BACTERIAL PATHOGENICITY

Increased gastric emptying induced by *Helicobacter heilmannii* type 1 infection in rats

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The association between *Helicobacter pylori* infection and gastric motility abnormalities is still controversial, partly because of the lack of an appropriate animal model. *H. heilmannii* type 1 (Hh1), a spiral bacterium that infects the stomach of both man and pigs, easily colonises and induces an inflammatory response in the gastric mucosa of rodents. For these reasons, the present study investigated the relationship between gastric motility in rats experimentally infected with Hh1 and correlated the results with serum gastrin and gastric somatostatin concentrations, as these hormones seem to be involved in gastric motility. Ten rats were inoculated with gastric mucus from an Hh1-positive pig and 10 animals with gastric mucus from an Hh1-negative pig (control group). After 56 days, gastric emptying was studied *in vivo* by scintigraphy. The animals were then killed, blood samples were collected for serum gastrin measurement, strips of the gastric wall were obtained for an *in-vitro* motor study and fragments of the gastric antrum were obtained for somatostatin content evaluation, Hh1 diagnosis and histological study. There was a significant increase in gastric emptying in the test group compared with the controls as demonstrated by the *in-vivo* and *in-vitro* studies. Serum gastrin levels were significantly higher and somatostatin levels were lower in the test group than in the controls. In addition, infected animals showed evidence of gastritis on histological examination. Gastric motility is altered in rats infected with Hh1, a fact possibly related to concurrent abnormalities of gastrin and somatostatin secretion.

Introduction

Helicobacter pylori is the most important cause of gastritis in man and has been associated with peptic ulcer disease and gastric cancer [1, 2]. Several factors have been implicated in the pathogenesis of the diseases associated with the micro-organism, such as a decrease in antral somatostatin content and an increase in serum and antral gastrin concentrations observed in *H. pylori*-infected patients [3, 4]. The mechanism by which *H. pylori* changes gastric hormone metabolism has been only partially elucidated. Possible explanations include alkalinisation of the micro-environment as a result of production of ammonia from urea by *H. pylori* urease, other metabolic noxious products of the bacterium and inflamma-

tion of the antral mucosa induced by the infection that may stimulate G cells or inhibit D cells [5, 6].

These hormonal changes may disrupt the complex gastric balance and may affect gastrointestinal motility, as it has been demonstrated that gastrin is a potent motility agonist acting on gastrointestinal smooth muscles, whereas somatostatin has an opposite effect on these muscles [7, 8]. However, the association between *H. pylori* infection and alterations in gastric motility is still controversial [9, 10]. Studies on this subject are scarce, partly because of the lack of an appropriate animal model.

Swine are frequently colonised by a spiral bacterium that was recently shown to belong to the *Helicobacter* genus by 16S ribosomal RNA sequencing and corresponds to *H. heilmannii* type 1 (Hh1), a potential human pathogen [11, 12]. These spiral bacteria have been observed in the gastric mucosa of patients with gastric symptoms and presenting with histologically

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proven gastritis [13]. The bacterium also seems to be a pathogen in swine, as it is associated with gastritis and peptic ulcer of the pars oesophagea of the pig stomach [14].

Hh1 easily colonises and induces an inflammatory response in the gastric mucosa of infected rodents [15, 16], thus providing an experimental model that permits the study of the relationship between gastric spiral bacterium infection and gastrointestinal motility. Therefore, the aim of this study was to evaluate the motor response of the oesophagus and stomach, serum gastrin and tissue somatostatin concentrations, and histopathological alterations in rats experimentally infected with Hh1.

Materials and methods

Animals

This work was performed according to international guidelines for animal laboratory care. Twenty 2–4-month-old female Wistar rats with free access to water and a commercial pellet diet were used in the study.

Inocula

Mucus was obtained from the gastric antral mucosa of Hh1-positive and -negative pigs. Mucus from an Hh1-positive pig was used only when a large number of tightly spiralled micro-organisms were observed on carbol fuchsin-stained smears. Mucus was considered negative when carbol fuchsin staining, urease test and PCR for Hh1 were negative. One part of the mucus was homogenised in three parts of saline 0.85% in a vortex blender and then used for rat inoculation. Twenty rats lightly anaesthetised with ether were inoculated *via* a stomach tube with 0.2 ml of the mucus homogenate as follows: 10 rats with mucus containing Hh1 (test group) and 10 with mucus containing no Hh1 (controls). All rats were maintained under the same conditions in separate cages.

In-vivo motor study [17]

Fifty-six days after inoculation, the animals were fasted overnight and maintained individually in metabolic cages. They were then anaesthetised with ketamine chloride. Peptone broth (100 μ l) mixed with 100 μ l of ^{99m}Tc phytate (18.5 MBq/ml) was administered by oesophageal cannulation.

Data were collected at one frame each 20 s for 10 min in a Siemens Orbiter gamma-camera/Microdelta, fitted with a general purpose collimator. The images were obtained immediately after the radioisotope administration. Fifteen images of the oesophagus and stomach of each animal were obtained during 10 min, at 40-s intervals. The oesophageal area was determined in the first and last images. The radioactivity in the first

image corresponded to 100% and the radioactivity in the last image (Remnant index) was determined by means of proportional calculation; this corresponded to the radioactive material that persisted inside the oesophagus after the end of the study (oesophageal remnant), according to the formula:

$$\text{ER} = \frac{\text{EL} \times 100}{\text{EF}}$$

ER = oesophageal remnant; EF = Radioactive count in first image; EL = Radioactive count in last image.

The gastric area was determined by the sum of the oesophageal and gastric areas in the same images, and the radioactivity counted in the first and last images. The total count of the first image was considered to be 100%, and the proportional value was calculated in the last image, according to the formula:

$$\text{GE} = \frac{100 \times (\text{SF} - \text{SL})}{\text{SF}}$$

GE = Gastric emptying; SF = Radioactive count in first image; SL = Radioactive count in last image.

The difference in proportional value between the first and last images was considered to be the gastric emptying index, i.e., the amount of radioactive material that was left in the stomach at the end of the study.

In-vitro motor study [18]

After the in-vivo motor study, the animals were anaesthetised with ketamine chloride and a midline laparotomy was performed. Blood samples were collected by puncture of the vena cava and the animals were then killed by aortic section.

The stomachs were separated from the rest of the alimentary tract, opened longitudinally and thoroughly washed with saline 0.85%. Strips of 3×0.5 cm in length of the gastric wall taken along the greater curvature of the stomachs from the corpus to the antrum were obtained from each animal for the in-vitro motor study. Each tissue was used only once for each group. The fragments were maintained in a nutrient solution containing NaCl 17.2 g, KCl 0.43 g, CaCl_2 0.43 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.215 g, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.1075 g, NaHCO_3 2.15 g and glucose 2.15 g in 2.150 ml of water.

The agonist used in this assay was acetylcholine hydrochloride (Sigma). The method used for study was the construction of dose–response curves to cumulative acetylcholine doses. Successive concentrations of the agonist increasing on a logarithmic scale were added to the perfusion bath containing 10 ml of nutrient solution and the maximal contractile response was recorded after each agonist dose. The next dose was then added without washing the preparation. Thus, a staggered dose–response curve was obtained. The

first dose capable of inducing a response was considered to be the threshold dose (TD). The greatest contractile amplitude obtained in response to the agonist was the maximal contraction (MC). The affinity of the muscle fibre for the agonist was calculated on the basis of the maximal contraction (pD₂). The values obtained for these parameters for each preparation are reported as the median (ranges).

Bacteriological study

Gastric fragments of the antral and oxyntic mucosa were also obtained and used for carbol fuchsin-stained smears [19], urease test [20], culture and PCR for Hh1. One specimen from each area was smeared on to a glass slide, heat-fixed, stained with carbol fuchsin and examined by oil immersion microscopy for the presence of spiral bacteria. The specimen for the urease test was inoculated into Christensen's urea 2% agar and examined within 24 h. Specimens for culture were plated on to Belo Horizonte medium [21] and Skirrow's medium and incubated under micro-aerobic conditions at 37°C for up to 10 days. Fragments for PCR were held at -80°C until tested.

PCR

Dissected tissue samples were thawed and suspended in a tube containing 1 ml of PBS. They were centrifuged twice in PBS at 13 000 *g* for 10 min and crude DNA was extracted from the resulting pellet as described by Fox *et al.* [22]. Briefly, the pellet was digested with lysozyme, proteinase K and other enzymes, and then the DNA was precipitated with hexadecyltrimethylammonium bromide (CTAB). After phenol-chloroform extraction, the DNA was precipitated with ethanol. The pellets were suspended in 50 µl of sterile distilled water and DNA was quantified by measuring the optical density at 260 nm.

The PCR was performed in a final volume of 50 µl containing Taq DNA polymerase, 25 pmol of each primer, 1.5 mM MgCl₂, 200 µM deoxyribonucleotides (dGTP, dATP, dTTP and dCTP) and 1× reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl). Amplification cycles were performed in a DNA thermal cycler (PTC-100™, MJ Research Inc.) as follows: denaturation at 94°C for 1 min, annealing at 65°C for 1 min, extension at 72°C for 1 min (30 cycles) and a final extension at 72°C for 10 min. PCR-amplified products were electrophoresed in agarose 1% gel, stained with ethidium bromide and examined under UV light.

The 16S rRNA cistrons were amplified with primers FH1 and FH2 (GenBank accession no. L10079) which were chosen on the basis of the 16S rRNA gene sequence of *H. heilmannii* [23].

Hormonal study

Blood samples obtained by puncturing the vena cava were used for serum gastrin determination and serum samples were frozen immediately and stored at -20°C until assayed.

Tissue samples of gastric antrum from the lesser curvature were obtained for the somatostatin assay. These fragments were frozen immediately and stored in liquid nitrogen until processed. Hormone was extracted, frozen immediately and lyophilised.

Somatostatin extraction from gastric mucosa. Only specimens weighing >0.01 g were used for somatostatin assay. Somatostatin was extracted from the antral mucosa by the method of Bryant and Bloom [24]. Capped 1.5-ml Eppendorf tubes were filled with 1.0 ml of distilled water, placed in a vigorously boiling water bath and left there for 20 min for temperature stabilisation. The tissue was weighed while thawing and transferred to the tube. Glacial acetic acid (30 µl) was added to each tube and mixed. After 15 min in the water bath, two 300-µl samples for somatostatin determination were removed, frozen immediately and lyophilised.

Somatostatin assay. Somatostatin concentration was measured by radioimmunoassay at the Royal Postgraduate Medical School, London, by a slight modification of the method of Williams *et al.* [25]. Briefly, the samples were reconstituted to their original volume with 0.5 M acetic acid and assayed in duplicate at various dilutions. Antiserum was raised in white rabbits immunised with synthetic somatostatin-14 and used at a final dilution of 1 in 80 000. The antiserum cross-reacted fully with somatostatin-28 and showed no cross-reactivity with any known brain regulatory peptides. The assay used iodinated synthetic (Tyr11) somatostatin purified by high-performance liquid chromatography and was done in 60 mM sodium phosphate buffer, pH 7.4, containing 10 mM ethylenediamine-tetraacetic acid, 7 mM sodium azide and bovine serum albumin 0.3% w/v, with incubation at 4°C for 4 days. The bound and free labels were separated after the addition of 6 mg of dextran-coated charcoal (BDH; Norit GSX) in assay buffer containing gelatin 0.25% and centrifugation for 20 min at 1600 *g*. Percentage binding was calculated from the counts from the bound and free labels obtained with a gamma-counter. The assay could detect up to 1 fmol difference between adjacent tubes with a 95% confidence limit.

Gastrin assay. Gastrin was assayed by a double-antibody ¹²⁵Iodine radioimmunoassay (Diagnostic Products, Los Angeles, CA, USA). This assay system uses an antibody that can detect active gastrin (G-17 and G-34), has very low cross-reactivity with other naturally occurring compounds and has a detection limit of

c. 12 pg/ml. The assays were done in duplicate during a single session after all samples had been collected.

The values obtained for gastrin and somatostatin concentrations are reported as median (ranges).

Histopathological study

Specimens of the oxyntic and antral mucosa of the stomach were taken from each animal for histopathological examination. The fragments were fixed in Bouin's fluid for 18–24 h, dehydrated in an alcohol-xylene series and embedded in paraffin. Sections (4 mm thick) were stained with haematoxylin and eosin for histological examination. The degree of inflammatory infiltrate was scored as mild, moderate or intense.

Statistical analysis

The two-tailed χ^2 test (histopathological study and microbiological study) and Kruskal-Wallis test (TD, MC, pD2, oesophageal remnant, gastric emptying, antral somatostatin content and serum gastrin levels) were used for statistical analysis. Differences were taken as significant when $p < 0.05$.

Results

Bacteriological study

The organism was present in the antral and oxyntic mucosa of all test rats by observation of smears and PCR. The infection was restricted to the stomach in all animals except four, in which small numbers of bacteria were also observed in the duodenum. The pre-formed urease test was also positive (within 3 h) in all test animals. Hh1 was not found in the antral or oxyntic mucosa of any control animal. Spiral bacteria morphologically resembling *H. muridarum* and *H. trogonum* were not observed in smears and were not isolated from the gastric mucosa of any animal.

In-vivo motor study

No significant differences were observed between the test and control groups with respect to the oesophageal remnant, as shown in Fig. 1 (median 56.5%, range 12.2–99.3% in test group *versus* 89.9%, range 24.0–104% in control group, $p = 0.06$), but a significant increase in gastric emptying was observed in Hh1-positive animals when compared with the control group (median 44.2%, range 30.9–70.8% in control group *versus* 67.1%, range 45.1–99.9% in test group), as shown in Figs. 2 and 3.

In-vitro motor study

Greater gastric contraction was measured in the test group than in the control animals ($p = 0.001$). With

Fig. 1. Oesophageal remnant (%) in rats negative and positive for Hh1.

Fig. 2. Gastric emptying (%) in rats negative and positive for Hh1.

regard to the TD and affinity of the muscle receptor for the agonist (pD2), no differences were observed between control and test groups (Table 1).

Hormonal study

The serum gastrin level was significantly higher in the infected animals than in the non-infected animals (median 30.0 pg/ml, range 20.9–84.1 pg/ml in control

Fig. 3. Gastric emptying scintigraphy in an Hh1-negative and an Hh1-positive rat. **1**, first image; **15**, last image; each sequence of three images (1–3, 4–6, etc.) is related to 2 min of study: 1–3, 2 min; 1–6, 4 min; 1–9, 6 min; 1–12, 8 min; 1–15, 10 min.

Table 1. Threshold dose (TD), maximal contraction (MC) and affinity (pD2) in muscle strips obtained from the stomach of Hh1-positive and -negative rats

Test animal	Median (range) results		
	TD (10^{-5})	MC (mm)	pD2
Hh1-negative	0.2 (0.01–35.0)	3.2 (0.1–9.6)	2.9 (0.1–8.6)
Hh1-positive	0.01 (0.001–1.0)	7.8 (0.1–18.5)	3.1 (1.3–4.3)
	p = 0.1	p = 0.001	p = 0.9

group *versus* 64.3 pg/ml, range 26.7–197.5 pg/ml in test group, $p = 0.03$). The antral somatostatin content was significantly lower in the test group than in the control group (median 3.2 ng/mg, range 1.5–9.1 ng/mg in control group *versus* 1.0 ng/mg, range 0.5–2.6 ng/mg in test group, $p = 0.003$).

Histological study

Variable degrees of antral gastritis were observed in the infected animals, ranging from moderate to intense mononuclear and polymorphonuclear cell infiltration, mainly in the lower half of the antral mucosa, but extending through the gastric glands in three animals. The intensity of the inflammatory infiltrate was moderate in four animals and intense in six. Lymphocyte aggregates were observed in four animals. Three

bacterium-positive rats had a normal oxyntic mucosa, three presented with mild cell infiltration and four had moderate cell infiltration. There were no inflammatory cells in the antral or oxyntic mucosa of most control animals ($p = 0.00001$). In three rats there were only scarce mononuclear cells in the lower half of the gastric mucosa. Small lymphocyte aggregates were observed in one animal.

Discussion

H. pylori is considered to be the major agent of gastritis and an essential factor in the pathogenesis of peptic ulcer [1, 2]. Furthermore, there is increasing evidence that the micro-organism is involved in the genesis of gastric cancer. However, studies concerning the physiological changes caused by *H. pylori* infection have been partly hindered by the scarcity of experimental models that reproduce the major characteristics of human infection in animals. The known hormonal changes induced by the infection may be responsible to a greater or lesser extent for the motor changes of the upper digestive tract, but reports concerning gastric emptying in human subjects with or without *H. pylori* infection are scarce and inconsistent [9, 10, 26–28]. Therefore, the search for an animal model for studies of this type is fully justified. A model has been developed for the study of gastric spiral bacteria infection by using Wistar rats infected with *H. heilmannii* type 1 (Hh1). This bacterium has been

observed in the gastric mucosa of pigs with peptic ulcer of the pars oesophagea and gastritis [14] and in patients with gastric symptoms and histological gastritis [13]. Therefore, in the present investigation Wistar rats were infected with Hh1 to study the motor changes induced by a micro-organism of the genus *Helicobacter*. Histopathological alterations, as well as alterations in the concentrations of gastrin and somatostatin possibly implicated in the motor changes, were also studied.

Among the factors that control digestive motility are smooth muscle cells, intercellular connections and the enteric nervous system [29]. Several extra-intestinal controls should also be mentioned, such as the central and extrinsic nervous system, neurotransmitters and the signals emitted by gastrointestinal hormones [30], with gastrin, acetylcholine, gastrointestinal polypeptide (GIP) and motilin being the major agonists [29].

The method for the evaluation of gastric emptying based on the use of radioactive drugs [17] used in the present study has been considered the gold standard in clinical studies, and continues to be the most feasible and the safest method for the study of gastric emptying in human subjects [31]. The method of proportional calculation obtained from the difference between the radioactivity measured at the beginning and at the end of a given period of time in a previously defined area was adopted in the present study because it is one of the methods used for investigations in man. This method confirms whether gastric emptying is delayed or accelerated, but does not reveal the cause of the abnormality, i.e., antral contraction or transpyloric flow [32], with the consequent need for complementary studies for the identification of the factor(s) involved in these changes. Thus, a motor study was performed *in vitro* to determine the changes in antral smooth muscle contractility that might affect gastric emptying. In the study of *in-vitro* motility through the evaluation of organs isolated in a bath, the peaks of the electric waves corresponded to the muscle contractions in response to stimulation with the agonist. The technique for the use of cumulative acetylcholine doses recommended by Van Rossum and Van Den Brink [33] provides data about the motor function of the loop in terms of receptors and of the contractile status of the fibres, and is well correlated with *in-vivo* motor activity.

The present study evaluated gastric emptying *in vivo* by intragastric infusion of a peptone liquid meal, a stimulus which is believed to provoke gastrin release due to distension, to food collision and to chemical stimulation, and to have a direct stimulatory action on gastric parietal cells [34]. Although it is generally accepted that a test with a solid meal is more adequate than a test with a liquid meal for the detection of abnormalities of gastric emptying [35], examination after a liquid meal is more accurate because it does not

depend on the spontaneous participation of the animal in the ingestion of solid particles.

In the *in-vivo* study, an increase in gastric emptying rate was observed in infected animals, a result that was confirmed by the *in-vitro* study, in which gastric motility was increased in the same group of animals, as shown by an increase in maximal contraction. These changes may be due to alterations of the gastrointestinal hormones gastrin and somatostatin, the former having an agonist action and the latter an antagonist action on digestive motility. Gastrin acts directly on smooth muscle cells, resulting in contraction, probably by stimulating acetylcholine secretion from the nerve endings and by direct stimulation [36]. Somatostatin mainly acts on acid secretion and gastrin secretion, strongly inhibiting gastrin secretion in the basal and postprandial stages and stimulated by the neural route [37]. It also acts by delaying gastric emptying and by reducing the motility of the small intestine [38], perhaps by inhibiting the normal occurrence of the inter-digestive motor cycle and the electric activity of the stomach and small intestine [36], or by directly or indirectly reducing basal acetylcholine secretion [39]. There is also the possibility that the relaxing response of the stomach results from the secretion of endogenous catecholamines or from a direct action of somatostatin on adrenergic receptors [40].

The present study demonstrated for the first time changes in gastrin and somatostatin concentrations induced by a *Helicobacter* species other than *H. pylori*. As observed in human patients infected with *H. pylori* [3, 4, 41], Hh1 induced a significant increase in serum gastrin concentration in rats. The hypergastrinaemia that occurs in *H. pylori*-infected patients may be secondary to a reduction in antral somatostatin levels [3, 4, 41], as observed in the infected animals. Possible explanations for the reduction in somatostatin concentration in the antrum of *H. pylori*-infected individuals include alkalinisation of the environment as a result of the production of ammonia from urea by the action of the potent urease produced by the bacteria. Also, the action of other toxic metabolites of the bacteria may play a role, as well as the inflammatory response of the antral mucosa induced by infection, which may directly or indirectly inhibit the D cells of the gastric antrum [40]. These same mechanisms may also explain the events observed in the present study, as Hh1 is urease positive and infection with the micro-organism progresses to antral gastritis.

The model of *Helicobacter* infection used in the current study is similar to human infection with *H. pylori*, as both *H. pylori* and Hh1 are phylogenetically close, both are urease positive and both are involved in the genesis of gastritis and peptic ulcer. Furthermore, changes in gastrin and somatostatin concentrations are observed in infection with the two agents. However, the present results cannot be fully extrapolated to man. For

example, animals were studied in the early phase of infection and it is not known whether the changes observed in them would be maintained over more prolonged periods of infection. On the other hand, an advantage of the model, which made it possible to observe the changes in motility so clearly, was the fact that all animals had been infected for the same period of time, a condition that would be difficult to evaluate in man, as it would be impossible to determine the length of infection for any given individual.

In conclusion, the present study demonstrated that experimental Hh1 infection of Wistar rats caused alterations in stomach motility characterised by hypermotility. Oesophageal hypermotility was also observed in the test group that was not statistically different from the control group. Among the factors that may separately or jointly contribute to these alterations are changes in gastrin and somatostatin concentrations, and inflammatory changes in the gastric antrum subsequent to infection with the bacteria. However, extensive further studies are needed for a better understanding of the mechanisms responsible for the alteration of gastric physiology observed in infection with microorganisms of the genus *Helicobacter*.

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