterol levels were higher

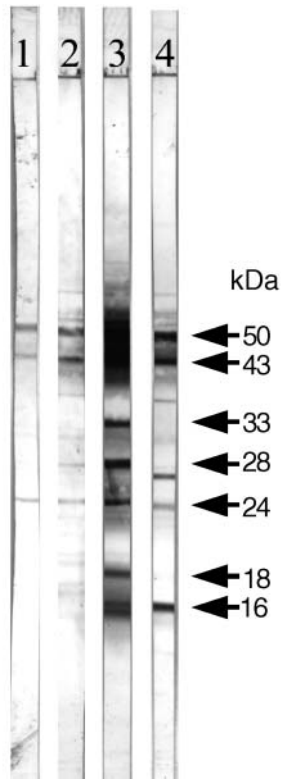


Fig. 1. Immunoblot to separate *H. pylori* cell surface proteins. 1: Prior to infection. 2: Control animal, 5 months. 3: *H. pylori*-infected animal, 5 months. 4: *Helicobacter* mixture challenged animal (*H. bilis*, *H. hepaticus*, *H. pullorum*, "*F. rappini*"), 3½ months.

in *H. pylori*-infected animals than in uninfected animals 5 months postchallenge. Mean C3 level was 132% in infected animals and 120% in uninfected animals, where 100% corresponds to the zone diameter with a pooled control serum from young animals. Mean total cholesterol level was 1.80 mmol/l in infected and 1.28 mmol/l in uninfected animals. Statistical analysis could not be performed due to the small number of animals used.

Three of the four animals infected with *H. pylori* for 5 months had severe antral gastritis, whereas the fourth infected animal showed no gastritis. Macroscopic lesions were not observed. Gastritis was absent in the challenged but uninfected 5-month animal and in one of the control animals. The other 5-month control animal displayed mild gastritis. The animal infected for 3½ months displayed a moderate antral gastritis, whereas gastritis was mild in three and absent in three of the six uninfected 3½ animals. Inflammatory infiltrates consisted

mostly of lymphocytes and plasma cells, but neutrophils were also present (Figs. 2–4). Inflamed mucosa displayed restricted areas of focal degeneration and necrosis of crypt epithelium and an increased number of mitotic figures, indicating a regenerative process in the epithel-

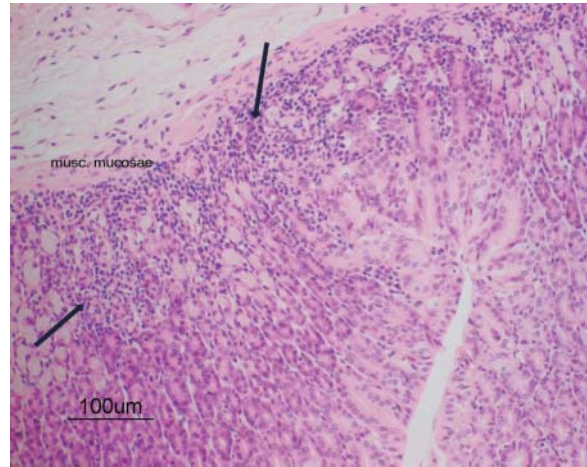


Fig. 2. Guinea pig stomach displaying a moderate leukocytic infiltrate, most prevalent in the basal areas of the gastric mucosa (arrows). Lymphocytes and plasma cells are the predominant cells in the cellular infiltrate. The picture is taken in the junction region between body and antrum. (Objective: ×20).

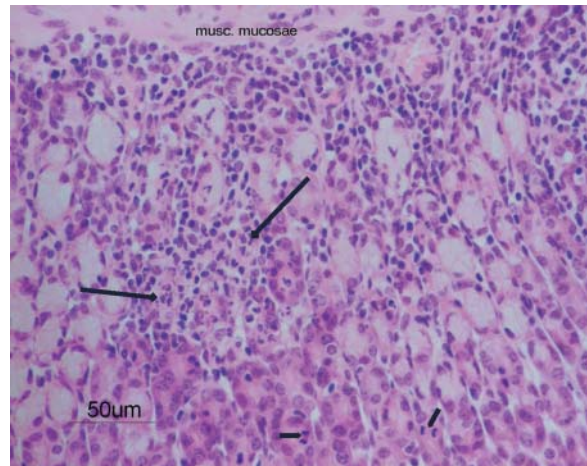


Fig. 3. Junction between body and antrum. Detail of histologic chronic, active gastritis, showing a restricted area of focal degeneration and necrosis of gland epithelium (long arrows). The affected area also exhibits moderate numbers of neutrophils, whereas the basal mucosa is infiltrated by abundant lymphocytes and plasma cells. The short arrows indicate mitotic figures in the neck of the glands. (Objective: ×40).

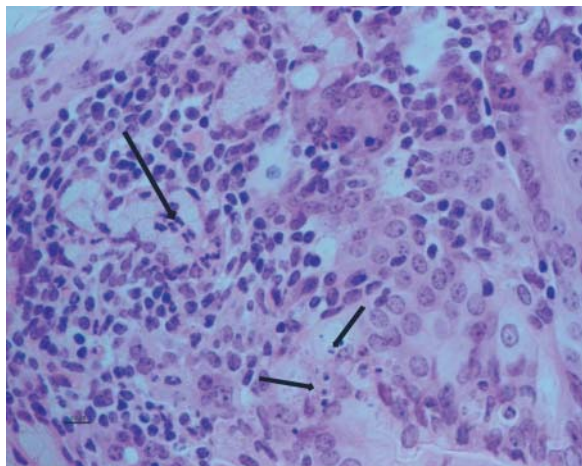


Fig. 4. Detail of histologic chronic antral gastritis in guinea pig. Moderate disruption of gastric mucosa by a cellular exudate composed predominantly of lymphocytes and plasma cells. The long arrow indicate at a few neutrophils positioned in the glandular lumen. Note degenerative and necrotic changes at the level of the surface epithelium (short arrows). (Objective:  $\times 60$ ).

ium (Fig. 3–4). Neither atrophic gastritis nor intestinal metaplasia was found. No gastritis was found in the corpus region of any animal.

## DISCUSSION

The guinea pig could be an important alternative to the gerbil model of *H. pylori* infection since both species develop severe gastritis upon experimental infection. Since severe inflammation seems to promote the development of gastric cancer (28), the guinea pig with pronounced gastritis may become a good candidate for a new *H. pylori*-induced gastric carcinoma model. We here show that an *H. pylori* infection persisted for 5 months in experimentally infected guinea pigs, with severe chronic antral gastritis in three of four infected animals. A focal degeneration and necrosis of the gastric crypt epithelium was seen and an increased number of mitotic figures indicated a regenerative process in the epithelium. Atrophic gastritis, intestinal metaplasia or malignant changes did not develop. Chronic infection for one year or longer, with a larger group of animals, is needed to evaluate the potential of the guinea pig as a model for gastric cancer.

Gastritis was confined to the antrum region

of the infected animals. In humans, the presence of inflammation in the corpus region of the stomach increases the risk of developing gastric atrophy, metaplasia and eventually gastric cancer (29), and in *H. pylori*-infected gerbils, inflammation was localized in the antrum initially, but extended to the corpus region after 12 months (11). In future guinea pig experiments it will be important to assess whether the inflammation extends to the corpus region over time.

A serological immune response in immunoblot and EIA to *H. pylori* cell-surface antigens was detected in *H. pylori*-infected animals after  $3\frac{1}{2}$  and 5 months. Two control animals exhibited high EIA titres. False-positive results limit the usefulness of EIA to monitor *H. pylori*-infected animals in long-term experiments. In contrast, using immunoblot the control animals showed no reaction. However, one of the *H. pylori*-challenged animals negative by culture, PCR and histopathology exhibited bands by immunoblot. This animal may initially have been infected, but later cleared the infection. Immunoblot revealed six immunoreactive cell-surface proteins with low molecular mass in sera from *H. pylori*-infected animals (Fig. 1). The immunogenic neutrophil-activating protein 15 kDa subunits (30) could be one of the double bands at approximately 16 kDa and a membrane-associated lipoprotein (Lpp20) (31) with a molecular mass of 18 kDa, also known to be immunogenic, could be the band found at 18 kDa. For the other proteins, with molecular masses of 24 kDa, 28 kDa and 33 kDa, several candidate proteins could be found in database and literature searches.

Serum C3 and cholesterol levels were higher in *H. pylori*-infected than in control animals 5 months postchallenge. The small number of animals used did not permit statistical analysis, but the results further support our findings from a previous work (9). Elevated serum acute-phase protein levels may directly contribute to cell-wall inflammation and adhesion or cause changes in blood lipid levels, which could explain the controversial link between *H. pylori* infection and cardiovascular disease (32–37).

Neither the HpSTAR faeces antigen test nor the semi-nested PCR assay for the *cagA* gene was able to detect *H. pylori* in guinea pig faeces. In contrast, these methods have been found use-

ful to detect *H. pylori* infection in mice (38). The digestive efficiency of the gastrointestinal tract of guinea pigs has been shown to be higher than that of rabbits, rats, hamsters and leaf-eared mice (39, 40). The high level of digestion may cause a breakdown of DNA and antigens during the passage through the guinea pig gut, thereby precluding detection in faeces. This explanation is further supported by the fact that *H. pylori* DNA or antigens were not detected 24 h after inoculation with a massive dose of *H. pylori*. It has been shown that significant concentrations of an orally given marker are present in the faeces of guinea pigs 24 h following single oral challenge (39, 41, 42).

The guinea pigs used were shown to be free of indigenous *Helicobacter* infection. The guinea pig is one of the few laboratory animals where such an infection has not been reported. Natural *Helicobacter* infections found in other laboratory animals, such as mice, Mongolian gerbils, rats, hamsters and cats, may be confounding factors in these animal models (3, 43). In the current study we also set out to assess the use of the guinea pig as an experimental animal for infection with *H. bilis*, *H. hepaticus*, *H. pullosum* and "*F. rappini*". We did not succeed in infecting the animals with these bacterial species and new strategies will now be tested. The use of broth cultures instead of agar cultures has been shown to be more efficient in experimental *H. pylori* infection and should be tried with these bacterial species as well (44).

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