JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1998, 49, 3, 387-403

T. BRZOZOWSKI, **P.Ch. KONTUREK, S.J. KONTUREK, E. KARCZEWSKA, R. PAJDO, *J. STACHURA, ***P. GHIARA, **E.G. HAHN

# GASTRIC SECRETION AND ULCER HEALING IN MOUSE STOMACH INFECTED WITH CYTOTOXIN EXPRESSING STRAIN OF *HELICOBACTER PYLORI*

Department of Physiology, *Department of and Pathomorphology, Jagiellonian University School of Medicine, Krakow, Poland and **Department of Medicine I, University of Erlangen-Nuremberg, Erlangen, Germany and ***Immunobiological Research Institute Siena, Siena, Italy

*Helicobacter pylori* (Hp) is a major risk factor of peptic ulcer but studies on the relation between Hp infection and gastric pathology are limited due to lack of convenient models resembling Hp infection in humans. We studied the effects of inoculation of conventional BALB/c mice with toxigenic type I Hp (cagA+ and vacA+) and non-toxigenic type II Hp (cagA- and vacA-) vs administration of vehicle on gastric secretion and healing of gastric ulcers. The gastric secretion studies were performed on mice with chronic gastric fistula before and after inoculation with toxigenic or non-toxigenic Hp strain or administration of vehicle (saline). Gastric ulcers were produced in mice inoculated with toxigenic and non-toxigenic Hp strain or vehicle and then sacrificed at day 0 and after 2, 4, 7, 14 and 28 days. Ulcer area and gastric blood flow (GBF), plasma gastrin and gastric luminal somatostatin were determined. Gastric mucosal biopsy specimens were also taken for the assessment of the presence of viable Hp using rapid urease test, the Hp-culture and the reverse transcriptase — polymerase chain reaction (RT-PCR) analysis of the signal for Hp CagA. Gastric acid output was reduced by over 50% immediately after Hp inoculation and this effect persisted during all time intervals tested, being significantly more pronounced in type I Hp-infected stomach. The area (7 $mm^2$ ) of ulcers in control mice decreased gradually and then continued to decline during 14 days to disappear almost completely after 28 days. In contrast, the ulcers were present till day 28 in all mice infected with type I or type II Hp strain being significantly larger especially with type I Hp-infection. The GBF in control mice showed gradual rise with decreasing ulcer size being significantly higher at the ulcer margin than the ulcer crater and reached after 14 and 28 days the value not significantly different from that in vehicle-administered mice. In contrast, the GBF in type I Hp-infected mice but to a lesser extent , in type II Hp infected mice was significantly lower than in the vehicle controls, both at the ulcer margin and the crater of ulcers at all tested days. Hp-infection was accompanied by significant increment in plasma gastrin and the fall in gastric somatostatin contents observed at all test days, particularly in mice infected with type I Hp strain. Edema of surface epithelium appeared after 7 days and weak but significant mucosal inflammatory infiltration occurred after 14 days to further increase after 28 days, especially in type I Hp and less in type II Hp infected mice. We conclude that conventional mice with gastric ulcers can be successfully infected by both toxigenic and non-toxigenic Hp strains and this infection markedly reduces gastric acid secretion and delays healing

of ulcers probably due to the fall in mucosal microcirculation in ulcer area, mucosal inflammation and impairment in gastric-somatostatin link.

Key words: *Helicobacter pylori, ulcer healing, CagA, VacA, gastric blood flow, gastrin, somatostatin, inflammation, gastritis, gastric secretion*

## INTRODUCTION

*Helicobacter pylori* (Hp) is recognized as a major pathogen in chronic type B gastritis and gastro-duodenal ulcers (1—3) that appear especially in patients infected with Hp strains expressing cagA and vacA encoded cytotoxins (4). The mechanism of Hp-induced gastric damage has not been fully explained but the disintegration of gastric mucus by Hp-produced enzymes, the release of aggressive factors including ammonia and lipopolysaccharides and activation of neutrophils enhancing the generation of oxygen reactive species have been proposed to explain the deleterious action of Hp on the stomach (5—11).

The studies on various aspects of Hp infection and gastric pathology are, however, limited due to the lack of adequate animal model resembling gastric Hp infection in human stomach (12—16). Recently, Marchetti *et al* (17) have proposed the model of Hp infection in mice after successful colonization of their stomach by Hp expressing cagA and vacA cytotoxins but the effect of this germ on gastric secretion and ulcer healing have not been examined.

This study was designed to determine the effect of cagA and vacA expressing and nonexpressing cytotoxin Hp strains as compared to vehicle (saline) on 1) gastric secretion, gastric blood flow (GBF) and plasma gastrin and gastric luminal somatostatin contents; 2) healing of gastric ulcers in mice and 3) the histological alterations in gastric mucosa (the presence of Hp and the inflammatory changes) during ulcer healing.

## MATERIAL AND METHODS

Male conventional BALBc mice, weighing 20—40 g and fasted for 24 h were used in all studies.

### *Studies on gastric secretion*

Gastric secretion was studied in 20 male mice weighing approximately 30 g and equipped with chronic gastric fistula (GF) about 1 month before secretory tests as described in our previous studies using rats (21). After the collection of two basal 30 min samples, the cannula of gastric fistula was closed and the mice received intragastrically (i.g.) *inoculum* containing toxigenic Hp (SPM 326, type I) or nontoxigenic (SPM 314, type II), supplied kindly by Prof. P. Ghiara, Department of Microbiology, Sienna, Italy obtained from the fresh clinical isolates or vehicle (saline). Hp strains (containing $2x10^9$ colony forming units, CFU, per milliliter) were inoculated in a total volume of 0.2 ml 15 min after alkalinization of the stomach with 0.2 ml of 100 mM

$NaHCO_3$ as described by Marchetti *et al.* (17) and remained in the stomach for 60 min. Then, the stomach was washed out with 0.5 ml of tap water and the gastric secretion was collected throughout the 2 h period. Acid output was measured in each 60 min collected aliquot and expressed as output per hour. At the end of experiment, the inoculation procedure was repeated immediately after closing the GF. Gastric secretory examination in these gastric fistula rats was repeated after 4, 14 and 28 days upon the inoculation or administration of vehicle. After completing the secretory studies, the rats were sacrificed and gastric mucosal samples were taken for the assessment of the presence of Hp using the methods described below.

## *Production of gastric ulcers*

Gastric ulcers were produced in 90 mice by our modification (22) of acetic acid method originally described in rats by Okabe *et al* (23). Briefly, under light ether anesthesia, the abdomen was opened, the stomach exposed and 50 µl of acetic acid was poured through the plastic mould onto serosal surface of the mouse stomach for 5 s. This resulted in an immediate necrosis of mucosa and submucosa within the area (about 7 $mm^2$ ) of the mould application and this lesion became chronic ulcer within about 3 days without perforation or penetration to surrounding organs (22).

## *Gastric inoculation with type I and type II Hp of mouse stomach*

Since acetic acid-induced ulcers healed completely within two weeks (22), we have chosen period of 0, 2, 4, 7, 14 and 28 days after ulcer induction to study the time course of healing of these ulcers in mice infected with toxigenic or administered with vehicle (saline) to serve as controls. In studies with inoculation with Hp three series of experiments (A, B and C) were performed; series A and B included gastric inoculation with Hp ($2 \times 10^9$ in type I Hp-infected mice CFU per milliliter) using toxigenic Hp strain (SPM 326 type I) and non-toxigenic Hp strain (SPM 314 type II). Series C was given intragastrically (i.g.) vehicle (saline) in the total volume of 0.2 ml in similar manner as described for secretory studies using a steel catheter. Inoculation in series A and B was started one day before ulcer production and this was repeated after day 1 upon the ulcer induction. Then, the Hp-inoculated mice as well as those vehicle-administered were sacrificed immediately (day 0) and after 2, 4, 7, 14 and 28 days upon the induction of ulcers.,

## *Determination of gastric ulcer area and gastric blood flow (GBF), plasma gastrin and gastric luminal concentration of somatostatin*

To evaluate the alterations in GBF during ulcer healing in Hp-infected and vehicle-administered mice, the animals were anesthetized with ether, the abdomen was opened and the stomach was exposed. The GBF was determined at the ulcer margin , the ulcer crater and the contralateral intact mucosa using $H_2$-gas clearance technique as described previously (24). Immediately after GBF measurement, a venous blood sample was withdrawn from the vena cava into the EDTA containing vials and used for the determination of plasma gastrin levels by radioimmunoassay (RIA) as described previously (21,24). In addition, somatostatin was measured by RIA in luminal content collected from the stomach after measurement of GFB using a commercially available RIA-kit as described previously (26). For this purpose, the gastric content was collected after washing out the stomach with 0.5 ml of saline injected into the stomach just after the GBF had been measured. The pyloric sphincter in these mice was ligated to prevent the escape of the gastric content into duodenum. The collected sample was then immediately neutralized with NaOH to pH 7.0, centrifuged and then frozen with liquid nitrogen and finally

stored in −80°C until RIA-somatostatin. The antiserum used for this measurement recognized only cyclic forms of somatostatin-14 and somatostatin-28 equally and did not cross react with any known gastrointestinal peptide. The detection limit was 0.5 pmol/ml. The intraassay and interassay variations were 8% and 12%, respectively.

The stomachs were then quickly removed and pinned open for the determination of the area of gastric ulcers by computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany) by two investigators under blinded conditions as described in our previous studies in rats (24, 25).

## *Quantification of viable Hp in mouse stomach*

In mice inoculated with type I Hp strain or administered with vehicle, the half of the stomach not containing the ulcer or its scar was excised, homogenized in 1 ml of phosphate-buffer saline with a homogenizer (Ultra Turax) followed by dilution with the same buffer. Aliquots (100 µl) of the dilutions were applied to Helicobacter agar plates (Becton-Dickinson UK.) that were incubated at 37°C under a microareophilic atmosphere for 7 days. The colonies were identified as Hp as determined from shape (spiral) under a microscope ( × 2000; Olympus, Tokyo, Japan). In addition, positive test for oxidase, urease and catalase was performed to indicate specificity for Hp. The number of colonies was determined and the viable Hp was expressed as CFU/g of gastric tissue weight.

## *Histological evaluation of gastric mucosa in Hp inoculated mice*

The samples of the gastric mucosa from ulcer area and grossly unchanged mucosa were excised in each mouse for the assessment of the presence of Hp and gastric inflammation using Warthin-Starry silver staining and hematoxylin-eosin (H-E) staining histological examination, respectively. The samples were fixed in 10% buffered formalin and embedded in paraffin (24). The paraffin sections were cut at a thickness of 5 µm and stained. Histological examination was performed on coded slides by two experienced pathologists unaware of the treatment given.

## *Determination of Cag A transcripts in the gastric mucosa by reverse transcriptase polymerase chain reaction (RT-PCR)*

The stomachs were removed from mice with intact gastric mucosa and from those with gastric ulcers treated with inoculated with vehicle, toxigenic or non-toxigenic Hp strains after 28 days upon ulcer induction. Mucosal specimens (about 200 mg) were scraped of on ice using a slide glass and immediately snap frozen in liquid nitrogen and stored at −70°C for further analysis. Total RNA was isolated from the gastric mucosa using a rapid guanidinum isothiocyanate/phenol chloroform single step extraction kit from Stratagene based on method described by Chomczynski *et al* (27). Following precipitation, the RNA was resuspended in RNAse-free TE buffer and the concentration was estimated by absorbance at 260 mm wavelength. Furthermore, the quality of each RNA sample was determined by running the agarose-formaldehyde electrophoresis. RNA samples was frozen at −80°C until analysis.

Single stranded cDNA was generated from total cellular RNA (5 µg) using StrataScript™ reverse transcriptase (Stratagene, La Jolla, USA) and oligo (dt) primers (Stratagene, La Jolla, USA). Briefly, 5 µg of total RNA was used as the template to synthesize complementary DNA with 2.5 U of Maloney murine leukemia virus reverse transcriptase in 5 µl of buffer containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl, 5 mM MgCl ; 1 mM of each deoxyribonucleoside triphosphate (dNTP); 2.5 mM of oligo (dt) primers and 1.4 U/µl RNAse block. RT was performed at room temperature for 20 min, then at 37°C for 15 min, at 90°C for 5 min and at 5°C for 5 min. The resulting complementary DNA was used as a template for subsequent PCR.

Bacterial gene expression was then analyzed by PCR using Hp specific primers. The nucleotide sequences of the Hp CagA were based on the published cDNA sequences encoding CagA (28). The primers were synthesized by Biometra (Gottingen, Germany). The CagA sense primer was

5'GAT AAC AGG CAA GCT TTT TGA GG3'

while the CagA antisense primer was

5'CTG CAA AAG ATT GTT TGG CAG A3' (28).

Concomitantly, amplification of control rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Clon Tech, Palo Alto, CA) (983 bp) was performed on the same samples to verify RNA integrity.

Reaction mixture for PCR contained cDNA template (2 µl), 50 pmol of each primer, and 2.5 U of *Termus aquaticus* DNA (Promega) in 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dNTP in a volume of 50 µl. RT blanks (no RNA included) and PCR blanks (no cDNA products included) were incubated in each analysis. The mixture was overlaid with 25 µl of mineral oil to prevent evaporation. Amplification was performed using a DNA thermal cycler (Perkin-Elmer-Cetus) for 33 cycles, each of which consisted of 1 min at 95°C for denaturation, 1 min at 45°C for annealing, and 2 min at 72°C for extension. The final cycle included extension for 7 min at 72°C to ensure full extension of the product. The PCR product in the volume of 8 µl each was electrophoresed on 1.5% agarose gel stained with ethidium bromide, and then visualized under UV light. Location of predicted PCR-product was confirmed by using DNA digest Phix 174/Hae II as a standard marker. The gel was then photographed under UV transillumination. To avoid PCR contamination, PCR reactions were prepared in a dedicated area used only for PCR and the PCR products were opened in a laminar flow hood separated from the PCR preparation area.

### *Statistical analysis*

Results are expressed as means ± SEM. Statistical comparisons were made by analysis of variance and, where appropriate, by unpaired Student t-test with p value $<0.05$ being considered significant.

## RESULTS

### *Effect of Hp strains on gastric secretion*

*Fig. 1* shows the effects of vehicle and toxigenic and non-toxigenic Hp strains on gastric acid and pepsin secretion in conscious mice prepared with GF. In vehicle-treated animals, the acid output averaged $38 \pm 5$ µmol/60 min while the pepsin output averaged $0.31 \pm 0.08$ mg/60 min. The introduction into the stomach of *inoculum* containing type I or type II Hp strain caused almost immediate reduction in gastric acid outputs by 60% and 26%, and pepsin outputs by 62% and 41%, respectively, as compared to the values obtained in vehicle-administered mice. This reduction in gastric acid and pepsin outputs was also observed after 4, 14 and 28 upon Hp inoculation and it was significantly greater in mice inoculated with toxigenic than with non-toxigenic Hp strain.

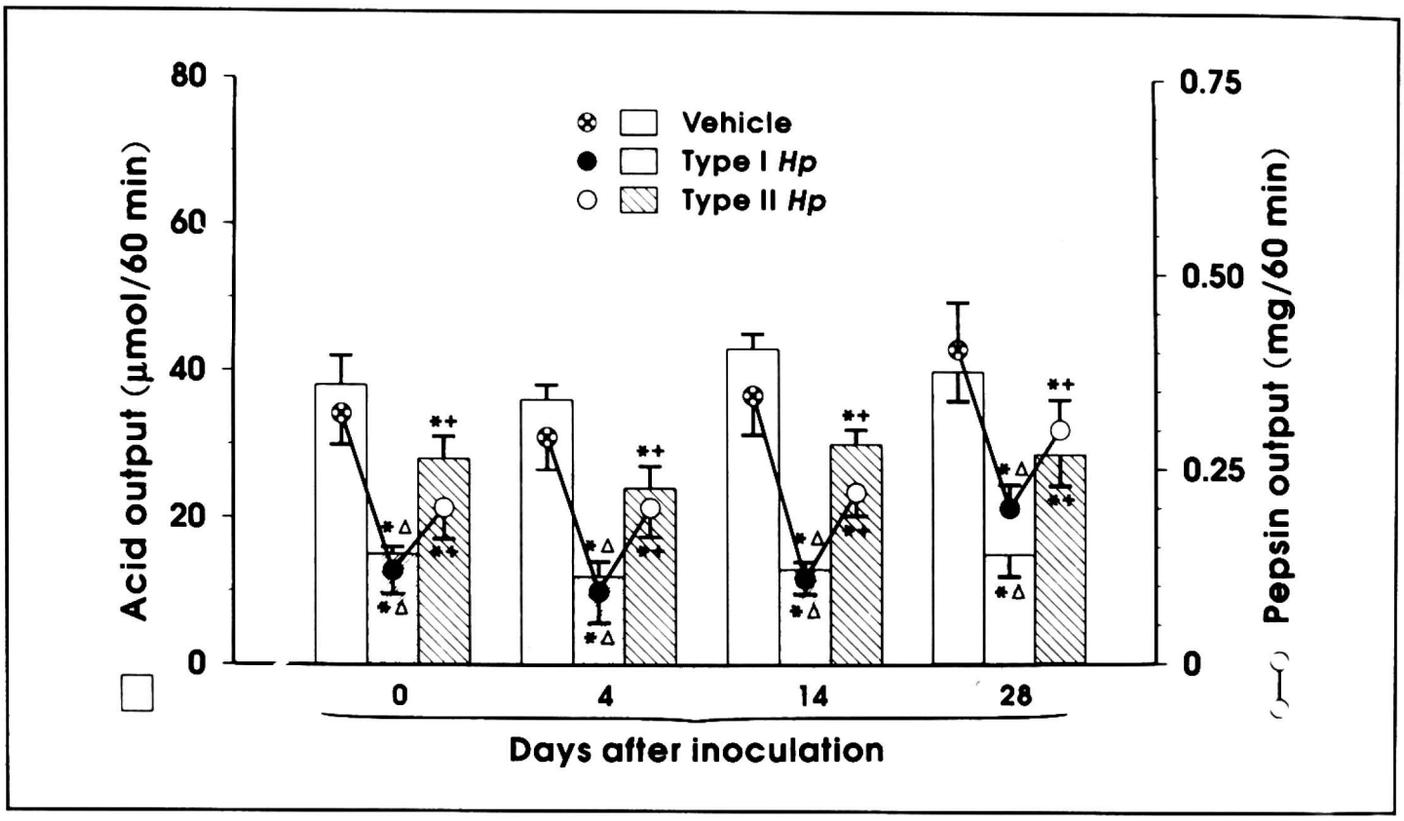


*Fig. 1.* The effect of toxigenic (type I) and non-toxigenic (type II) Hp strains or vehicle (saline) on the gastric acid and pepsin outputs in mice equipped with gastric fistula 60 min after inoculation (day 0) and following 4, 14 and 28 days postinoculation. Results are mean ± SEM of 6—8 mice. Asterisk indicates significant change as compared with the value obtained at day 0. Cross indicates significant change as compared with the value obtained in control mice given vehicle. Delta indicates significant change as compared to the value obtained in mice inoculated with type II Hp strain.

*Effects of toxigenic Hp strain on ulcer healing and GBF*

Immediately after serosal application of acetic acid the ulcer area was about 7 $mm^2$ and it was significantly decreased already after 2 days in vehicle-treated controls as compared to that in Hp inoculated mice (*Fig. 2*). There was a significant difference in the ulcer area between type I or type II Hp-infected and non-infected vehicle-administered mice after 4, 7, 14 and 28 days. In mice inoculated with type I Hp strain, the area of ulcers remained significantly larger at all tested days, than that in animals inoculated with type II Hp strain. At 28 day upon ulcer induction, the ulcers were completely healed in vehicle-treated mice whereas in Hp inoculated mice the ulcers were observed even after 28 days. Such a macroscopic appearance of an ulcer in mice after 7 days upon inoculation with vehicle or type I Hp strain is presented in *Fig. 3* showing that the area of ulcer is larger in Hp-infected stomach than in that treated with vehicle.

The GBF in non-ulcerated intact mucosa of mice treated with vehicle averaged 42 ± 3 ml/min-100 g tissue (taken as 100%) and it was significantly decreased immediately after ulcer induction (day 0) to about 26% at ulcer crater and to about 32% at the ulcer margin (*Fig. 4*). After 2 days, when the

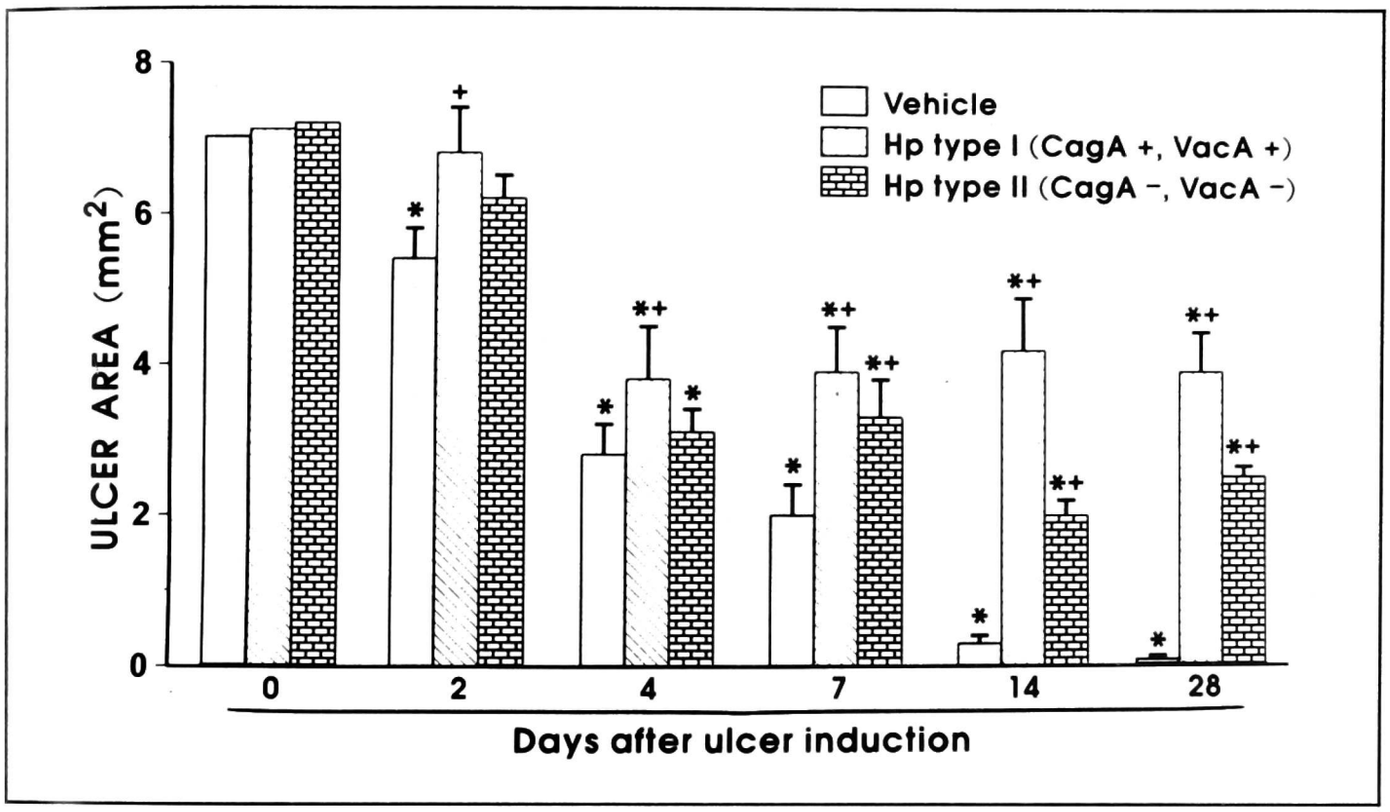


*Fig. 2.* The effect of toxigenic (type I) and non-toxigenic (type II) Hp strains or vehicle (saline) on the area of gastric ulcer at day 0 (initial) and following 2, 4, 7, 14 and 28 days upon the ulcer production in Hp-infected or vehicle-administered stomach. Results are mean ± SEM of 6—8 mice. Asterisk indicates a significant change as compared with the value obtained at day 0. Cross indicates a significant change as compared with the value obtained in control mice given vehicle.

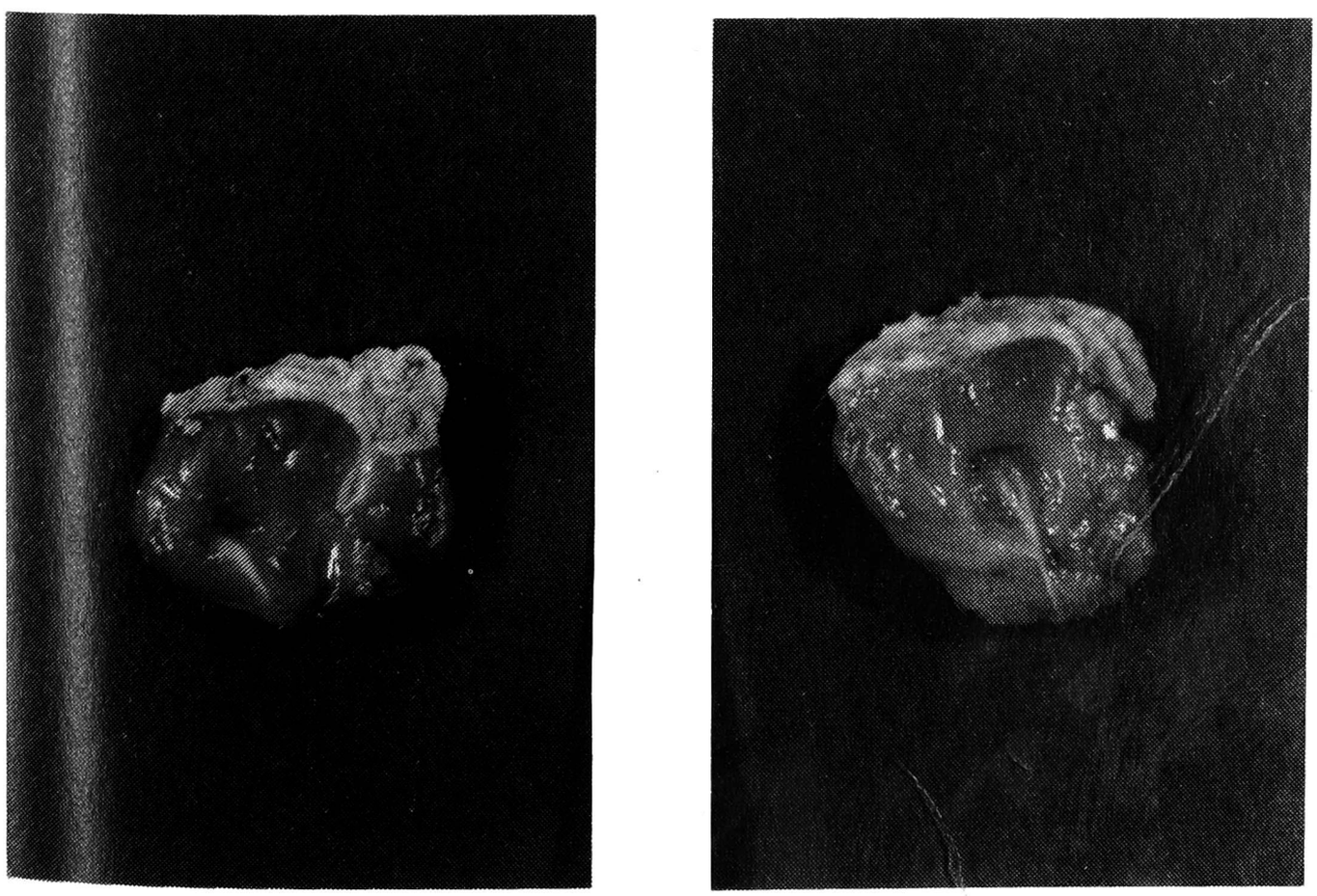

*Fig. 3.* The gross appearance of 7-day old gastric ulcer in mouse inoculated with vehicle (on the left) and type I Hp strain (on the right). Note, larger ulcer in the stomach of the mouse infected with type I Hp strain as compared to that in vehicle-treated stomach.

ulcer area showed significant and gradual decrease at subsequent days, the GBF at the ulcer crater and ulcer margin in vehicle-administered mice initially was not significantly different from that observed at day 0 but with subsequent observation days, the GBF at both sides of ulcer showed a gradual and significant increase to reach after 28 days the value not significantly different from that recorded in the intact mucosa (*Fig. 4*). Moreover, the GBF values at ulcer margin were significantly higher than those at ulcer crater at all days of examination except day 0. In contrast, mice inoculated with type I Hp strain which showed significantly larger ulcer area at all time periods, the GBF was significantly lower, both the ulcer crater and the ulcer margin when compared to that recorded in vehicle-controls throughout all study periods and failed to return to the value observed in intact gastric mucosa (*Fig. 4, Table 1*).

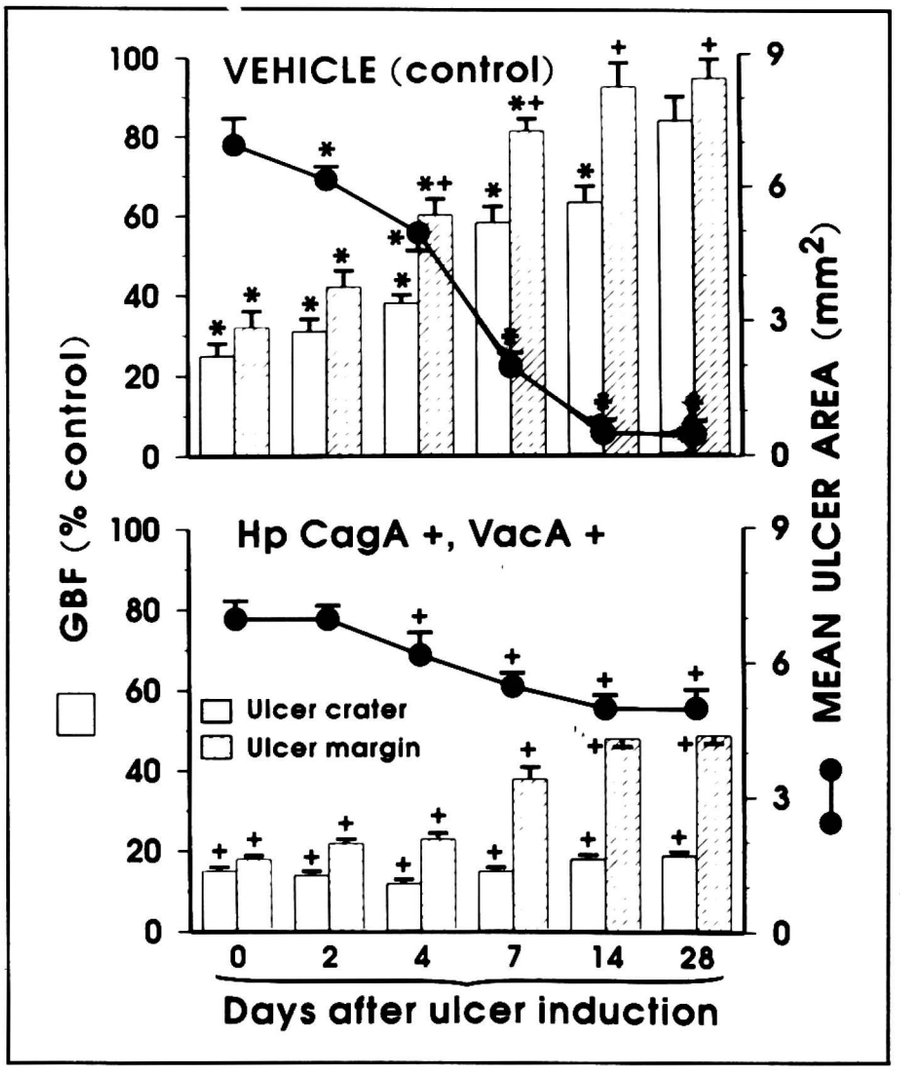


*Fig. 4.* Gastric blood flow (GBF) in the crater and margin of gastric ulcers on day 0 and 2, 4, 7, 14 or 28 days upon ulcer induction in mice administered with vehicle (control) (upper panel) or type I Hp strain expressing CagA and VacA (lower panel). Results are mean ± SEM of 6—8 mice. Asterisk indicates significant change as compared with the value obtained in intact gastric mucosa (GBF) or with that attained at day 0 (ulcer area). Cross indicates a significant change as compared with the value obtained in ulcer crater at corresponding test days (upper panel) or significant change as compared to the values obtained in vehicle-treated animals (lower panel).

*Effect of vehicle or Hp on plasma gastrin and luminal somatostatin levels*

The plasma concentrations of gastrin and gastric luminal somatostatin concentrations in vehicle-treated control mice averaged 22 ± 2 and 68 ± 9 pM/L, respectively (*Table 1*). In mice infected with type I Hp strain, the significant increment in plasma gastrin and the significant fall in luminal somatostatin levels were already observed at day 0 upon the inoculation with

type I Hp strain and ulcer formation. The increments in plasma gastrin and the fall in gastric luminal somatostatin concentrations in mice infected with toxigenic type I Hp strain were significantly greater at all test days upon ulcer production than those observed in mice inoculated with type II Hp strain (*Table 1*).

*Table 1* The gastric blood flow (GBF) in the ulcer crater and ulcer margin (expressed as percent of that recorded in the intact mucosa), the plasma gastrin levels and luminal concentration of somatostatin (SOM) in mice inoculated with vehicle, type I or type II Hp strain at day 0 and after 7, 14 and 24 days postinoculation. Results are means ± SEM of 6—8 mice. Asterisk indicates a significant change as compared to the values obtained with vehicle (saline) at respective days postinoculation. Cross indicates a significant change as compared to the values obtained in mice inoculated with type II Hp strain.

| Treatment | GBF (% Control) Ulcer crater | Ulcer margin | Gastrin pmol/L | SOM pmol/L |
|---|---|---|---|---|
| | | At day 0 | | |
| Vehicle | 26±3 | 32±4 | 22±2 | 68±9 |
| type I | 16±2*+ | 18±2*+ | 34±3* | 52±6* |
| type II | 18±3* | 20±8* | 28±2 | 54±12 |
| | | At day 7 after ulcer induction | | |
| Vehicle | 56±5 | 80±6 | 20±3 | 65±5 |
| type I | 15±3*+ | 38±5*+ | 37±6*+ | 32±4*+ |
| type II | 28±6* | 63±4* | 29±4 | 49±6* |
| | | At day 14 after ulcer induction | | |
| Vehicle | 62±4 | 92±6 | 22±3 | 68±9 |
| type I | 18±2*+ | 38±4*+ | 41±5*+ | 38±6*+ |
| type II | 39±4* | 67±5* | 32±3 | 54±4 |
| | | At day 28 after ulcer induction | | |
| Vehicle | 83±8 | 94±5 | 20±3 | 72±10 |
| type I | 19±5*+ | 48±2* | 31±3*+ | 45±6*+ |
| type II | 39±4* | 56±6* | 34±5 | 62±12 |

*Verification of the presence of viable Hp in mice infected with Hp*

As shown in *Table 2*, mice infected with type I and type II Hp strain showed positive rapid urea test already at day 2 after gastric inoculation with type I and at 7 day in mice inoculate with type II Hp strain. The presence of viable Hp strains was confirmed by Hp culture of gastric homogenate showing an increase in the number of bacteria after 7 days to about $1 \times 10^4$ CFU/g with

type I Hp and to $1 \times 10^3$ CFU/g with type II Hp infection. The number of viable Hp plateaued after 14 and 28 days, reaching the level of about $1 \times 10^5$ with type I Hp and $1 \times 10^4$ with type II Hp inoculation. Most of the bacteria were observed in the mucus layer but some microorganisms were adherent to the surface epithelium (*Fig. 5*). The bacteria existed diffusely over the antrum but some were also present in fundus of the stomach (*Fig. 5*). No Hp was detected in the vehicle-administered animals, as determined by the histology, rapid urea test or Hp culture (*Table 2*).

*Table 2* Rapid urease test and histological detection of Hp in the stomach as well as mucosal inflammation in mice administered saline (control) or injected with type I (toxigenic) or type II (non-toxigenic) Hp strain on mouse stomach with gastric ulcers as determined after 2, 7, 14 and 28 days upon ulcer formation.

| | Vehicle | Type of inoculation | |
|---|---|---|---|
| | | Type I Hp | Type II Hp |
| | Rapid urease test | | |
| Initial | — | — | — |
| After 2 days | — | + | — |
| After 7 days | — | + + | + |
| After 14 days | — | + + + | + + |
| After 28 days | — | + + + | + + |
| | Histological Hp detection (Hp culture) | | |
| Initial | — | — | — |
| After 2 days | — | + | + |
| After 7 days | — | + + | + |
| After 14 days | — | + + + | + + |
| After 28 days | — | + + + | + + |
| | Mucosal inflammation | | |
| Initial | — | — | — |
| After 2 days | — | — | — |
| After 7 days | — | ± | ± |
| After 14 days | — | + | ± |
| After 28 days | — | + + | + |

Rapid urease test: —, negative, +, color change during 30 min; + +, color change during 15 min; + + +, color change during 5 min;

Histological Hp detection: 0, — no Hp present; + $1 \times 10^3$ CFU/g; + + $1 \times 10^4$ CFU/g; + + + $1 \times 10^5$ CFU/g; Mucosal infiltration —, inflammatory cells hardly detectable by H-E stain; ± several inflammatory cells in a field; + More than several inflammatory cells; + + severe mucosal inflammation.

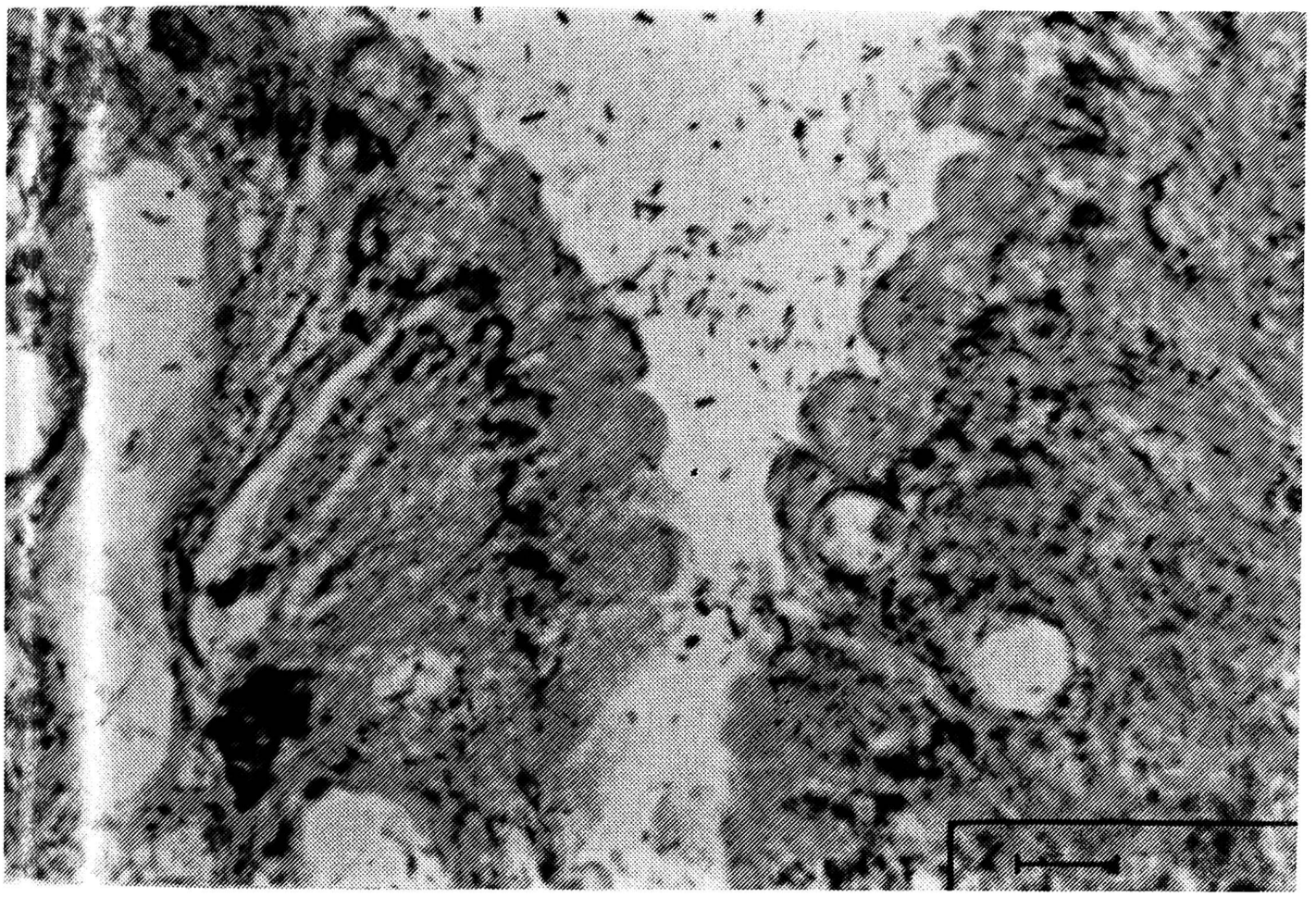

*Fig. 5.* The Warthin-Starry silver stained cross section through the fundic mucosa of a mouse 2 weeks postinfection with type I Hp strain expressing vacA and cagA encoded cytotoxins showing large numbers of bacteria distributed from the mucus layer down to the glandular pits; H&E, original magnification ×260.

*Histological evaluation of the gastric mucosa infected with Hp strains*

Histological examination revealed that the ulcer crater in mice infected with type I Hp strain was heavily infiltrated with neutrophils and focal regeneration of the surface epithelium at the ulcer margin was observed (*Fig. 6*). The mucosa distant to the ulcer was mostly normal after 7 days with only mild damage of surface epithelium and edema/congestion. After 14 and 28 days, in addition to superficial damage, the inflammatory (neutrophil/monocyte) infiltration was observed in the gastric mucosa (*Table 2*). After 28 days of infection with Hp the mucosal infiltration became more severe (*Table 2*).

*Expression of CagA mRNA in gastric mucosa of mice infected with type I or type II Hp strain by RT-PCR*

As demonstrated in *Fig. 7A*, the internal control with GAPDH gene showed intense signals in all samples tested indicating good quality of RNA isolated from the stomachs of vehicle-control mice as well as those inoculated with type I or type II Hp strain. The expression of CagA mRNA was

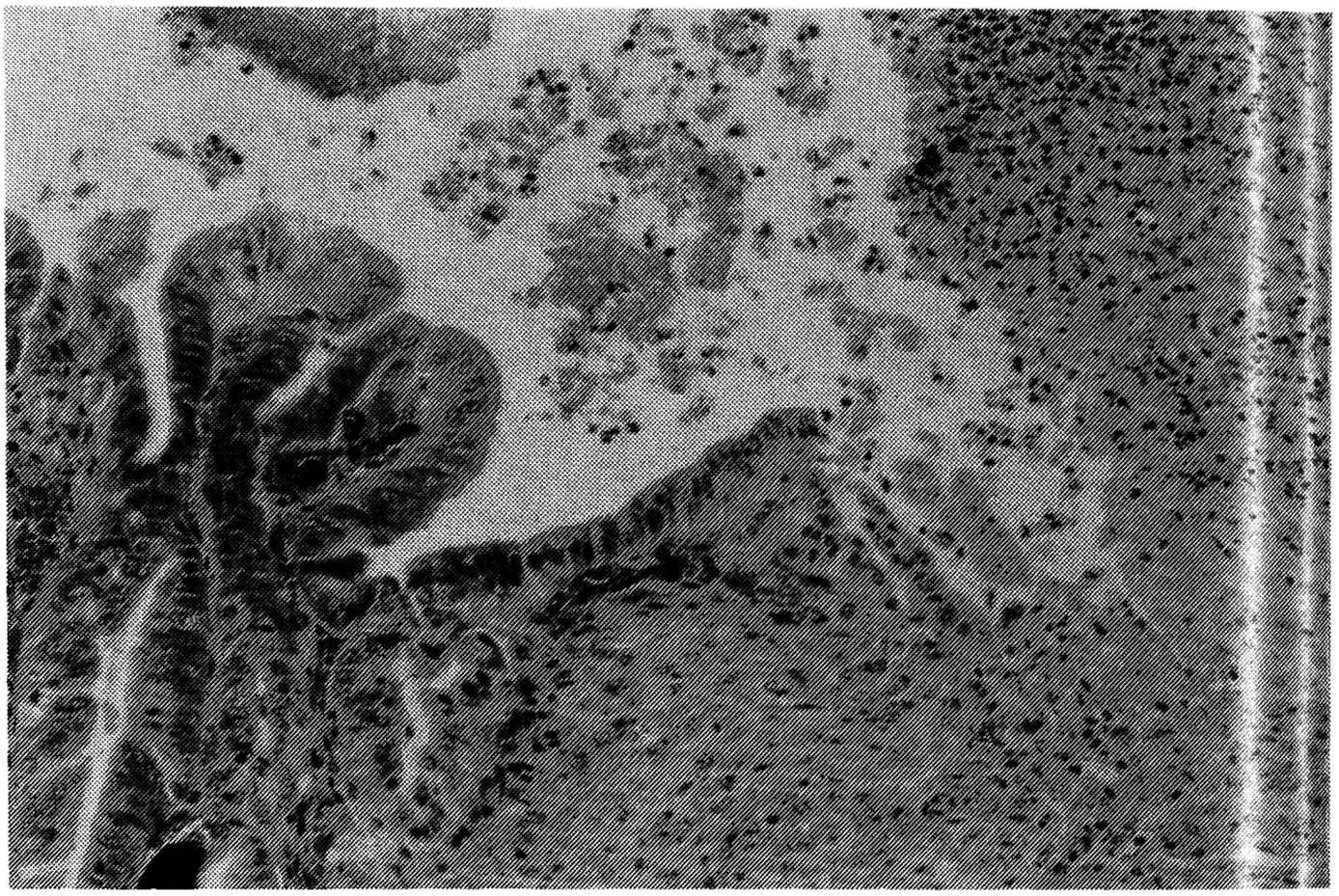

*Fig. 6.* Histological appearance of gastric ulcer in mouse gastric mucosa 7 days after ulcer induction in mouse inoculated with type I Hp strain. Note, that ulcer is filled with heavy neutrophil infiltration; H&E, magnification ×260.

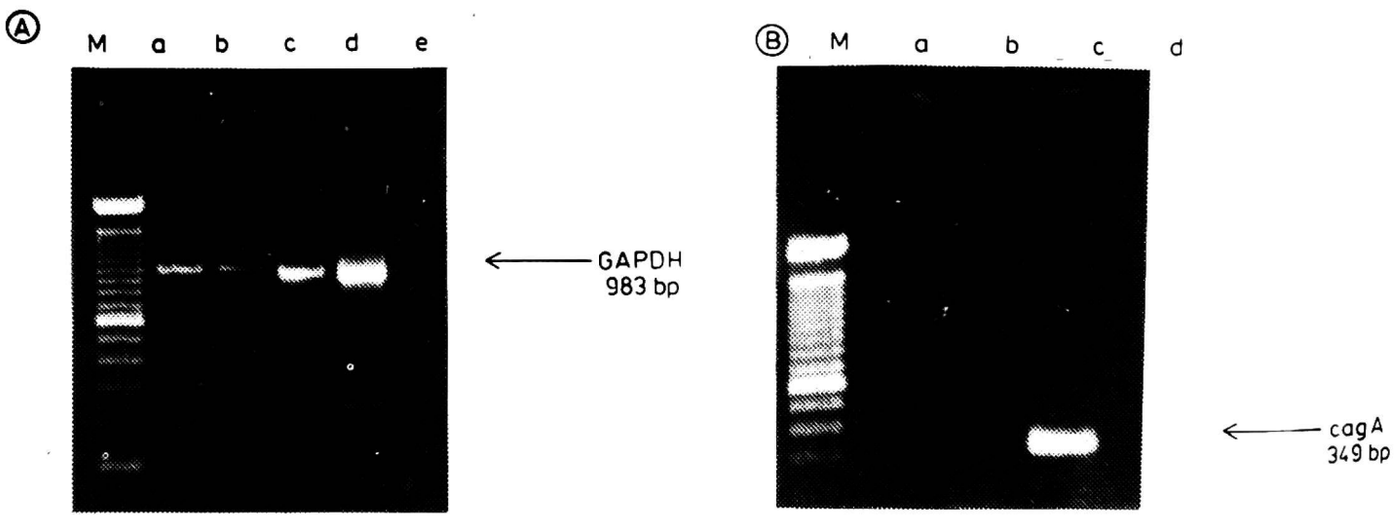


*Fig. 7.* A; Messenger RNA expression for mice GAPDH with RT-PCR in gastric mucosa of mice inoculated with vehicle (lane a) or with type II Hp strain (CagA−, VacA−) (lane b) or with type I Hp strain (CagA+, VacA+) (lane c). Lane d-positive control, lane e-negative control (water), M —size marker DNA, Arrow-expected PCR product (bp).

B; Messenger RNA expression for CagA with RT-PCR in gastric mucosa of mice inoculated with vehicle (lane a) or with type II Hp strain (CagA−, VacA−) (lane b) or with type I Hp strain (CagA+, VacA+) (lane c). Lane d- negative control (water), M-size marker DNA, Arrow-expected PCR product (bp).

undetected either in vehicle-treated mice or in those inoculated with type II Hp strain as measured 7 days after Hp inoculation (*Fig. 7B*). On the contrary, strong signal for CagA mRNA expression was detected in the gastric mucosa of mice inoculated with type I Hp strain (*Fig. 7B*).

## DISCUSSION

This study confirms (18-21) that the gastric mucosa of conventional BALBc mice can be successfully colonized by toxigenic Hp strain as determined by the rapid urease test, the Hp culture and the histological examination of gastric mucosa using the Warthin-Starry staining. The major finding of this study is that Hp strain expressing cagA and vacA encoded cytotoxins, delayed significantly the healing of chronic gastric ulcers and raised significantly plasma gastrin concentration while decreasing luminal release of somatostatin, similarly as in Hp infected human stomach (26).

The hipergastrinemia in Hp infected animals could be attributed to the reduction in gastric acidity and/or an impaired feedback inhibition of gastrin release by paracrine somatostatin. The deleterious effect of Hp on ulcer healing could be attributed, at least in part, to the mucosal inflammation and a significant fall in the GBF in the ulcer margin possibly induced by Hp-related cytokines. This suggests that the reduction of gastric microcirculation in the ulcer area might contribute to the delay in ulcer healing observed in the Hp-infected mice.

Hp appears to be the most frequent cause of gastritis in man (29), and was also implicated in the pathogenesis of gastric and duodenal ulcers, gastric lymphomas and gastric cancer (30). The mechanism of this action is not fully understood but Hp cytotoxins such as 94 kDa vacuolating toxins encoded by the gene vagA, and a 120—128 kDa protein encoded by the gene cagA were proposed to explain the deleterious action of Hp on the stomach (31). The gene encoding cytotoxins has been cloned and called cytotoxin associated gene A (32), because it is to contribute to the severity of inflammation in the gastric mucosa of humans.

Previous studies (17, 33) revealed that the most prevalent Hp strain in clinical isolates is that expressing both cagA and vacA genes. The role of toxigenic Hp strains in the pathogenesis of peptic ulcer disease has not been fully explained but, as shown in previous studies, over 60% of Hp strains produce toxins causing vacuolization of the cell and tissue damage (30—33).

Using such toxigenic Hp strain to inoculate the mouse stomach, we demonstrated for the first time that the ulcer healing was significantly delayed in these animals when compared to the healing in vehicle-treated mice. This Hp inoculation of animals was accompanied by gradual increase in mucosal damage first involving surface epithelium and then resulting after 28 days in

severe inflammatory infiltration of the mucosa. In all of these animals, the ulcerations were present even after 28 days and the quality of ulcer healing was poor as indicated by impaired restoration of glandular structure and the presence of neutrophil infiltration.

One striking difference between patients infected with toxigenic and non-toxigenic Hp strains was that those who were infected with Hp expressing cagA product showed greater degeneration of the surface epithelium and a denser neutrophil infiltration and finally were more likely to develop peptic ulcers (3, 4, 30). Our finding in mice that delay in healing induced by toxigenic Hp strain was accompanied by initially mild inflammatory changes followed later on by more severe inflammatory reaction in the gastric mucosa, suggests that neutrophils or their associated factors such as free oxygen species may be primary mediators of the impaired ulcer healing observed in these animals. This notion is in keeping with recent observation of Telford *et al* (36) that oral administration of Hp-expressing cytotoxin to mice caused initially only the vacuolization of epithelial cells and mucosal erosion without inflammation. Marchetti *et al* (17) showed prominent gastritis in mice at 4 and 8 weeks postinfection with Hp. In study by Lee *et al.* (37) chronic gastritis in mice had developed in a period of 6—8 month postinfection indicating that they do not exactly resemble human Hp infection (37).

Our study supports the notion that conventional mouse is an useful model for Hp infection and would be helpful to clarify the pathogenesis of this infection, especially in the early stage of bacteria colonization, at the same time, serving as a suitable animal model to study an association of Hp and ulcer healing in humans.

It is of interest that almost immediately after Hp infection the gastric acid and pepsin secretion was markedly suppressed and this was accompanied by the rise in the serum gastrin level and the fall in gastric release of somatostatin. Specific inhibition of gastric acid secretion is believed to facilitate acute Hp infection, and might explain the transient hypochlorhydria observed in individuals infected with Hp (3). It is of interest that such an inhibition of gastric secretion in mice infected with toxigenic Hp strain, was observed throughout 4 weeks of observation period upon the Hp inoculation. The observation that Hp strains inhibited gastric secretion is also in keeping with previous observations in humans documenting that successful Hp inoculation resulted in acute gastric infection that continued for several weeks and resulted in achlorhydria possibly due to acute gastritis. The restoration of gastric secretion occurred after the eradication of the bacteria (3, 38). The mechanism of this inhibition of gastric secretion remains to be tested but this could be due to the release of acid-suppressing factors such as lipopolysaccharides or by inflammatory cytokines as proposed recently (28, 39, 40). It is also not excluded that ammonia generated locally by Hp, could also contribute to the damage of

parietal cells and the suppression of gastric secretory response while raising plasma gastrin level as observed previously (25, 41) and found in our present study. An alternative explanation could be that the infection with toxigenic Hp strain attenuated gastric release of somatostatin (26) that is known to inhibit *via* paracrine pathway the G-cells producing gastrin. This alteration in luminal somatostatin release could be also explanatory for the impairment of the control of gastrin release during gastric infection with Hp strains with subsequent rise in serum gastrin level as observed in our study.

## REFERENCES


1. Blaser MJ. Hypotheses on the pathogenesis and natural history of *Helicobacter pylori*-induced inflammation. *Gastroenterology* 1992; 102: 720—727.
2. Graham DY, Go MF. *Helicobacter pylori*: current status. *Gastroenterology* 1993; 105: 279—282.
3. Konturek PC, Konturek SJ, Konturek JW. Gastric secretion and the pathogenesis of peptic ulcer in the *Helicobacter pylori* infection. *J Physiol Pharmacol* 1996; 47: 5—12.
4. Crabtree JE, Figura N, Taylor JD, Bugnoli M, Armellini D, Tompkins DS. Expression of 120 kilodalton protein and cytotoxicity in *Helicobacter pylori*. *J Clin Pathol* 1992; 45: 733—734.
5. Murakami M, Saita H, Teramura S *et al.* Gastric ammonia has a potent ulcerogenic action on the rat stomach. *Gastroenterology* 1993; 105: 1710—1715.
6. Megraud F, Neman-Simha SV, Brugmann D. Further evidence of toxic effect of ammonia produced by *Helicobacter pylori* urease in human epithelial cells. *Infect Immun* 1992; 60: 1858—1863.
7. Figura N, Crabtree IE. *Helicobacter pylori* vacuolating toxin. In: *Helicobacter pylori*: Basic Mechanism to Clinical Cure, eds. Hunt, 1994.
8. Suzuki M, Miura S, Suematsu M *et al. Helicobacter pylori* elicits gastric mucosal cell damage associated with neutrophil-derived toxic oxidants. *Eur J Gastroenterol Hepatol* 1993, 5 (Suppl 1): S35—S39.
9. Kurose I, Granger DN, Evans DJ *et al. Helicobacter pylori*-induced microvascular protein leakage in rats: role of neutrophils, mast cells and platelets. *Gastroenterology* 1994, 107, 70—79.
10. Tsujii M, Kawano S, Tsuji S, Fusamoto H, Kamada T, Sato N. Mechanism of gastric mucosal damage induced by ammonia. *Gastroenterology* 1992; 102: 1881—1888.
11. Wallace JL. Possible mechanisms and mediators of gastritis associated with *Helicobacter pylori* infection. *Scand J Gastroenterol* 1991; 26 (Suppl 187): 65—70.
12. Engstrand L, Gustavsson S, Jorgensen A, Schwan A, Scheynius A. Inoculation of barrier-born pigs with *Helicobacter pylori*: a useful animal model for gastritis type B. *Infect Immun* 1990; 58: 1763—1768.
13. Eaton KA, Morgan DR, Krakowka S. Persistence of *H. pylori* in conventionalized piglets. *J Infect Dis* 1990; 161: 1299—1301.
14. Fox JG, Correa P, Taylor NS *et al. Helicobacter mustelae-associated* gastritis in ferrets. An animal model of *Helicobacter pylori* gastritis in humans. *Gastroenterology* 1990; 99: 352—361.
15. Euler AR, Zurenko GE, Moe JB, Ulrich RG, Yagi Y. Evaluation of two monkey species (Macaca mulatta and Macaca fascicularis) as possible models for human *Helicobacter pylori* disease. *J Clin Microbiol* 1990; 28: 2285—2290.

16. Dubois A, Fiala N, Heman Ackah LM *et al.* Natural gastric infection with *Helicobacter pylori* in monkeys a model for spiral bacteria infection in humans. *Gastroenterology* 1994; 106: 1405—1417.
17. Marchetti M, Arico B, Burroni D, Figura N, Rappuoli R, Ghiara P. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science* 1995; 267: 1655—1658.
18. Lee A, Fox JG, Otto G, Murphy J. A small animal model of human *Helicobacter pylori* active chronic gastritis. *Gastroenterology* 1990; 99: 1315—1323.
19. Dick-Hegedus E, Lee A. Use of a mouse model to examine anti-*H. pylori* agents. *Scand J Gastroenterol* 1991; 26: 909—915.
20. Karita M, Li Q, Canfero D, Okita K. Establishment of a small animal model for human *Helicobacter pylori* infection using germ-free mouse. *Am J Gastroenterol* 1994; 89: 208—213.
21. Brzozowski T, Konturek SJ, Sliwowski Z, Pytko-Polonczyk J, Szlachcic A, Drozdowicz D. Role of capsaicin-sensitive sensory nerves in gastroprotection against acid-independent and acid-dependent ulcerogens. *Digestion* 1996; 57: 424—432.
22. Konturek SJ, Radecki T, Piastucki I, Drozdowicz D. Studies on the gastroprotective and ulcer healing effect of colloidal bismuth subcitrate. *Digestion* 1987; 37 (Suppl 2): 8—15.
23. Okabe S, Roth JLA, Pfeiffer CJ. A method for experimental penetrating gastric and duodenal ulcers in rats. *Am J Dig Dis* 1971; 16: 277—284.
24. Brzozowski T, Konturek PC, Konturek SJ, Ernst H, Stachura J, Hahn EG. Gastric adaptation to injury by repeated doses of aspirin strengthens mucosal defense against subsequent exposure to various strong irritants in rats. *Gut* 1995; 37: 749—757.
25. Brzozowski T, Konturek PC, Konturek SJ, Ernst H, Sliwowski Z, Hahn EG. Mucosal irritation, adaptive cytoprotection and adaptation to topical ammonia in the rat stomach. *Scand J Gastroenterol* 1996; 31: 837—846.
26. Konturek JW, Bielanski W, Konturek SJ, Domschke W. Eradication of *Helicobacter pylori* and gastrin-somatostatin link in duodenal ulcer patients. *J Physiol Pharmacol* 1996; 47: 161—175.
27. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinum thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156—159.
28. Peek RM, Miller GG, Tham KT *et al.* Heightened inflammatory response and cytokine expression in vivo to CagA *Helicobacter pylori* strains. *Lab Invest* 1995; 73: 760—770.
29. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; 1: 1311—1315.
30. Peterson WL. *Helicobacter pylori* and peptic ulcer disease. *N Engl J Med* 1991; 324: 1043—1048.
31. Cover TL, Dooley CP, Blaser MJ. Characterization of and human serologic response to proteins in *Helicobacter pylori* broth culture supernatants with vacuolizing cytotoxin activity. *Infect Immun* 1990; 58: 603—610.
32. Tummuru MKR, Cover TL, Blaser MJ. Cloning and expression of a high molecular weight major antigen of *Helicobacter pylori*; evidence of linkage to cytotoxin production. *Infect Immun* 1993; 61: 1799—1809.
33. Ghiara P, Marchetti M, Blaser MJ *et al.* Role of Hp virulence factors, vacuolating cytotoxin, CagA and urease in a mouse model of disease. *Infect Immun* 1995; 63: 4154—4160.
34. Karita M, Kouchiyama T, Okita K, Nakazawa T. New small animal model for human gastric Hp infection: success in both nude and euthymic mice. *Am J Gastroenterol* 1991; 86: 1596—603.
35. Graham DY, Go MF, Evans DJ Jr. Review article: urease gastric ammonium/ammonia, and *Helicobacter pylori* — the past, the present and recommendations for future research. *Aliment Pharmacol Ther* 1992; 6: 659—669.
36. Telford JL, Ghiara P, Dell'Orco M *et al.* Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J Exp Med* 1994; 179: 1653—1658.

37. Lee A, O'Rourke J, De Ungria MC, Robertson B, Daskalopoulos G, Dixon MF. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* 1997; 112: 1386—1397.
38. Sipponen P, Hyvarinen H, Siurala M. *H. pylori* corpus gastritis — relation to acid output. *J Physiol Pharmacol* 1996; 47: 151—159.
39. Michetti P, Corthesy-Theulaz I, Davin C *et al.* Immunization of BALB/c mice against *Helicobacter* felis infection with Hp urease. *Gastroenterology* 1994; 107: 1002—1011.
40. Crabtree JE, Shallcross TM, Heatley RV, Wyatt JI. Mucosal tumor necrosis factorαand interluekin-6 in patients with *Helicobacter pylori* associated gastritis. *Gut* 1991; 32: 1473—1477.
41. Dial EJ, Hall LR, Romero JJ, Lichtenberger LM. Rats with gastritis have increased sensitivity to the gastrin stimulatory effects of luminal ammonia. *Gastroenterology* 1996; 110: 801—808.

Received: July 3, 1998
Accepted: July 9, 1998

Autor's address: T. Brzozowski Department of Physiology, University School of Medicine, ul. Grzegórzecka 16, 31-531 Krakow, Poland