Helicobacter pylori infection density and gastric inflammation in duodenal ulcer and non-ulcer subjects

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Abstract

The factors that determine which *Helicobacter pylori* infected subjects develop duodenal ulcer (DU) are unclear. This study tested the hypothesis that infection density and urease activity are higher in DU than non-DU subjects. Fifty five DU and 55 age and sex matched non-DU subjects were studied. Quantitative methods were used for measuring infection density (viable organism count) and urease activity (Berthelot reaction). DU subjects had a greater antral infection density (geometric mean of colony forming units/mg biopsy protein; $10{\cdot}5\times10^5$ *v* $1{\cdot}3\times10^5$, $p<0{\cdot}001$). They also had higher biopsy urease activity (geometric mean of NH_3 nmol/min^{-1}/mg protein^{-1}; 103 *v* 25, $p<0{\cdot}001$). Urease activity per organism, however, was similar in the two groups showing that high antral urease activity in DU was a reflection of organism density. DU was not present in subjects with an antral infection density less than 10^5 colony forming units/mg protein. A correlation was present between *H pylori* viable counts and the severity and activity of gastritis. Both severity and activity of gastritis were greater in the antrum of DU compared with non-DU subjects but there was no difference in the body between the two groups. It is concluded that antral *H pylori* infection density is probably an important determinant of DU development, and that there is a baseline of infection density that is necessary for ulcer formation.

(*Gut* 1995; **37**: 319–324)

Keywords: *Helicobacter pylori*, infection density, gastric inflammation.

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Accepted for publication 31 January 1995

Since the initial identification and isolation of *Helicobacter pylori* in the early 1980s,[1] it has become clear that this organism plays a key part in the pathogenesis of duodenal ulceration.[2 3] Over 95% of duodenal ulcer (DU) patients are infected with *H pylori*[4] and eradication results in a cure for DU disease.[5] However, while all infected subjects develop gastritis, most do not develop DU.

Studies have attempted to explain the outcome of *H pylori* infection on the basis of differences in strain virulence.[6–8] A cytotoxin obtained from some strains of *H pylori* has been shown to produce vacuolation in cultured cell lines.[9] The association between DU and carriage of cytotoxic strains of *H pylori* is strong, however, such strains also occur in most subjects without DU[7] and therefore can only provide part of the explanation for the development of ulceration.

H pylori produces large amounts of urease, the ammonia generated by this has been shown to have cytopathic effects on different cultured cell lines in a concentration dependent manner,[10 11] and to be toxic to the gastric mucosa of experimental animals.[12] It is possible that strains of *H pylori* associated with ulceration produce greater amounts of urease.

Histological assessment of the distribution and density of *H pylori* in the stomach has suggested that there may be greater colonisation of the gastric antrum in DU patients than subjects without DU,[13] however, one study has produced conflicting results, showing no significant difference between the two groups.[14] The patchy nature of the distribution of the organism and the tendency of *H pylori* to colonise gastric glands in large clumps renders semi-quantitative histological studies inadequate for the measurement of a potentially immense range of infection densities in vivo. In addition, no assessment of organism viability is possible on histological sections.

In this study we aimed to assess by quantitative methods *H pylori* infection density and urease activity in patients with DU and in those without; and the relation of infection density to factors associated with DU.

Methods

Subjects

Subjects were recruited from an open access endoscopy service. All were *H pylori* positive on the basis of serological tests performed prior to endoscopy as part of a separate *H pylori* screening study. Fifty five DU patients and 55 age and sex matched subjects with no evidence of DU disease were recruited. Exclusion criteria were: acid suppressive treatment, anti-inflammatory drugs or antibiotics in the previous month; gastric ulcers, previous gastric surgery or duodenitis

Patient characteristics

	Duodenal ulcer (n=55)	*Non-ulcer (n=55)*	*p Value*
Median age (range)	49 (22–72)	46 (22–74)	NS
Male (%)	33 (60)	33 (60)	NS
Smokers (%)	28 (50)	6 (11)	<0·001
Family history of DU (%)	16 (29)	5 (9)	<0·01
Ethnic group (white/Asian/black)	35/12/8	34/12/9	NS

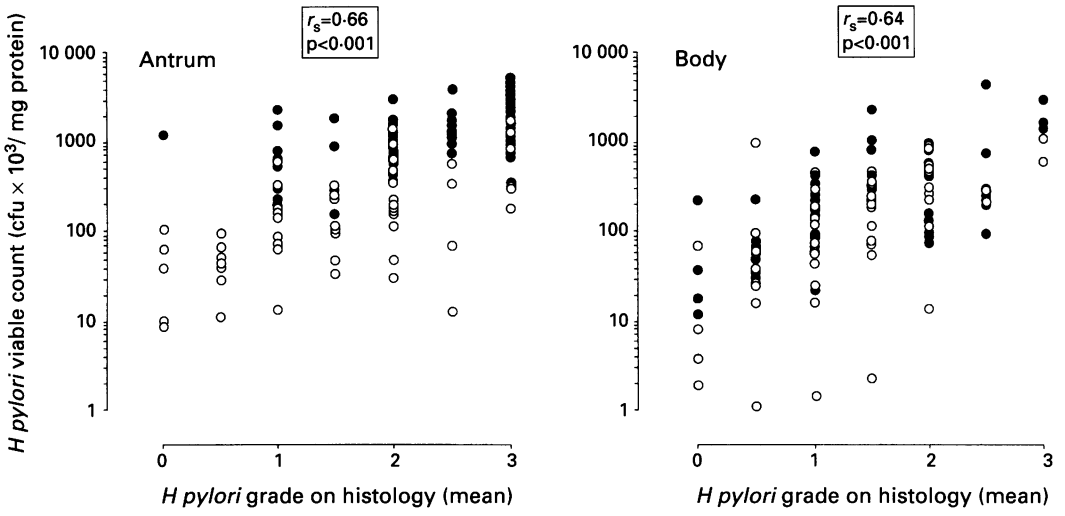

Figure 1: Relation between infection density assessed by histological grading and that measured by viable count. (Duodenal ulcer ●, non-ulcer ○ subjects).

with no ulcer. Eight non-DU subjects were also excluded because no growth could be detected on culture of their gastric biopsy specimens and three who were *H pylori* negative on both histological examination and culture. *H pylori* was cultured from all DU subjects recruited. Detailed patient information including previous history of DU, family history, smoking habit, and drug ingestion was obtained by a research nurse using a questionnaire before endoscopy. The DU and non-DU groups were of similar ethnic origins; smoking and a family history of peptic ulceration were, however, significantly more common in DU subjects (Table). The study was approved by the St George's Hospital ethics committee and informed consent was obtained from all patients.

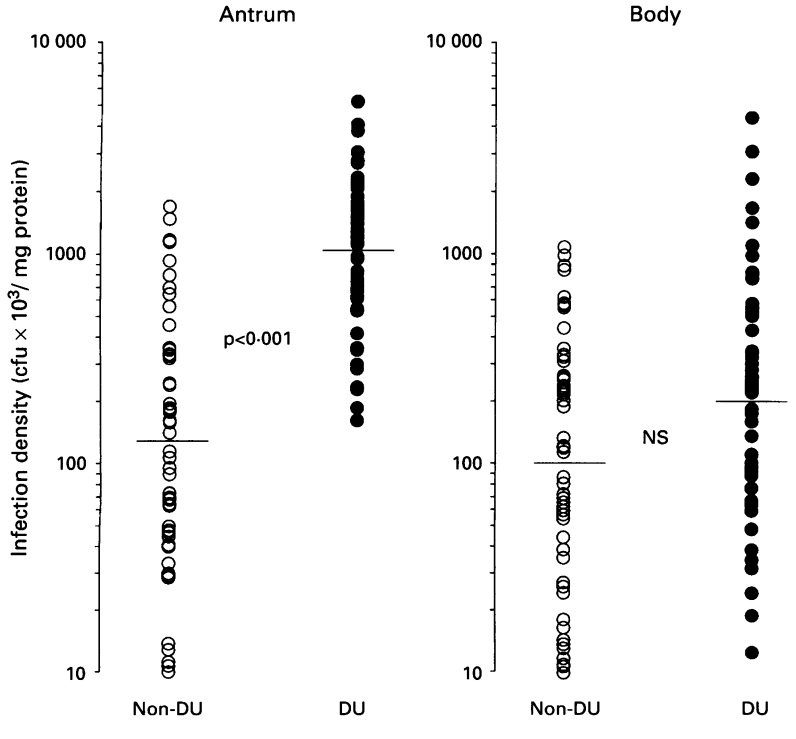

Figure 2: Gastric antral and body infection density in duodenal ulcer and non-ulcer subjects.

Infection density

Three gastric antral and three body biopsy specimens were obtained using the same size forceps from similar topographical sites at each endoscopy. Specimens were from the inferior surface (2 and 3 cm away from the pylorus) and from the superior surface of the antrum (2 cm from the pylorus); and body biopsy specimens from 5 cm above the angulus on the lesser curve and two sites from the greater curve opposite the angulus. The specimens were covered with a drop of saline in individual containers, maintained at 4°C, and processed within two hours. Specimens were homogenised separately in 2 ml of normal saline, and all samples were diluted to a protein concentration of 50–100 μg/ml. Four aliquots of each diluted homogenate were plated on Columbia agar enriched with 5% lysed horse blood, and incubated for five days. *H pylori* colonies were confirmed on the basis of morphology, Gram stain, catalase test, and urease activity. They were counted and the viable count expressed as colony forming units (cfu) per mg of biopsy protein.

Biopsy urease activity

Duplicate aliquots of homogenate were also incubated with 50 mM urea at 37°C for one hour. The ammonia produced was measured by a photometric method using the Berthelot reaction,[15] giving a quantitative measurement of biopsy urease activity, which was expressed as nmol of ammonia per mg of protein per minute.

Gastric histology

Two biopsy specimens from the antrum and two from the body were obtained from each patient. The specimens, from sites adjacent to those used for viable count measurement, were fixed in formal saline and embedded in paraffin wax within 12 hours. Sections were cut and stained with haematoxylin and eosin, and assessed subjectively by one histopathologist for *H pylori* density; sections were graded

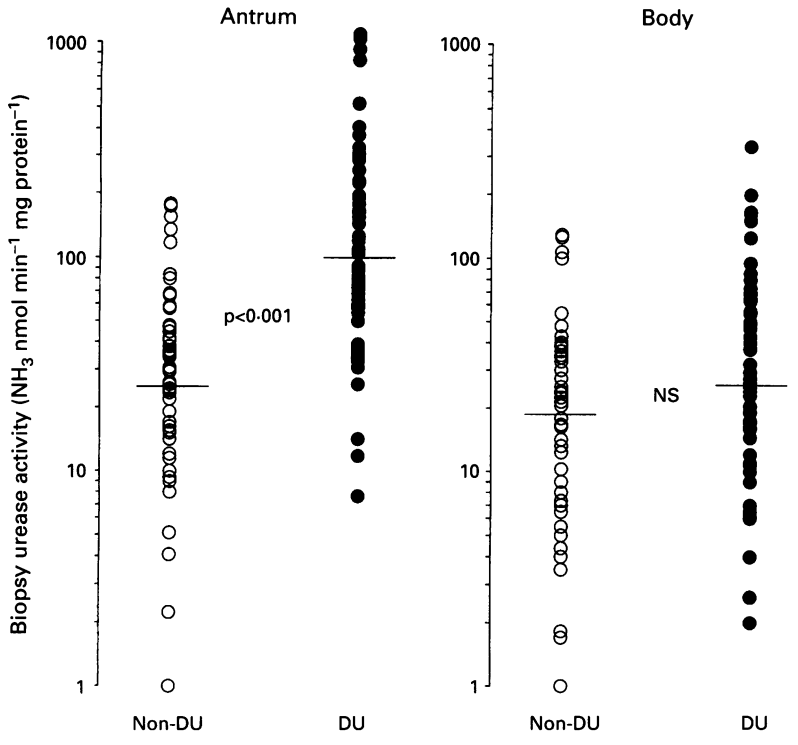

Figure 3: Gastric antral and body biopsy urease activity in duodenal ulcer and non-ulcer subjects.

0–3, corresponding to absent, scant, moderate, and heavy colonisation. Severity and activity of gastritis in the same specimens were also scored 0–3, corresponding to nil, mild, moderate, and severe mononuclear cell infiltration, and neutrophil infiltration respectively.[16] The mean scores of the two specimens obtained from each area from the same subject were used, giving values on a seven point scale.

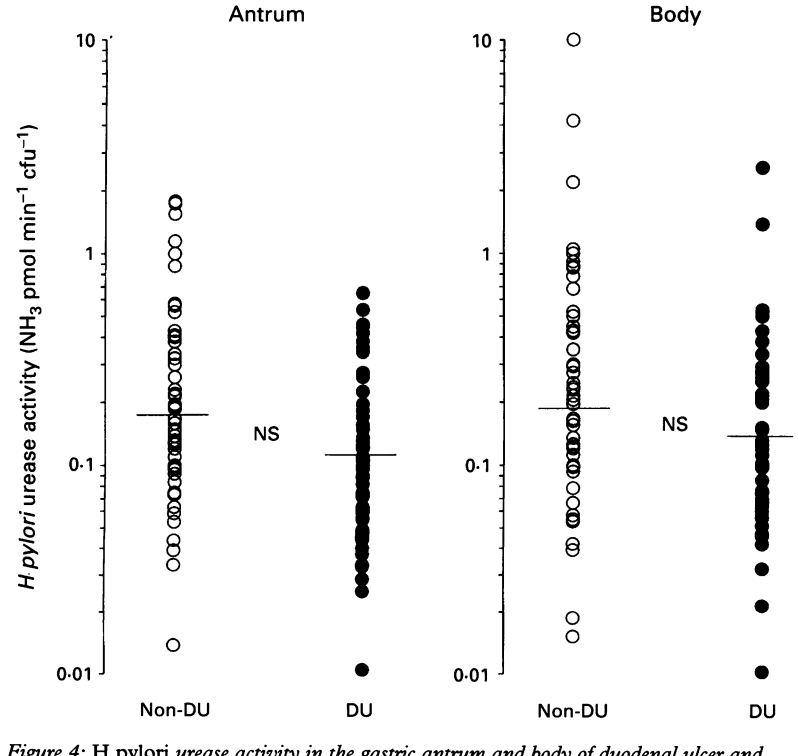

Figure 4: H pylori *urease activity in the gastric antrum and body of duodenal ulcer and non-ulcer subjects.*

Statistical methods

Infection density in the antrum and in the body of each subject was expressed as the geometric mean of the viable counts from respective biopsy specimens. Urease activity was likewise expressed as the geometric mean of measurements from antral and from body biopsy specimens. Differences between means were compared by Student's *t* test following log transformation of the data. Multiple regression was used to determine the relation between infection density and the conventional risk factors for DU. The relation between viable count and histological grade of infection density was assessed by Spearman's rank correlation coefficient. This test was also used to assess the relation between viable counts and the histological parameters of inflammation.

Results

Reproducibility

To assess the effect of clumping at high infection densities, the reproducibility of viable counts at varying dilutions was assessed. Tenfold dilutions were performed on 20 of the specimens, and the counts were found to vary by 12%. The intra-assay coefficient of variation (CV) at the lowest concentration used was 10%. Intra-assay CV for the urease assay was 5%.

Infection density

Figure 1 shows that there was a close relation between infection density assessed by histological grading and that measured by viable count from the same area.

Figure 2 shows that mean *H pylori* density was more than seven times greater in the antrum of DU than non-DU subjects. Furthermore, there was a critical value of infection density (10^5 cfu/mg) below which DU was not found. Infection density was significantly greater in the antrum than in the body of DU patients. In non-DU subjects, however, there was no difference in infection density between body and antrum. Gastric body infection density did not differ between the two groups.

Assessment of the conventional risk factors for DU showed that age, male sex, smoking, family history of DU, and ethnic group were not associated with infection density independently of DU.

Urease activity

Figure 3 shows that there was a significantly greater biopsy urease activity in the antrum of DU than non-DU subjects; and in the antrum compared with the body in DU subjects. As with infection density, however, there was no difference between either body and antral urease activity in non-DU subjects, or in the gastric body between DU and non-DU subjects.

Dividing biopsy urease activity by the viable count from the same biopsy we obtained the

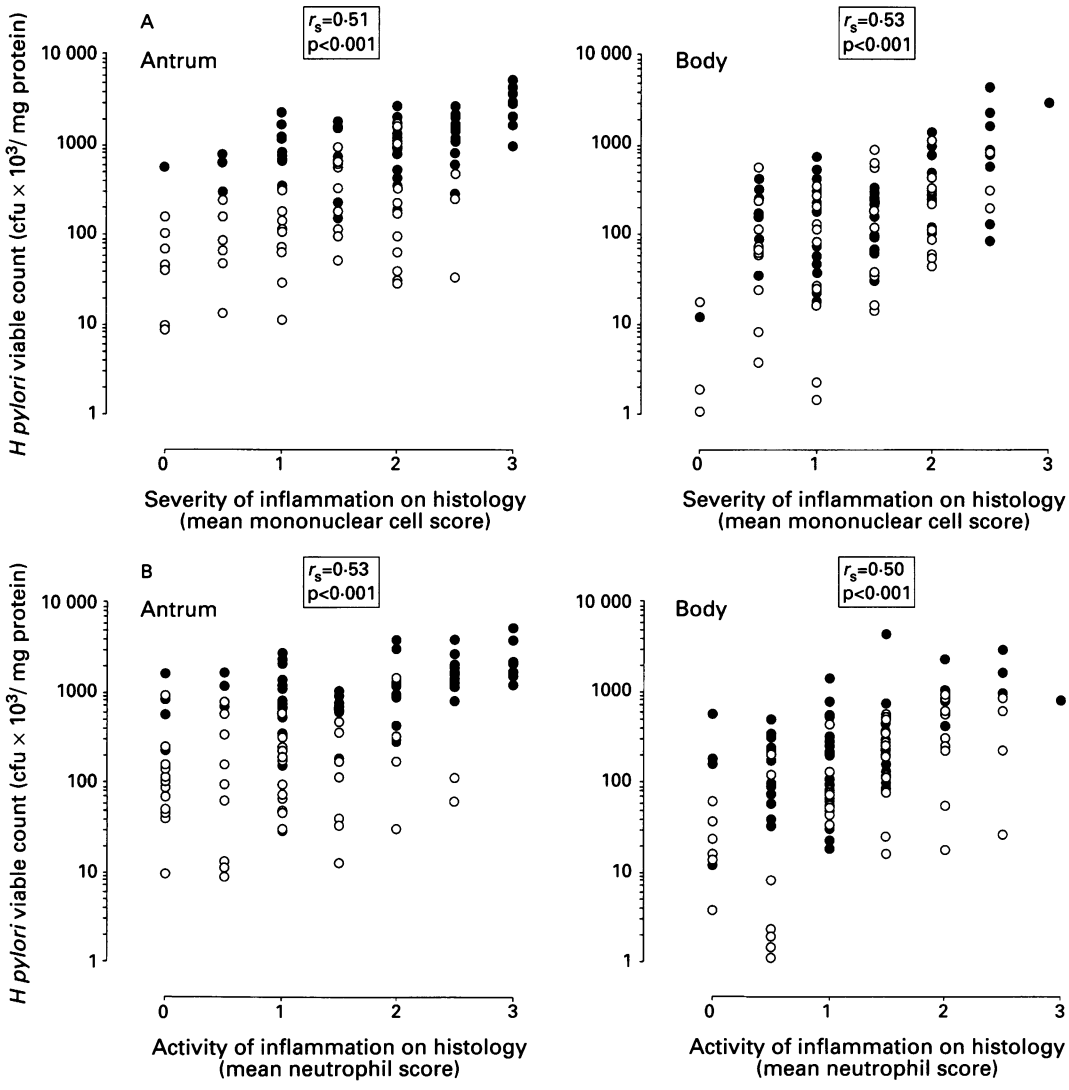

Figure 5: (A) Relation between viable count and the severity of gastritis. (B) Relation between viable count and the activity of gastritis. (Duodenal ulcer ●, non-ulcer ○ subjects).

urease activity/cfu, which was similar in the antrum and in the body of DU and non-DU subjects (Fig 4).

Severity and activity of gastritis

Figure 5(A) and (B) shows the correlation between viable count and the severity and activity of gastritis. Both severity and activity of gastritis were significantly greater in the antrum of DU compared with non-DU subjects (Mann-Whitney U test, $p<0·001$), but there was no significant difference in the body between the two groups (Fig 6).

Discussion

We have shown that both *H pylori* infection density and biopsy urease activity are significantly greater in the antrum of DU subjects than those without DU. Urease activity per organism, however, is similar in these two groups showing that the higher antral biopsy urease activity is a reflection of greater organism density. Our data also suggest that there is a threshold level of infection density, which is necessary for ulcer formation.

The main limitation to enumerating *H pylori* arises from the distribution of the organism at the mucosal surface. A high density of organisms results in a clumping effect, which causes more than one organism to contribute to the formation of a single colony when incubated on agar. Dilution of homogenates before incubation reduced this effect and further dilution made no additional difference, suggesting that at the concentrations used in this study clumping was not an important factor. A further limitation is definition of the two groups, as a number of subjects in the non-DU group may have a DU diathesis and will progress to ulceration in the future. These subjects cannot be considered as true controls, and could have biased our results against finding a greater difference between DU and non-DU subjects. Negative *H pylori* cultures from eight non-DU patients probably resulted from low density of infection, and exclusion of results from these patients may have had a similar effect.

It is possible that an increased infection density in the antrum of DU subjects is an epiphenomenon arising as a result of an inflammatory or pathophysiological process associated with DU. In this study the severity

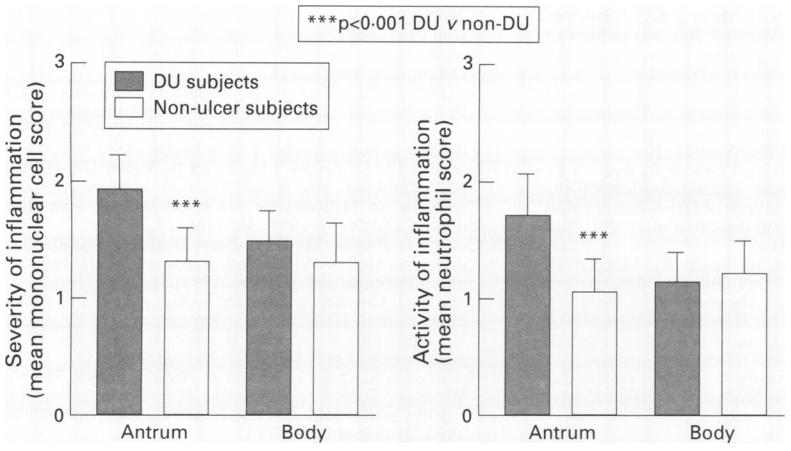

Figure 6: Severity and activity of gastritis in duodenal ulcer and non-ulcer subjects.

and activity of antral gastritis were greater in DU than non-DU patients. Antral inflammation could increase the availability of nutrients for *H pylori* as a result of higher gastric mucosal cell turnover, and so may contribute to an increase in the numbers of organisms. Gastric colonisation, however, almost undoubtedly precedes gastritis, making it less likely that inflammation determines infection density. There are a number of mechanisms whereby *H pylori* may produce gastritis. Its cytotoxin and urease[11] have both been implicated in causing damage to the gastric mucosa both by toxic effects on mucosal cells and also by initiation of an ineffective chronic local immune response against the organism through the highly immunogenic nature of these proteins.[17] Ammonia has been shown to have an additive effect to that of the cytotoxin on vacuolation of HeLa cells in vitro[18] showing that these two virulence factors have a synergistic effect, which may be important in vivo. A 128 kD *H pylori* protein, associated with cytotoxin production has also been shown to produce a mucosal inflammatory response.[19] It is possible that more severe inflammation results from greater and more damaging concentrations of ammonia, cytotoxin or 128 kD protein in contact with the mucosa.

Physiological abnormalities of gastric acid secretion found in DU could determine the distribution of *H pylori*. Acid production in the body of the stomach may explain the greater numbers of organism in the non-acid producing antrum compared with the body, however, it is difficult to explain how the raised gastric acid secretion found in DU could favour a greater density of antral organisms compared with non-DU subjects. It seems more probable that *H pylori* itself is responsible for the increased acid secretion. McColl's group[20] have shown that sucralfate treatment, which in normal subjects has no inhibitory effects on acid secretion, produced a significant reduction in *H pylori* colonisation both on histological assessment and ^{14}C urea breath test in DU subjects, and no change in corpus or antral gastritis scores. This was accompanied by a 50% reduction in the basal acid output, suggesting that infection density is an important determinant of the basal level of acid production. McColl's group[21] have also shown that gastrin releasing peptide stimulated a sixfold increase in gastrin and acid secretion in duodenal ulcer patients and only a threefold increase in *H pylori* infected non-DU subjects compared with uninfected controls. Difference in stimulated acid secretion between *H pylori* infected DU and non-DU subjects may also reflect the difference in antral infection density.

In this study we were unable to find any difference in the urease activity of strains from DU and non-DU subjects, which argues against there being ulcerogenic strains of *H pylori* with greater urease activity per organism. Other differences in *H pylori* strains, however, may be important in determining infection density. Expression of the 128 kD protein, which is associated with DU but whose function remains unclear, may cause more severe gastritis by promoting greater *H pylori* colonisation.

H pylori density may be important in explaining how some infected subjects develop DU. We can speculate that a sufficiently high antral infection density and inflammatory response are needed to increase gastric acid secretion, an important factor in the development of gastric metaplasia in the duodenum.[22] Colonisation of areas of metaplasia, which is more likely with a greater antral organism density then leads to chronic duodenitis, and this may further increase the extent of gastric metaplasia.[23] Increasing duodenal colonisation and inflammation, in the presence of acid, ultimately results in mucosal disruption and ulceration.

The determinants of infection density may be important in explaining why some *H pylori* infected subjects develop complications such as DU. Apart from severity of inflammation we were unable to show any other convincing associations with infection density. More conventional risk factors for DU such as age, smoking, and male sex were not found to be independent determinants of infection density. Other factors, however, such as diet, blood group, and secretor status not considered in this study may influence the density of *H pylori* colonisation.

In conclusion this study has shown that DU is associated with increased *H pylori* infection density and suggests that a threshold of infection density in the gastric antrum is necessary for ulcer formation. Identification of the determinants of infection density may help to clarify the differences in outcome with *H pylori* infection.

Part of this study has been published in abstract form in *Gut* 1993; **34** (suppl 4): S50.

SK was supported by the Astra Foundation Research Fellowship for Gastroenterology.

1 Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; i: 1311–5.
2 Graham DY. Campylobacter pylori and peptic ulcer disease. *Gastroenterology* 1989; **96:** 615–25.
3 Tytgat GN, Noach L, Rauws EA. Helicobacter pylori. *Scand J Gastroenterol Suppl* 1991; **187:** 1–8.
4 Blaser MJ. Helicobacter pylori and the pathogenesis of gastroduodenal inflammation. *J Infect Dis* 1990; **161:** 626–33.
5 Rauws EA, Tytgat GN. Cure of duodenal ulcer associated with eradication of Helicobacter pylori. *Lancet* 1990; **335:** 1233–5.

6 Crabtree JE, Taylor JD, Wyatt JI, Heatley RV, Shallcross TM, Tompkins DS, *et al.* Mucosal IgA recognition of Helicobacter pylori 120 kDa protein, peptic ulceration, and gastric pathology. *Lancet* 1991; **338:** 332–5.
7 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:** 603–10.
8 Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, *et al.* Molecular characterization of the 128-kDa immunodominant antigen of Helicobacter pylori associated with cytotoxicity and duodenal ulcer. *Proc Natl Acad Sci USA* 1993; **90:** 5791–5.
9 Leunk R, Johnson P, David B, Kraft W, Morgan R. Cytotoxic activity in broth-culture filtrates of Campylobacter pylori. *J Med Microbiol* 1988; **26:** 93–9.
10 Xu J-K, Goodwin S, Cooper M, Robinson J. Intracellular vacuolation caused by the urease of Helicobacter pylori. *J Infect Dis* 1990; **161:** 1302–4.
11 Megraud F, Neman SV, Brugmann D. Further evidence of the toxic effect of ammonia produced by Helicobacter pylori urease on human epithelial cells. *Infect Immun* 1992; **60:** 1858–63.
12 Eaton KA, Brooks CL, Morgan DR, Krakowka S. Essential role of urease in pathogenesis of gastritis induced by Helicobacter pylori in gnotobiotic piglets. *Infect Immun* 1991; **59:** 2470–5.
13 Eidt S, Stolte M. Differences between helicobacter associated gastritis in patients with duodenal ulcer, pyloric ulcer, other gastric ulcers and gastritis without ulcer. In: Malfertheiner P, Ditschuneit H, ed. *Helicobacter pylori gastritis and peptic ulcer.* Berlin: Springer-Verlag, 1990: 228–36.
14 Genta R, Hubermann R, Graham D. The microscopic topography of Helicobacter pylori in patients with different expression of the infection. *Acta Gastroenterol Belg* 1993; **56:** 124.
15 *Quantitative urease/Berthelot determination.* Sigma Diagnostics, 1985: 2–11.
16 Fitzgibbons PL, Dooley CP, Cohen H, Appleman MD. Prevalence of gastric metaplasia, inflammation, and Campylobacter pylori in the duodenum of members of a normal population. *Am J Clin Pathol* 1988; **90:** 711–4.
17 Crabtree JE, Shallcross TM, Wyatt JI, Taylor JD, Heatley RV, Rathbone BJ, *et al.* Mucosal humoral immune response to Helicobacter pylori in patients with duodenitis. *Dig Dis Sci* 1991; **36:** 1266–73.
18 Cover TL, Puryear W, Perez-Perez G, Blaser MJ. Effect of urease on HeLa cell vacuolation induced by Helicobacter pylori cytotoxin. *Infect Immun* 1991; **59:** 1264–70.
19 Crabtree J, Covacci A, Farmery S, Xiang Z, Tompkins D, Perry S, *et al.* Helicobacter pylori induced gastric epithelial interleukin-8 secretion is associated with the CagA positive phenotype. *Gut* 1994; **35** (suppl 2): S34.
20 Banergee S, El-Omar E, Mowat A, Ardill J, Park R, Watson W, *et al.* Sucralfate suppresses H pylori infection and reduces gastric acid secretion by 50% in DU patients. *Gut* 1994; **35** (suppl 2): S35.
21 El-Omar E, Penman I, Dorrian C, Ardill J, McColl K. Eradication of H pylori lowers gastrin-mediated acid secretion by 70% in DU patients. *Gut* 1992; **33:** S28.
22 Wyatt JI, Rathbone BJ, Dixon MF, Heatley RV. Campylobacter pylori and acid induced gastric metaplasia in the pathogenesis of duodenitis. *J Clin Pathol* 1987; **40:** 841–8.
23 Khulusi S, Mendall M, Badve S, Patel P, Finlayson C, Northfield T. The effect of Helicobacter pylori eradication on gastric metaplasia of the duodenum. *Gut* 1995; **36:** 193–7.