Breakdown of gastric mucus in presence of *Helicobacter pylori*

R L Sidebotham, J J Batten, Q N Karim, J Spencer, J H Baron

Abstract

The potential of *Helicobacter pylori* to degrade gastric mucus was examined. Colonies of *H. pylori* cultured from antral mucosal biopsy specimens of patients with non-autoimmune gastritis were washed with sterile saline, passed through a sterilisation filter, and the filtrate examined for urease, protease, and mucolytic activity. The filtrate failed to hydrolyse bovine serum albumin, or to degrade stable mucus glycoprotein structures of high particle weight that had been separated from human gastric mucus on Sepharose 2B. The high particle weight mucus glycoprotein was, however, extensively degraded when incubated with *H. pylori* filtrate (which possessed urease activity) in the presence of 2 M urea, to release fragments of $M_r \sim 2 \times 10^6$. The high particle weight mucus glycoprotein was also broken down to a comparable extent when incubated with Jack bean urease in the presence of 2 M urea, or 1 M ammonium carbonate, or 40 mM carbonate-bicarbonate buffer (pH 8.7), but not when treated with 4 M urea alone, or Jack bean urease alone. These results indicate that the loss of high particle weight mucus glycoprotein in gastric mucus from patients with gastritis and gastric ulcers is unlikely to be due to the mucolytic action of an extra-cellular protease produced by *H. pylori*, but it may result from the destabilising effects of a carbonate-bicarbonate buffer, generated at the mucosal surface when *H. pylori* urease hydrolyses transuded plasma urea.

The proportion of mucus glycoprotein incorporated in stable structures of sufficiently high particle weight, to be excluded from Sepharose 2B gel, is considerably less in gastric mucus from patients with gastric ulcer than from those without ulcers.^{1,2} We have concluded that this structural change, which may be an aetiological factor in peptic ulcer,³ is a result of defective biosynthesis or breakdown at the mucosal surface, because it is not significantly correlated with output or concentration of acid or pepsin.²

A recent report^{4,5} specifically implicated an extra-cellular protease produced by *H. pylori*, a Gram negative bacterium that infects the gastric mucosa in most patients with gastric ulcer.^{6,7} We examined this claim and tested the

hypothesis that decreased high particle weight mucus glycoprotein in mucus from patients with gastric ulcer is a consequence of the urease activity of *H. pylori*.

Methods

Gastric juice was aspirated continuously from healthy volunteers and collected on ice in 10 minute fractions for 90 minutes during intravenous infusion of pentagastrin ($6 \mu\text{g kg/hour}$).⁸ The nine 10 minute fractions of stimulated juice were adjusted to pH 8 within five minutes of the end of the collection period, pooled, homogenised to disperse insoluble mucus, and clarified by centrifugation at $2000 \times g$ for 10 minutes. The juice was concentrated (when necessary) to about 200–300 ml by evaporation in a vacuum at 40°C and dialysed for 72 hours against three lots of 5 litres of deionised water at 4°C to remove salts, peptides, amino acids and phenol red marker dye.⁸ The non-dialysable material, consisting of mucus, pepsin, and plasma macromolecules, was then lyophilised. Non-dialysable material (400 mg) was dispersed in 0.01 M NaCl (40 ml) and the high particle weight mucus glycoprotein separated from lower molecular weight mucus glycoprotein structures of smaller size, pepsin, and plasma macromolecules by chromatography on a column ($100 \times 5 \text{ cm}$) of Sepharose 2B eluted with 0.01 M NaCl plus 0.02% sodium azide. The separation was monitored by quantitative reaction of fractions (each 10 ml) with phenol-sulphuric acid reagent.⁹ Fractions containing the high particle weight mucus glycoprotein, which eluted in the void volume (V_0) of the column, were combined, concentrated (as described) until the neutral hexose content was 2 mg/ml (measured with phenol-sulphuric acid), and stored at 4°C . Yields were equivalent to 50 mg mucus glycoprotein. The high particle weight mucus glycoprotein was not purified further, to avoid the possibility of structural breakdown.

H. pylori cultured from antral mucosal biopsy specimens obtained from patients with type B (non-autoimmune) gastritis, were initially cultured on 7% blood agar, made selective by the incorporation of IsoVitalax (1%), vancomycin (6 mg/l), nalidixic acid (20 mg/l) and amphotericin (2 mg/l). The bacteria were incubated in a microaerophilic atmosphere, established with the use of an Oxoid gas generating kit (No BR 60), at 37°C for three days.

Isolates were subcultured on 7% non-selective blood agar under identical conditions

Department of Surgery,
Royal Postgraduate Medical School,
Hammersmith Hospital, Du Cane Road, London W12 0NN
R L Sidebotham
J J Batten
J Spencer
J H Baron

Department of Bacteriology,
St Mary's Hospital Medical School, London
Q N Karim

Correspondence to:
Dr J H Baron

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before the plates were gently washed with sterile saline (5 ml a plate) and the wash solution centrifuged ($2000 \times g/10$ minutes) and passed successively through Millipore sterilisation filters (0.45 and $0.2 \mu\text{m}$) to retain the bacteria. The filtrate was stored at 4°C with sterility confirmed by culture. The protein content of the *H. pylori* filtrate was determined by the Lowry method, as modified¹⁰ with bovine serum albumin as standard.

N,N dimethylated¹¹ bovine serum albumin (5 mg) was incubated for 20 hours at 37°C in 0.1 M TRIS-HCl buffer containing 5 mM calcium chloride, pH 7.8 (10 ml), with (i) *H. pylori* filtrate (1 ml), (ii) pronase (BDH 70 000) proteinase activity unit of kakem (units/g; $125 \mu\text{g}$) as a control, and (iii) boiled filtrate or boiled pronase, serving as reagent blanks. Aliquots ($500 \mu\text{l}$) were removed from these mixtures at suitable intervals, and proteolytic breakdown monitored by measuring (a) released α amino residues with ninhydrin,¹² and (b) low molecular weight peptides as described,¹⁰ after removal of undigested protein from the aliquot by precipitation with trichloroacetic acid (2% w/v).

Gel electrophoresis of (i) *H. pylori* filtrate, (ii) bovine serum albumin (BSA), and (iii) BSA after incubation with *H. pylori* filtrate for 20 hours at 37°C (as described) was performed according to the method of Laemmli.¹³ Samples (10 – $200 \mu\text{g}$ protein per lane) were preincubated in 1% sodium dodecyl sulphate and M mercaptoethanol, and separated at a constant current (80 mA) for 12 hours. Gels were stained for protein with polyacrylamide gel electrophoresis Blue G-90 (0.04% w/v) in perchloric acid (3.5% w/v), and diffusion destained in 2.5% aqueous methanol containing 7.5% acetic acid.

The urease activity of the *H. pylori* filtrate was determined by measuring the release of ammonia¹⁴ from urea substrate at 37°C in the presence of 0.085 M phosphate buffer, pH 8 . Mean urease activity was $2 \mu\text{M}$ units/ml of filtrate (when $1 \mu\text{M}$ unit catalyses the hydrolysis of $0.5 \mu\text{M}$ urea/minute at 37°C , pH 8), and was not greatly affected by storage at 4°C for two weeks.

Gastric mucosal pH (≤ 7.4) should rise in *H. pylori* infection as transuded plasma urea (between 5 – 10 mM) is hydrolysed by the bacterial urease in presence of mucosal/transuded bicarbonate (between 20 – 30 mM). The following experiment was designed to assess how hydrolysis of urea might affect mucosal pH in the vicinity of the bacterium.

Changes in pH when urea-bicarbonate solutions are incubated with *H. pylori* filtrate

Incubation time (hours)	pH		
	5 mM urea–30 mM bicarbonate solution	10 mM urea–30 mM bicarbonate solution	
0	7.4	7.4	7.4*
1.5	8.3	8.5	7.8
3	8.7	8.8	7.9
5	8.8	9.1	8.1
24	8.7	9.0	8.1

**H. pylori* filtrate omitted in control.

Sodium bicarbonate solution (30 mM; 10 ml) was gassed to pH 7.4 with carbon dioxide, and sufficient urea was added to make the solution either 5 mM or 10 mM with respect to the urea. The urea-bicarbonate solution was then incubated with *H. pylori* filtrate ($250 \mu\text{l}$; $7 \mu\text{M}$ urease units/ml: sufficient enzyme to hydrolyse all the urea in the 10 mM solution in two hours at the optimal reaction rate) in a sealed drum phial at 37°C , and the pH recorded after one and a half, three, five and 24 hours. The results are summarised in the table.

High particle weight mucus glycoprotein (V_0 concentrate from the Sepharose 2B column; 1 ml) was incubated for 20 hours at 37°C (i) alone, and (ii) in the presence of the following: (a) *H. pylori* filtrate ($200 \mu\text{l}$); (b) *H. pylori* filtrate plus 2 M urea; (c) Jack bean urease (BDH 95 000 μM units/g; $100 \mu\text{g}$); (d) Jack bean urease plus 2 M urea; (e) 4 M urea; (f) 1 M ammonium carbonate; (g) a buffer (pH 8.7), consisting of 10 mM ammonium carbonate plus 30 mM NaHCO_3 ; and (h) Sigma porcine pepsin (2% w/w). The volume of each solution was adjusted to 2.5 ml before incubation, by addition of an appropriate volume of buffer or deionised water. This reduced the concentration of NaCl (from the Sepharose 2B column eluant) to about 20 mM. Breakdown of the high particle weight mucus glycoprotein was assessed by quantitative reaction with phenol-sulphuric acid⁹ after separation of high particle weight mucus glycoprotein (V_0) low molecular weight structures of smaller size on a column (1×70 cm) of Sepharose 2B eluted with 0.05 M NaCl. The column was calibrated with "highly polymerised" calf thymus DNA

Figure 1 Chromatography on Sepharose 2B (1×70 cm column eluted with 0.05 M NaCl) of porcine gastric mucus before (—) and after (----) proteolysis (Sigma pronase 2% w/w in 0.1 M TRIS-HCl buffer; pH 7.8 at 37°C for 24 hours) with reduction (dithiothreitol 2% w/w for four hours). Void volume (V_0), total volume (V_t), dextran 2000 (D_2), dextran T500 (D_5). Separations were monitored with phenol-sulphuric acid.

Figure 2 SDS-PAGE of bovine serum albumin (BSA), *H pylori* filtrate, and BSA after incubation with *H pylori* filtrate. After electrophoresis the gel was stained for protein. Lane 1 BSA, lane 2 BSA after incubation with *H pylori* filtrate for 20 hours at 37°C, and lane 3 *H pylori* filtrate (200 µg protein; about 10 times the amount of filtrate-protein applied to lane 2).

(Vo marker), glucose (Vt marker), dextran 2000 ($M_r \sim 2 \times 10^6$), dextran T500 ($M_r \sim 5 \times 10^5$), and with porcine gastric mucus (fig 1). Mean distribution coefficients (K_{av}) of components on Sepharose 2B were calculated as described.¹⁵

Results

A sterile filtrate of *H pylori* was found to contain seven protein bands when examined by SDS-polyacrylamide electrophoresis (fig 2). The major band corresponded to a molecular weight of 60 kilodaltons, with less prominent

bands at 74, 58, 57, 54, 30 and 23 kilodaltons. At least three of these proteins seem to be associated with urease activity in the filtrate.¹⁶ When incubated with 5 or 10 mM urea-bicarbonate solution *H pylori* filtrate raised the pH from 7.4 to (at least) 8.8 in five hours (table).

No hydrolysis of bovine serum albumin could be discerned after incubation with *H pylori* filtrate (even when freshly prepared) for 20 hours at 37°C, when breakdown was monitored by SDS-polyacrylamide electrophoresis (fig 2), or spectrophotometric methods (fig 3).

Breakdown of high particle weight mucus glycoprotein was negligible (when compared with that achieved with pepsin) after incubation with *H pylori* filtrate for 20 hours at 37°C (fig 4B). In contrast, extensive breakdown of high particle weight mucus glycoprotein was observed on incubation with *H pylori* filtrate to which had been added 2 M urea (fig 4C). A comparable breakdown of high particle weight mucus glycoprotein occurred after incubation with Jack bean urease in the presence of 2 M urea, 1 M ammonium carbonate (fig 4C), and a buffer (pH 8.7) containing 10 mM ammonium carbonate and 30 mM NaHCO_3 (fig 4D), but was negligible when high particle weight mucus glycoprotein was treated for the same period of time with 4 M urea, or Jack bean urease alone (fig 4B). The major fragment arising from breakdown of high particle weight mucus glycoprotein had a mean distribution coefficient (K_{av}) of 0.26. This was considerably less than that of dextran T500 (K_{av} 0.69), or of the product of limit proteolysis/reduction of gastric mucus (fig 1), but equivalent to that of dextran 2000, and of a mucus glycoprotein structure isolated from porcine gastric mucus with $M_r \sim 2 \times 10^6$.^{17,18}

We noted that the elution profile shown in fig 4D was exactly reproduced when the gel-excluded (Vo) and gel-included mucus glycoprotein structures depicted in fig 4D were separately chromatographed again on Sepharose 2B. This indicated that the gel-excluded mucus glycoprotein structure in fig 4D was not identical with the original high particle weight mucus glycoprotein shown in fig 4A, but a less stable aggregate formed by association of mucus glycoprotein structures of $M_r \sim 2 \times 10^6$. Allen *et al* have also reported in vitro aggregation of mucus glycoprotein structures of $M_r \sim 2 \times 10^6$.¹⁹ Comparison of fig 4C and fig 4D further suggested that the potential for aggregation of mucus glycoprotein structures of $M_r \sim 2 \times 10^6$ is determined by conditions under which the high particle weight mucus glycoprotein was initially fragmented. It is noteworthy that the ratio of the Sepharose 2B gel-excluded and gel-included mucus glycoprotein structures, after incubation of high particle weight mucus glycoprotein in carbonate-bicarbonate buffer (fig 4D), is similar to that seen in the elution profile on Sepharose 2B of mucus aspirated from patients with gastric ulcer or gastritis (fig 5), and mucus removed from the gastric mucosal surface of rats with increased susceptibility to ulceration induced by aspirin.²⁰

Figure 3 The release of (A) α -amino residues (A570 after reaction with ninhydrin) and (B) low molecular weight peptides (A650 after reaction with Folin-Ciocalteu reagent) from bovine serum albumin on incubation with pronase (■), and *H pylori* filtrate (●) in 0.1 M TRIS-HCl; pH 7.8, at 37°C.

Figure 4

Chromatography on Sepharose 2B (1×70 cm column eluted with 0.05 M NaCl) of high particle weight mucus glycoprotein (A) before and after incubation alone (—), and after (---) proteolysis with Sigma porcine pepsin (2% w/w in 0.1 M KCl-HCl buffer; pH 2); (B) after incubation with *H. pylori* filtrate, or Jack bean urease or 4 M urea; (C) after incubation with *H. pylori* filtrate + 2 M urea, or Jack bean urease + 2 M urea, or 1 M ammonium carbonate; (D) after incubation with ammonium carbonate- NaHCO_3 buffer; pH 8.7.

Figure 5

Chromatography on Sepharose 2B (1×70 cm column eluted with 0.05 M NaCl) of mucus (non-dialysable) aspirated from (A) volunteers and (B) patients with gastric ulcer material and gastritis. Fractions containing plasma glycoprotein denoted (■) and mucus glycoprotein (□). The mucus glycoprotein was delineated by reference to the elution profile of porcine gastric mucus (fig 1), and was excluded from Bio-Gel A 0.5 m after digestion with Sigma pronase, 2% w/w in 0.1 M TRIS-HCl buffer; pH 7.8, at 37°C for 24 hours. Separations were monitored with phenol-sulphuric acid.

Discussion

A sterile filtrate of *H. pylori*, prepared in our laboratory in accordance with the procedure outlined by Slomiany *et al.*,⁴ failed to hydrolyse bovine serum albumin, or to break down high particle weight mucus glycoprotein, as described.^{4,5} These findings cast further doubt²¹ on the authenticity of a recent report of an extra-cellular *H. pylori* protease with mucolytic activity,^{4,5} and prompted us to seek an alternative cause of the degradative effect of *H. pylori* filtrate on gastric mucus.

We noted that Fitzgerald and Murphy had administered 8 M urea to patients with peptic ulcer anticipating that ammonium carbonate, released by the activity of urease in the gastric mucosa, would neutralise stomach acid.²² The treatment was not well tolerated, and there was evidence of an irritant effect of the ammoniacal milieu on the gastric mucosa. Since then, the destructive effects of urea in the presence of urease²³ and of exogenous^{23,24} and endogenous^{25,26} ammonia on the gastric mucosal barrier have been detailed by other authors. These observations suggested that the in vitro mucus breakdown reported by Slomiany *et al.* might be attributable to a mucolytic action of ammonium carbonate, released by hydrolysis of 6 M urea which had been incorporated into the column eluant when fractionating *H. pylori* filtrate-incubation mixtures.⁴ This hypothesis was tested by incubating high particle weight mucus glycoprotein with *H. pylori* filtrate in the presence of 2 M urea. Under these conditions breakdown of high particle weight mucus glycoprotein was extensive, although fragments were larger ($M_r \sim 2 \times 10^6$) than are produced by pepsin proteolysis, and exhibited a limited potential for aggregation. Incubation of high molecular weight mucus glycoprotein with Jack bean urease in the presence and absence of 2 M urea, 4 M urea, and 1 M ammonium carbonate confirmed that the breakdown was mediated through urease activity in the *H. pylori* filtrate.

The extent to which *H. pylori* urease was able to degrade mucus in our in vitro experiments was of particular interest, as a potential cause of decreased high particle weight mucus glycoprotein in mucus aspirated from patients with gastric ulcer or gastritis.² Human plasma nor-

mally contains 5–10 mM urea.^{27,28} Marshall has indicated that a mean of 85% of transuded plasma urea is hydrolysed (to ammonium carbonate) by urease in patients with *H. pylori* infection,²⁹ and indeed the concentration of ammonia is reported to reach 25 mM (equivalent to 12.5 mM ammonium carbonate) in mucus from such patients.³⁰ In solution ammonium carbonate partially decomposes to form a carbonate-bicarbonate buffer system. When the contribution of plasma/mucosal bicarbonate²⁷ is taken into consideration, the hydrolysis of transuded plasma urea could, in theory, generate a 25–50 mM carbonate-bicarbonate buffer (pH 9) at the gastric mucosal surface. We therefore examined the in vitro effects of such a buffer (10 mM ammonium carbonate plus 30 mM NaHCO_3 ; pH 8.7) on high particle weight mucus glycoprotein. Breakdown of high particle weight mucus glycoprotein was extensive, causing fragments of $M_r \sim 2 \times 10^6$ (with a noticeable potential for aggregation) to be released.

These data support a role for *H. pylori* urease in the in vivo breakdown of human gastric mucus. This may be brought about by the mechanism summarised in fig 6. Gastric mucus is a gel formed from mucus glycoproteins. These macromolecules are bound into complex structures: mucus glycoprotein of $M_r \sim 5 \times 10^5$ are cross-linked through proteins to form mucus glycoprotein complexes (structure II) of $M_r \sim 2 \times 10^6$,³¹ which are incorporated with lipids^{5,32} into very large spherical or ellipsoidal micelles.^{33–34} The micelles then interact to form the mucus layer and endow it with the properties of elasticity, hydrophobicity, and resistance to proteolysis. In *H. pylori* infection we envisage that (i) pH is increased to about 9 in the vicinity of the bacterium, by the action of urease on transuded plasma urea, (ii) pH sensitive protein-lipid interactions,³⁵ that normally stabilise structure I are broken, and (iii) the now destabilised structure I is disassembled, perhaps by the “corkscrewing” motility of *H. pylori*³⁶ to release structure II. This mechanism may be of pathological importance in facilitating *H. pylori* colonisation of gastric mucosa (which seems to be urease dependent³⁷) and promoting peptic ulceration.³

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Figure 6 The in vivo breakdown of gastric mucus by *H. pylori*. It is envisaged that micellar structure I is destabilised and disaggregated when the mucosal pH rises to 8.7 by the action of *H. pylori* urease on transuded plasma urea. Hydrophobic structure (lipids) O, mucus glycoprotein (MG), “link” protein dimers (P).

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