Chemotactic response of *Helicobacter pylori* to human plasma and bile

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To clarify further the role of chemotaxis in *Helicobacter pylori* colonization, the in vitro bacterium response to human plasma and bile (secretions containing chemo-effector compounds that are present in the gastric mucus layer) was examined. Human plasma, after dilution to 1% (v/v) with buffer, was found to be a chemoattractant for the motile bacillus. Human gall-bladder bile, after dilution to 2% (v/v) with buffer, was found to be a chemorepellent, but did not cause the motility of the bacillus to be diminished after prolonged exposure. The basis of the chemoattractant effect of plasma was explored by examining how urea and 12 amino acids found in plasma affected the taxis of *H. pylori*. Urea and the amino acids histidine, glutamine, glycine and arginine were the strongest chemoattractants. Other amino acids were chemoattractants, with the exceptions of aspartic and glutamic acids, which were chemorepellents. The basis of the chemorepellent effect of bile was explored by examining how the six most abundant conjugated bile acids in human bile affected the taxis of *H. pylori*. All the bile acids were chemorepellents, with the greatest effects being demonstrated by taurocholic and taurodeoxycholic acids. The implications of these findings for *H. pylori* colonization of gastric epithelium are discussed.

INTRODUCTION

Within the gastric microenvironment, *Helicobacter pylori* is found to be most frequently congregating at inter-epithelial junctions, and associating with the epithelial surface by cell attachment and mucus entrapment (Hazell *et al.*, 1986; Thomsen *et al.*, 1990). The incentive to identify the factors that control this distinctive pattern of colonization is considerable, since it has been shown that infection density at the epithelial surface correlates directly with the severity of gastritis and risk of peptic ulceration (Khulusi *et al.*, 1995; Talamini *et al.*, 1997).

Freter (1980) has indicated that effective colonization of the epithelial surface only occurs when bacteria with normal motility and chemotaxis behaviour are directed towards the mucosa by an appropriate chemotactic stimulus. In the *H. pylori*-infected stomach, the important chemotactic gradients across the mucus layer, which draw the bacterium into the epithelial surface, have yet to be identified. However, they are likely to be formed from biliary surfactants refluxing into the stomach, and constituents of plasma transuding from gastric mucosa.

Abbreviations: CLV, curvilinear velocity; CR, chemotactic response.
more particularly should exist within a plasma concentration gradient that decreases from the epithelial to the luminal surface of the mucus layer. This gradient might be expected to draw the bacterium into the epithelial surface, since amino acids found in plasma are reported to act as chemoattractants for other bacteria (Hugdahl et al., 1988; Mesibov & Adler, 1972; Moulton & Montie, 1979).

In this preliminary communication we have tested these ideas, by examining how the movements of H. pylori in an in vitro chemotaxis system are affected by human plasma and bile and some of their constituents.

METHODS

Gall-bladder bile was collected by routine puncture from a prospective (adult, male) surgical patient. The sample was sterilized by filtration through a 0.22 μm syringe filter (Acrodiscs) and stored at 4 °C until required. Blood plasma was obtained from a healthy volunteer and used immediately. Conjugated bile acids, amino acids and urea were from Sigma.

Bacteria. H. pylori was isolated from endoscopic biopsies of patients with duodenal ulcer or non-ulcer dyspepsia. The bacterium was initially plated out on 7 % (v/v) defibrinated horse blood agar, which included H. pylori-selective supplement (Oxoid), and incubated in a microaerobic environment (10 % CO2, 18 % O2 and 72 % N2) within a CampyPak gas jar (Oxoid) at 37 °C for 48 h. Colonies from these plates were suspended in sterile saline to a turbidity equivalent to that of McFarland’s No. 4 standard (108 bacteria ml−1). From this suspension, 100 μl was transferred to 2-9 ml brain heart infusion broth (BBB) supplemented with 10 % (v/v) new born calf serum (Sigma) and H. pylori-selective supplement. The broth was then incubated with agitation in a 50 ml capacity loose-capped container (Bibby Sterilin) in an CampyPak gas jar (Oxoid) at 37 °C for 48 h. Colonies from these plates were suspended in sterile saline to a turbidity equivalent to that of McFarland’s No. 4 standard (108 bacteria ml−1). From this suspension, 100 μl was transferred to 2-9 ml brain heart infusion broth (BBB) supplemented with 10 % (v/v) new born calf serum (Sigma) and H. pylori-selective supplement. The broth was then incubated with agitation in a 50 ml capacity loose-capped container (Bibby Sterilin) in a microaerobic atmosphere (see above) at 37 °C for about 48 h (bacilli) or over 72 h (coci). Aliquots were withdrawn periodically for assessment of bacterial motility and viable count by the method of Miles et al. (1938). Gram stain, oxidase and catalase tests were used to confirm absence of contamination.

Motility measurements. The culture (containing bacilli or cocci) was initiated with fresh medium to a concentration not exceeding 108 bacteria ml−1. Aliquots (10 μl) of the diluted culture were then added to 90 μl of 100 mM phosphate/citrate buffer pH 6.5, or gall-bladder bile [diluted to 2.2 % (v/v) with phosphate/citrate buffer]. The resulting bacterial suspension was drawn by capillary action into an optical microslide (CamLab) of 0.1 mm path length. The microslide was sealed at one end with vinyl plastic putty (Oxford Labware), transferred to the warm stage (37 °C) of a phase-contrast microscope (X40 magnification) and allowed to equilibrate for 5 min before observations commenced with a Hobson BacTracker (Hobson Tracking Systems).

In this study, a single representative parameter was used to assess H. pylori motility: curvilinear velocity (CLV); the distance in microns travelled along the path of the bacterium in each second between two stops. To ascertain the contribution of Brownian movement to bacterial motility, H. pylori was killed (no growth on horse blood agar) by exposure to 10 % (v/v) formalin for 10 min at room temperature, before examination with the BacTracker. Data collected with the BacTracker were analysed by Mann–Whitney U-test with an SPSS (Social Sciences Version 6) statistics package. A detailed description of the Hobson BacTracker and its operation may be found in the manufacturer’s literature.

Chemotaxis measurements. The technique used to determine how H. pylori accumulates in gradients formed by bile, plasma or their constituents was a modification of that described by Adler (1973). Microcapillary tubes of 10 μl capacity (Sigma) were filled with blood plasma or gall-bladder bile, diluted to 1 and 2 % (v/v), respectively, in 100 mM phosphate/citrate buffer pH 6.5, or with blood plasma components (amino acids and urea) or selected bile acids, at concentrations of 1 and 2 mM, respectively, in phosphate/citrate buffer, and then sealed at the upper end with vinyl plastic putty (Oxford Labware). Control microcapillaries, used to assess background accumulations of bacteria, were filled with phosphate/citrate buffer alone. The open end of each microcapillary tube was inserted into an Eppendorf tube of 0.5 ml capacity, containing a suspension of the bacterium (bacilli or cocci) in buffer (300 μl) at a concentration of 105 cells ml−1, and incubated horizontally under microaerobic conditions for 45 min at 37 °C. After this time, the contents of the upper two-thirds of the tube were expelled onto a glass slide, and numbers of bacteria that had accumulated into the microcapillary counted with a Hobson BacTracker. Each assay was performed on seven different isolates (unless stated otherwise) and repeated at least six times. To normalize data from different experiments, results were expressed as the chemotactic response (CR), calculated as the number of bacteria in the test microcapillary, divided by the number of bacteria in the control microcapillary.

Statistical analyses. The significance of results was tested using the Mann–Whitney U-test. Differences at the P < 0.05 level were considered to be statistically significant.

RESULTS

Bacterial motility

The motility of the H. pylori bacillus was not significantly diminished (when compared to that in phosphate/citrate buffer), when the bacterium was exposed to diluted gall-bladder bile for up to 6 h (Fig. 1).

Accumulation of bacilli and cocci in microcapillary tubes

Bacillary and coccoidal forms of H. pylori were isolated from the same culture, and their accumulations in microcapillary tubes, containing phosphate/citrate buffer, 1 % blood plasma or 2 % gall-bladder bile, were compared under identical test conditions. Results are summarized in Fig. 2. The accumulation of the motile bacillary form of H. pylori in plasma was found to be significantly greater (P < 0.001) and in gall-bladder bile was found to be significantly less (P < 0.01) than in buffer. In contrast, the accumulation of the non-motile, non-flagellate (Worku et al., 1999) coccoidal form of H. pylori was not significantly different in buffer, plasma or bile.

Chemotactic response to human plasma, urea and some amino acids

Human plasma, diluted to 1 % (v/v) in phosphate/citrate buffer, was found to be a significant chemoattractant for the H. pylori bacillus (Table 1). The responsiveness of the bacillus was also tested towards urea, which is a major constituent of plasma (Snook, 1986), and 12 amino acids that occur in human plasma (Stein & Moore, 1954). Results are sum-
The chemotactic response of *H. pylori* to human plasma, human gall-bladder bile and selected constituents

Chemotaxis was measured under microaerobic conditions with a modified Adler apparatus in which the control microcapillary contained 100 mM phosphate/citrate buffer, pH 6.5. Plasma and bile were diluted in buffer. Amino acids and urea were at a concentration of 1 mM, and bile acids were at a concentration of 2 mM in buffer. Data are results from seven different isolates. Abbreviations: GCA, glycocholic acid; TCA, taurocholic acid; GDCA, glycodeoxycholic acid; TDCA, taurodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid.

### Table 1. Chemotactic response of *H. pylori* to human plasma, human gall-bladder bile and selected constituents

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CR (mean ± SD)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma 1 % (v/v)</td>
<td>4.6 ± 0.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Bile 2 % (v/v)</td>
<td>0.64 ± 0.2</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><strong>Plasma constituents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>3.6 ± 0.4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>2.3 ± 0.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>4.8 ± 0.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>0.7 ± 0.25</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>2.3 ± 0.4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>0.9 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>6.4 ± 0.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.2 ± 0.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>6.1 ± 0.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>1.6 ± 0.35</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>L-Proline</td>
<td>2.2 ± 0.35</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>1.3 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>L-Valine</td>
<td>1.7 ± 0.4</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><strong>Bile acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCA</td>
<td>0.65 ± 0.14</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>TCA</td>
<td>0.53 ± 0.13</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>GDCA</td>
<td>0.69 ± 0.15</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>TDCA</td>
<td>0.34 ± 0.10</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>GCDCA</td>
<td>0.66 ± 0.11</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>TCDCA</td>
<td>0.72 ± 0.19</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

* Mann–Whitney U-test of means compared with control. NS, not significant.

Urea and all the amino acids (apart from aspartic acid and glutamic acid) acted as chemoattractants at a concentration of 1 mM in phosphate/citrate buffer.

**Chemotactic response to human bile and some bile acids**

Human gall-bladder bile, diluted to 2 % (v/v) in phosphate/citrate buffer, was found to be a significant chemorepellent for the *H. pylori* bacillus (Table 1). The responsiveness of the bacillus was also tested towards the six most abundant conjugated bile acids in human bile (Lentner, 1981). Results are summarized in Table 1. All the bile acids were found to be significant chemorepellents at a concentration of 2 mM in phosphate/citrate buffer, with the strongest chemorepellent being taurodeoxycholic acid.
**DISCUSSION**

*H. pylori* is a chemotactic bacterium in which sensor proteins on the cell membrane are coupled to the flagellar motors through two CheY response regulator proteins, three CheV proteins (of uncertain function) and a histidine kinase sensor CheA (Foynes et al., 2000; Pittman et al., 2001). The importance of this chemoreceptor complex for effective gastric colonization has been demonstrated in the failure of motile, but non-chemotactic deletion mutants to survive in an animal model (Foynes et al., 2000). In this study, we have sought to clarify further the role of chemotaxis in *H. pylori* colonization by examining how the bacterium responds *in vitro* to human plasma and bile, secretions containing chemoeffector compounds that are present in the gastric mucus layer.

Comparative accumulation of bacillary and coccoidal forms of *H. pylori* in microcapillary tubes, containing buffer, plasma and gall-bladder bile, indicated that the motile bacillus responds chemotactically (in our test system) to both human plasma and bile. Highly diluted human plasma was found to be a significant chemotaxant for the bacillus, and highly diluted gall-bladder bile a significant chemorepellent.

Bile is reported to be bactericidal for *H. pylori*, although at higher concentrations than those used in our experiments (Tompkins & West, 1987). Our finding, that the motility of the *H. pylori* bacillus is not significantly diminished on prolonged exposure to diluted bile, is therefore of importance, because it established that the chemorepellent effect of bile in our experiments was not simply an artefact due to loss of bacterial viability.

The basis of the chemotaxant effect of diluted plasma was explored by examining the chemotactic behaviour of urea and several plasma amino acids. Measurements were conducted at a concentration of 1 mM, selected because it lies well below the mean concentration range for free amino acids [2.5–4 mM (Stein & Moore, 1954)] and for urea [5–10.5 mM (Snook, 1986)] in human plasma, and was the amino acid concentration that most often gave an optimum chemotactic response with other bacteria (Mesibov & Adler, 1972; Moulton & Montie, 1979).

Urea and the amino acids histidine, glutamine, glycine and arginine were the most potent chemotaxants amongst the plasma components tested. All the other amino acids were more moderate chemotaxants, except for aspartic and glutamic acids, which were chemorepellents.

For most of the amino acids we could discern no relationship between the magnitude of the mean chemotactic response and the nature of the substituent group R in the generalized amino acid formula: R, CH₃, NH₂, COOH. Exceptions were the chemorepellents aspartic and glutamic acids, where R includes a carboxyl group. Our experiments suggest that the carboxylamide group, -CO.NH₂, might be an important binding structure for *H. pylori* receptors, because (i) conversion of aspartic and glutamic acids to their respective amides turned chemorepellents into chemotaxants and (ii) this functional group is present in urea, one of the strongest chemotaxants tested.

Our finding that arginine and urea are significant attractants for *H. pylori* agrees with earlier reports (Mizote et al., 1997; Cerda et al., 2003). The response of the bacterium to these two plasma constituents (and to bicarbonate ion) has recently been linked with expression of the HP0099 gene (Cerda et al., 2003).

The basis of the chemorepellent effect of diluted gall-bladder bile was explored by examining the chemotactic behaviour of the six most abundant conjugated bile acids in the secretion (Lentner, 1981). Measurements were conducted at a concentration of 2 mM, reflecting the mean concentration range for combined bile acids in human gall-bladder bile (Arianoff, 1966; Nakayama, 1967) after dilution to 2 % (v/v) in buffer.

All the bile acids were significant chemorepellents for the *H. pylori* bacillus at this concentration, with the greatest effects being demonstrated by taurocholic and taurodeoxycholic acids. We are uncertain of the structural basis of this repellent action, although our findings with aspartic and glutamic acids (see above) suggest that there might be an involvement of free or esterified carboxyl groups in these surfactant molecules.

**Implications for gastric colonization**

The manner in which *H. pylori* colonizes the gastric microenvironment (Hazell et al., 1986; Thomsen et al., 1990) implies that chemotactic gradients, which draw the bacterium into the epithelial surface, are present across the mucus layer.

Our *in vitro* experiments strengthen the view that an important chemotactic gradient across the mucus layer is formed from components of plasma that transude from microcapillaries in the gastric mucosa. They showed, for instance, that plasma is a significant chemotaxant for the *H. pylori* bacillus at a dilution greater than that which normally occurs in the gastric lumen [between two and four times, when based on a urea marker (Piper et al., 1967; Snook, 1986)], and therefore at a dilution greater than that which must occur at the epithelial surface of the *H. pylori*-infected mucus layer.

Our *in vitro* experiments also strengthen the view that an important chemotactic gradient across the mucus layer is formed from components of bile that reflux into the stomach. They showed, for instance, that bile acids are significant chemorepellents for the *H. pylori* bacillus at a concentration of 2 mM. This concentration is likely to be attained post-prandially by combined bile acids in the pyloric antrum (Han et al., 1996), although less so in the body of the normal acid-secreting stomach (Dixon et al., 1986; Schindlbeck et al., 1987).

Bile and plasma chemotactic gradients across the gastric mucus layer may, therefore, be influential in directing...
H. pylori to the pyloric antrum, and in enabling this important pathogen to attain high population densities at the epithelial surface of the gastric mucus layer.

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REFERENCES


