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Relation between clinical presentation, *Helicobacter pylori* density, interleukin 1β and 8 production, and *cagA* status

Y Yamaoka, T Kodama, M Kita, J Imanishi, K Kashima, D Y Graham

Abstract

***Background*—It is not known whether *cagA*+ *Helicobacter pylori* in duodenal ulcer (DU) have enhanced virulence compared with non-DU *cagA*+ *H pylori*.**

***Aims*—To investigate the relation between presentation, *H pylori* density, interleukin 1β (IL-1β) and IL-8 production, and *cagA* status.**

***Methods*—Fifty DU and 50 gastritis patients with *cagA*+ *H pylori* and 11 with *cagA*− infections were studied. Bacterial density and cytokine production were assessed using the same biopsies. Cytokine production was also measured from supernatants of medium following coculture of *H pylori* with MKN-45 cells.**

***Results*—There was no relation between *H pylori* density and *cagA* status. There was a dose dependent relation between mucosal cytokine levels and density of *cagA*+ *H pylori*. *H pylori* density increased to a threshold, followed by a rapid increase in cytokines and then a plateau. IL-1β and IL-8 levels in the antrum were greater in DU than in gastritis; in the corpus the cytokine level/*H pylori* differed irrespective of similar *H pylori* densities. However, cytokine production was similar in vitro, independent of presentation or biopsy site, suggesting that host factors are critical determinants of the inflammatory response. Mucosal IL-8 and IL-1β levels were low with *cagA*− and *cagA*+, *cagE*− *H pylori* infections.**

***Conclusions*—The increase in antral IL-1β and IL-8 production and inflammation in DU is related to increased numbers of bacteria and not to an increase in cytokine production per *cagA*+ isolate. There was no evidence of enhanced virulence of *H pylori* from DU compared with *cagA*+ non-DU *H pylori*.**

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Keywords: duodenal ulcer; *Helicobacter pylori*; interleukin 1β; interleukin 8; *cagA*

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Attempts to identify virulent strains of *Helicobacter pylori*, which are more likely to result in clinically important outcomes, have focused on two groups of potential bacterial virulence factors, the *cag* pathogenicity island (for which *cagA* is a marker) and the vacuolating cytotoxin, VacA. The *cagA* gene is located in the most downstream portion of the *cag* pathogenicity island, a 40 Kb DNA region, containing open reading frames that code for a putative *H pylori* secretion system.[1] Although the mechanism is unknown, infection with strains that possess the *cag* pathogenicity island are associated with greater production of interleukin 8 (IL-8) than infection with strains without the island.[1] The CagA protein itself is not directly responsible for IL-8 production as inactivation of the *cagA* gene does not eliminate the ability of isolates containing the pathogenicity island to stimulate IL-8 in vitro.[1–3]

It was recently reported that the density of infection with *cagA* positive *H pylori* (*cagA*+, *vacA* s1 type strains) was greater than with *cagA* negative strains (*cagA*−, *vacA* s2 strains).[4] This suggests that the level of inflammation associated with *cagA*+ *H pylori* may be related to, or possibly the result of, an increase in *H pylori* density. This notion is also consistent with our previous observation that mucosal IL-1β and IL-8 production was closely related to *H pylori* density based on histology.[5 6] We previously reported that production of IL-8 and IL-1β in the antral mucosa was greater in patients with *cagA*+ *H pylori* compared with those with *cagA*− *H pylori;* IL-8 production was closely related to IL-1β production in the antrum.[5] We also reported that IL-8 production in the antral mucosa was greater in DU patients with *cagA*+ *H pylori* compared with *cagA*+ infections in patients with non-ulcer dyspepsia.[5]

This study was designed to explore further the relation between *H pylori* density, IL-1β and IL-8 secretion in the antrum and corpus, and the presentation of *H pylori* infection (DU or non-DU gastritis). The relation between *H pylori* density and IL-1β and IL-8 production in gastric mucosa was investigated using the same biopsy specimens for quantitative culture and measurement of cytokine production. In addition, the ability of *H pylori* from DU and non-DU gastritis patients to produce IL-1β and IL-8 in vitro was compared using gastric cancer cell line MKN-45 in order to address the question of possible enhanced virulence among DU strains.

Methods

POPULATION

Fifty DU patients infected with *cagA*+ *H pylori* (27 men, 23 women; mean age 52.7 years) and 50 age and sex matched *cagA*+ *H pylori*

Abbreviations used in this paper: DU, duodenal ulcer; IL, interleukin; MNC, mononuclear cell; PCR, polymerase chain reaction; PMN, polymorphonuclear cell.

infected patients without endoscopic evidence of DU (27 men, 23 women; mean age 53 years) were studied. All gastritis patients had histological gastritis without gastric or duodenal ulcer, gastric cancer, or oesophageal disease. In addition, 11 individuals with *cagA*− *H pylori* infection were studied (one DU and 10 gastritis; seven men, four women; mean age 53.5 years). Controls consisted of 50 age and sex matched asymptomatic volunteers who were negative for *H pylori* infection by the combination of negative culture, histology, and serology (27 men, 23 women; mean age 52.8 years).

Patients were excluded if they had received non-steroidal anti-inflammatory drugs, proton pump inhibitors, or antibiotics within the previous three months, as were those who had received recent blood transfusions or had undergone gastric surgery. Sixteen (32%) DU patients, 20 (33%) gastritis patients, and 15 (30%) volunteers were smokers. Informed consent was obtained from all patients and the protocol was approved by the ethics committee of Kyoto Prefectural University of Medicine. Forty four patients were included in our previous studies of inflammatory mediators in *H pylori* infection.[5] All were Japanese.

The presence of *H pylori* was determined by culture of the biopsy specimens and the *cagA* status was evaluated by polymerase chain reaction (PCR), as described previously.[5–7] In cases where strains were *cagA* gene negative by PCR, *cagA* status was confirmed as negative for CagA protein by immunoblotting, as described previously.[8]

Three biopsy specimens were taken, using the Olympus biopsy forceps FB-24KR, from the greater curve of the antrum (pyloric gland area) and corpus (fundic gland area). One specimen was used for quantitative culture and measurement of cytokines, while the other two specimens were used for histology. For the quantitative culture and measurement of cytokines, all cases in the antrum and 81 cases in the corpus were analysed from which 35 cases in each *cagA*+ DU and gastritis (sex and age matched) were randomly selected; all 11 *cagA*− cases were also analysed.

QUANTITATIVE CULTURE OF *H PYLORI*

Biopsy specimens were immediately placed in 1.5 ml of phosphate buffered saline (PBS; pH 7.4), and homogenised using a tissue homogeniser (Kontes, Vineland, New Jersey); serial tenfold dilutions in 1.0 ml of PBS were performed within 10 minutes. Ten μl aliquots were spread on Skirrow agar medium containing horse blood (8%), vancomycin (10 μg/ml), polymyxin B (2.5 U/ml), and trimethoprim (5 μg/ml), and incubated at 37°C under microaerophilic conditions for up to five days. The organisms were identified as *H pylori* by Gram staining, colony morphology, and positive oxidase, catalase, and urease reactions. Viable counts were recorded and expressed as colony forming units (cfu) per mg of biopsy protein. In tenfold dilutions, the counts were found to vary within 13%. Multiple colonies were collected together and all stock cultures were maintained at −80°C in Brucella broth (Difco, Detroit, Michigan, USA) supplemented with 20% glycerol (Sigma Chem Co., St Louis, Missouri, USA). The passage number of the *H pylori* used in this study averaged three. Genomic DNA was extracted using the QIAamp Tissue kit (QIAGEN Inc., Santa Clarita, California, USA) according to the manufacturer's instructions.

POLYMERASE CHAIN REACTION

cagA status and *vacA* genotype were evaluated by PCR, as described previously.[5–7 9–11] Furthermore, the primers 5'-TGCTGATACGATTAG AGA-3' (CAGEF) and 5'-TAGTCCCTTAGT GATGAT-3' (CAGER), and 5'-GCCATGTT AACACCCCCTAG-3' (CAGGF) and 5'-TTA ATGCGCTAGAATAGTGC-3' (CAGGR) were used to amplify the *cagE* and the *cagG* gene, respectively.[1 12] PCR was performed using a DNA Engine (MJ Research Inc., Watertown, Massachusetts, USA) for 35 cycles, consisting of one minute at 95°C, one minute at 50°C, and one minute at 72°C. The final cycle included a seven minute extension step to ensure full extension of the PCR product.

GASTRIC HISTOLOGY

Two biopsy specimens, from within 5 mm of sites used for culture were embedded in paraffin wax, stained with haematoxylin and eosin and Giemsa stains, and examined by one histologist blinded to the patient's clinical diagnosis or the characteristics of the *H pylori* strain. The following features were evaluated on each slide: *H pylori* density, and degree of mononuclear cell (MNC) and polymorphonuclear leucocyte (PMN) infiltration. All variables were graded using the visual analogue scale graded from 0 (absent/normal) to 5 (maximal intensity), as described by El-Zimaity *et al*.[13] The *H pylori* density was scored based on the average density on the surface and the foveolar epithelium. If areas with widely different scores were present on the same specimen, an average based on the general evaluation of the biopsy was used. Only areas without metaplasia were evaluated for the presence of *H pylori*.

IL-1β AND IL-8 PROTEIN MEASUREMENT IN BIOPSY SPECIMENS (IN VIVO CYTOKINE PROTEIN)

For IL-1β and IL-8 production from the biopsy specimens, the same specimen was studied as had been used for quantitative culture of *H pylori*. Aliquots of homogenate supernatants in PBS (pH 7.4), obtained by centrifugation (10 000 *g* for 10 minutes) after sampling for culture, were stored at −80°C until use. Total protein in biopsy homogenate supernatants was assayed by a modified Lowry method; IL-8 protein was measured by ELISA using commercially available assay kits (Research and Diagnostic Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions. In our laboratory, the ELISA sensitivities of IL-1β and IL-8 were approximately 10.5 pg/ml and 10 pg/ml, respectively. The mucosal levels of cytokines were expressed as pg/mg biopsy protein.

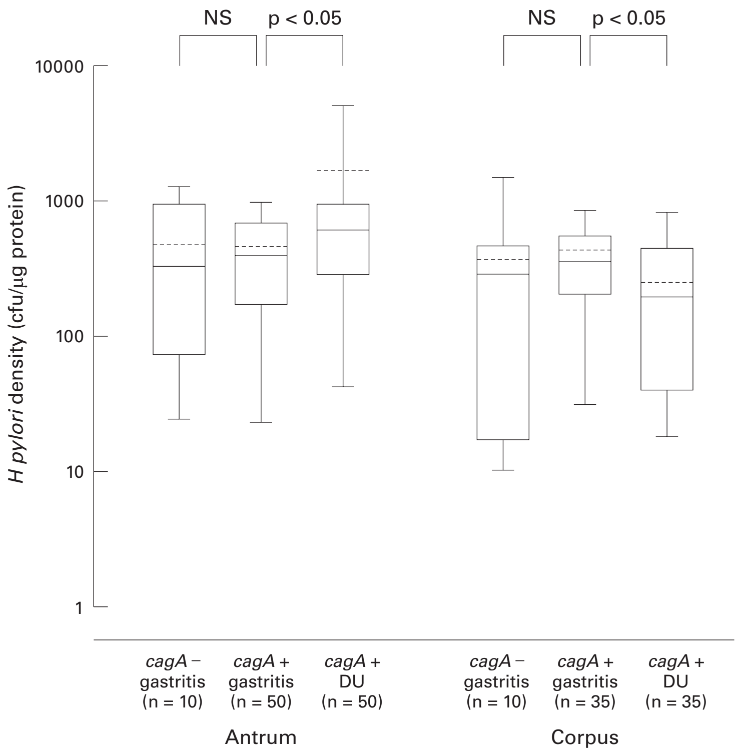

Figure 1 Helicobacter pylori density in antrum and corpus. The end of the bars indicates the 25th and 75th percentiles. The 50th percentile (median) is indicated with a solid line in the box; the broken line indicates mean value. The 10th and 90th percentiles are indicated with error bars. DU, duodenal ulcer.

IL-1β AND IL-8 PROTEIN MEASUREMENT FROM GASTRIC CANCER CELL LINE (IN VITRO CYTOKINE PROTEIN)

The human gastric cancer cell line MKN-45 was obtained from the Japanese Cancer Research Resources Bank. MKN-45 cells were routinely maintained in RPMI 1640 supplemented with 10% heat inactivated foetal calf serum and 40 µg/ml gentamicin. MKN-45 cells were plated into 24 well plates at a density of 1×10^5/ml and cultured for two days to confluence (about 5×10^5/ml). Each well was washed twice with antibiotic free tissue culture medium. Multiple *H pylori* colonies were collected together and all stock cultures were maintained at −80°C in Brucella broth (Difco, Detroit, Michigan) supplemented with 20% glycerol (Sigma). Stock frozen *H pylori* (pooled isolates from multiple colonies) was cultured in brain-heart infusion broth containing 5% horse serum with a gyratory shaker at 220 rpm for 24–48 hours, representing growth phases.

H pylori were harvested in PBS and suspended to yield a concentration of 5×10^7 cfu/ml in antibiotic free tissue culture medium, and added to the cultured cells immediately (bacterium to cell ratio 100:1). Epithelial cells were cultured with bacteria preparations for 24 hours at 37°C in a 95% air and 5% CO_2 humidified incubator. At the end of culture, supernatant fluids were aspirated and frozen at −80°C until assayed; cell viability was determined by trypan blue exclusion. No differences in epithelial cell viability in experimental and control culture without *H pylori* were evident. IL-1β and IL-8 in the supernatant were assayed in duplicate by ELISA (R&D Quantikine, R&D Systems Inc., Minneapolis, Minnesota, USA) and median concentrations were expressed as pg/ml. *cagA* gene/CagA protein positive strain, 88–23 (60190) and *cagA* gene/CagA protein negative strains 88–22 (Tx30) and 93–68 (kindly provided by Professor M J Blaser) were used as positive and negative controls for IL-8 production.

DATA ANALYSIS

The major groups were *cagA*+ DU, *cagA*+ gastritis, and *cagA*− gastritis. In the results and discussion the gastritis cases are designated as "gastritis". In general the data were not normally distributed and comparisons were made using the Mann-Whitney rank sum test. Spearman's rank test order correlation was used to test for relations between independent variables. A p value of less than 0.05 was accepted as significant.

Results

H PYLORI GENOTYPE

All *H pylori* isolates studied were *vacA* genotype s1-m1. The majority of *cagA*+ cases were also *cagE* and *cagG* positive; two gastritis cases (JK47A, B and JK72A, B) were *cagE* and *cagG* negative. All *cagA* negative strains were also *cagE* and *cagG* negative. There were no inconsistencies in *cagA* status between isolates from the antrum and corpus (antrum *cagA* positive and corpus *cagA* negative).

INFECTION DENSITY AND PRESENTATION

H pylori density was similar when assessed by histological grading or by quantitative culture both in the antrum and corpus (r=0.89, p<0.0001 and r=0.74, p<0.0001, respectively).

H pylori density was not directly related to *cagA* status. In the antrum and corpus the *H pylori* density in gastritis cases was similar in *cagA*+ and *cagA*− patients (antrum: 5.1 (0.6) versus 5.2 (1.9) $\times 10^5$ cfu/mg protein, p=0.65; corpus: 5.7 (1.1) versus 5.0 (2.4) $\times 10^5$ cfu/mg protein, p=0.22, respectively; fig 1).

In the antrum the average *H pylori* density in *cagA*+ DU patients was significantly greater than in *cagA*+ gastritis patients (14.4 (3.3) versus 5.1 (0.6) $\times 10^5$ cfu/mg protein, p<0.05). In contrast, in the corpus *H pylori* density was significantly higher in *cagA*+ gastritis patients compared with *cagA*+ DU patients (5.7 (1) versus 4.3 (1) $\times 10^5$ cfu/mg protein, p<0.05; fig 1).

The density of *H pylori* in both *cagA*+ DU and *cagA*+ gastritis was significantly correlated with antral cellular infiltration with MNC (r=0.83 and 0.67 for gastritis and DU, respectively) and PMN (r=0.80 and 0.79 for gastritis and DU, respectively; fig 2). In contrast, in the corpus the correlation between *cagA*+ *H pylori* density and cellular infiltration with MNC or PMN was not significant (r=0.2 or less for both gastritis and DU; fig 2).

CLINICAL OUTCOME, *cagA* STATUS, AND HISTOLOGY

Antral cellular infiltration was significantly higher in *cagA*+ DU cases than in *cagA*+ gastritis cases and corporal cellular infiltration

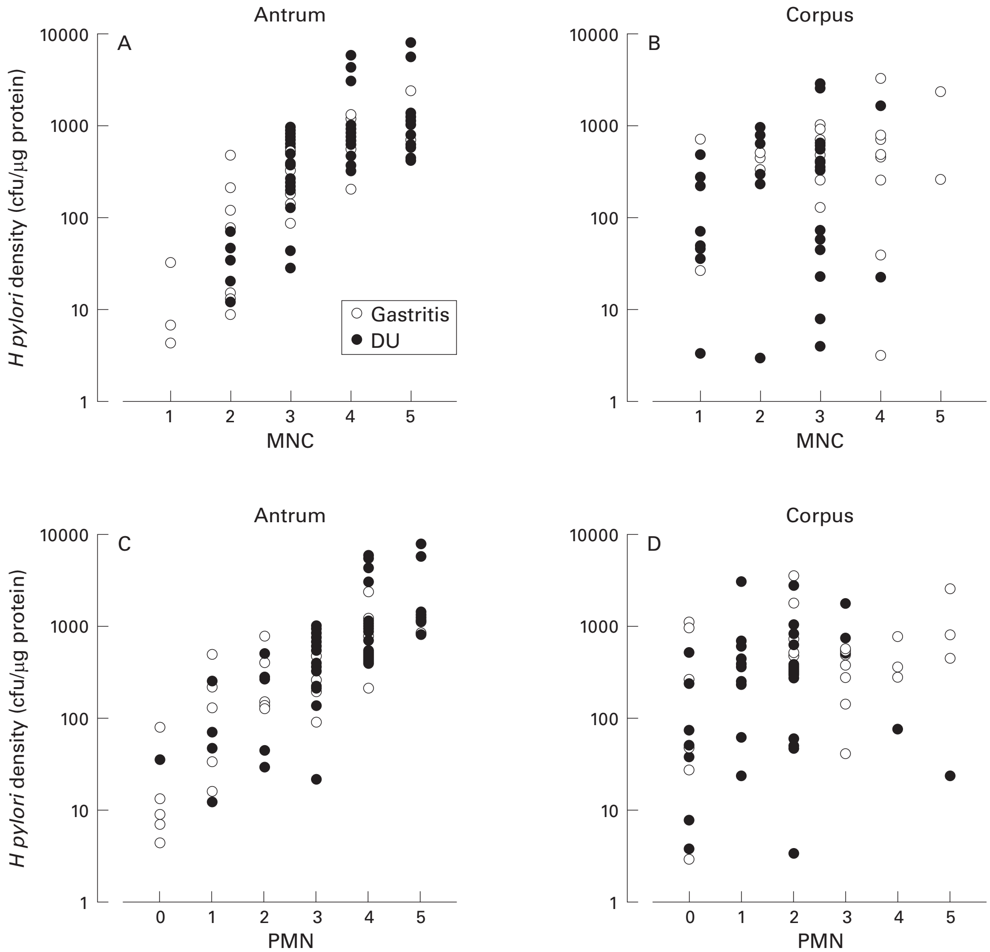

Figure 2 Relation between Helicobacter pylori density by culture and cellular infiltration in cagA+ cases. DU, duodenal ulcer; MNC, mononuclear cell; PMN, polymorphonuclear cell.

was significantly lower in *cagA*+ DU cases than in *cagA*+ gastritis cases (table 1). The degree of mucosal atrophy in both the antrum and corpus was significantly higher in *cagA*+ gastritis cases than in *cagA*+ DU cases. Cellular infiltration and atrophy was present but significantly less in *cagA*− gastritis cases than in *cagA*+ gastritis cases.

IN VIVO IL-1β AND IL-8 PRODUCTION FROM BIOPSY SPECIMENS

Consistent with the findings regarding cellular infiltration, the *cagA*+ DU patients had greater antral IL-1β and IL-8 production than *cagA*+ gastritis patients (IL-1β: median = 81.6 versus 56.5 pg/mg protein, p<0.0001; IL-8: median = 120.8 versus 59.3 pg/mg protein, p<0.001; table 2, fig 3). In contrast, *cagA*+ gastritis patients had significantly higher corporal IL-1β and IL-8 production than *cagA*+ DU patients (IL-1β: median = 79.5 versus 33.6 pg/mg protein, p<0.0001; IL-8: median = 79.2 versus 25.9 pg/mg protein, p<0.0001). None of the *cagA*+ DU patients had antral mucosal IL-8 levels of zero (below detectable levels), whereas 10% of those with *cagA*+ gastritis *H pylori* infection did. In contrast, corporal level of IL-8 was zero in seven of 35 (20%) *cagA*+ DU cases.

In *cagA*− cases, the IL-8 production in biopsy specimens was frequently below the level of detection in both the antrum and the corpus (median = 0 pg/mg biopsy protein; table 2) irrespective of *H pylori* density. The mucosal level of IL-1β was also significantly lower in *cagA*− gastritis cases than in *cagA*+ gastritis cases, both in the antrum and corpus.

Table 1 cagA status, clinical outcome, and histology

	cagA+ DU (n=50)	*cagA+ gastritis (n=50)*	*cagA− gastritis (n=10)*	*cagA+ DU v gastritis p value**	*Gastritis cagA+ v cagA− p value**
Antrum					
MNC	4	3	2	<0.03	<0.005
PMN	4	3	1	<0.04	<0.02
Atrophy	2	3	1	<0.005	<0.001
Corpus					
MNC	2.5	3	1.5	<0.02	<0.03
PMN	2	2.5	1	<0.04	<0.003
Atrophy	1	2	1	<0.0001	<0.008

*By Mann-Whitney sum test.
Mononuclear cell (MNC), polymorphonuclear cell (PMN), and atrophy scores presented as median (score 0–5).
DU, duodenal ulcer.

RELATION BETWEEN MUCOSAL IL-8 AND IL-1β PRODUCTION

In *cagA*+ *H pylori* infections, the antral and the corporal mucosal levels of IL-8 correlated with the levels of IL-1β (r=0.91 and 0.89 for gastritis and DU, respectively in the antrum; r=0.74 and 0.69 for gastritis and DU, respectively in the corpus; p<0.0001 for each).

Table 2 In vivo and in vitro interleukin (IL) 1β and IL-8 production

	Unit	*cagA+ DU*	*cagA+ gastritis*	*cagA− gastritis*	*cagA+ DU v gastritis p value**	*Gastritis cagA+ v cagA− p value**
IL-8						
Antrum						
In vivo	pg/mg protein	120.8	59.3	0.0	<0.001	<0.003
In vivo per *H pylori*	pg/cfu × 10^5	18.8	13.7	0.0	0.12	<0.0001
In vitro	pg/ml	3471	3278	724	0.67	<0.0001
Corpus						
In vivo	pg/mg protein	25.9	79.2	0.0	<0.0001	<0.0001
In vivo per *H pylori*	pg/cfu × 10^5	7.3	15.1	0.0	<0.01	<0.0001
In vitro	pg/ml	3420	3468	622	0.78	<0.0001
IL-1β						
Antrum						
In vivo	pg/mg protein	81.6	56.5	24.8	<0.0001	<0.0001
In vivo per *H pylori*	pg/cfu × 10^5	5.6	3.8	4.2	0.11	<0.0001
In vitro	pg/ml	–	–	–	–	–
Corpus						
In vivo	pg/mg protein	33.6	79.5	34.4	<0.0001	<0.0001
In vivo per *H pylori*	pg/cfu × 10^5	1.3	10.4	0.0	<0.0001	<0.003
In vitro	pg/ml	–	–	–	–	–

*By Mann-Whitney test.
All values presented as medians.
DU, duodenal ulcer.

The mucosal levels of IL-1β and IL-8 protein in both the antrum and corpus were significantly related to *H pylori* density of *cagA*+ strains (antrum: IL-1β: *r*=0.72; IL-8: *r*=0.82; p<0.0001 for each; corpus: IL-1β: *r*=0.63; IL-8: *r*=0.72; p<0.0001 for each; figs 4 and 5). In *cagA*− strains, the antral mucosal level of IL-8 protein was significantly related to *H pylori* density (*r*=0.63, p<0.05) and the corporal mucosal level of IL-1β and IL-8 protein was significantly related to *H pylori* density (IL-1β: *r*=0.67, p<0.05; IL-8: *r*=0.85, p<0.01).

RELATION OF MUCOSAL IL-1β AND IL-8 PRODUCTION TO *H PYLORI* DENSITY

To address the relation between *H pylori* density and cytokine production, biopsy IL-1β and IL-8 production for the *H pylori* density was normalised. The mucosal levels of IL-1β and IL-8 were adjusted by subtracting the

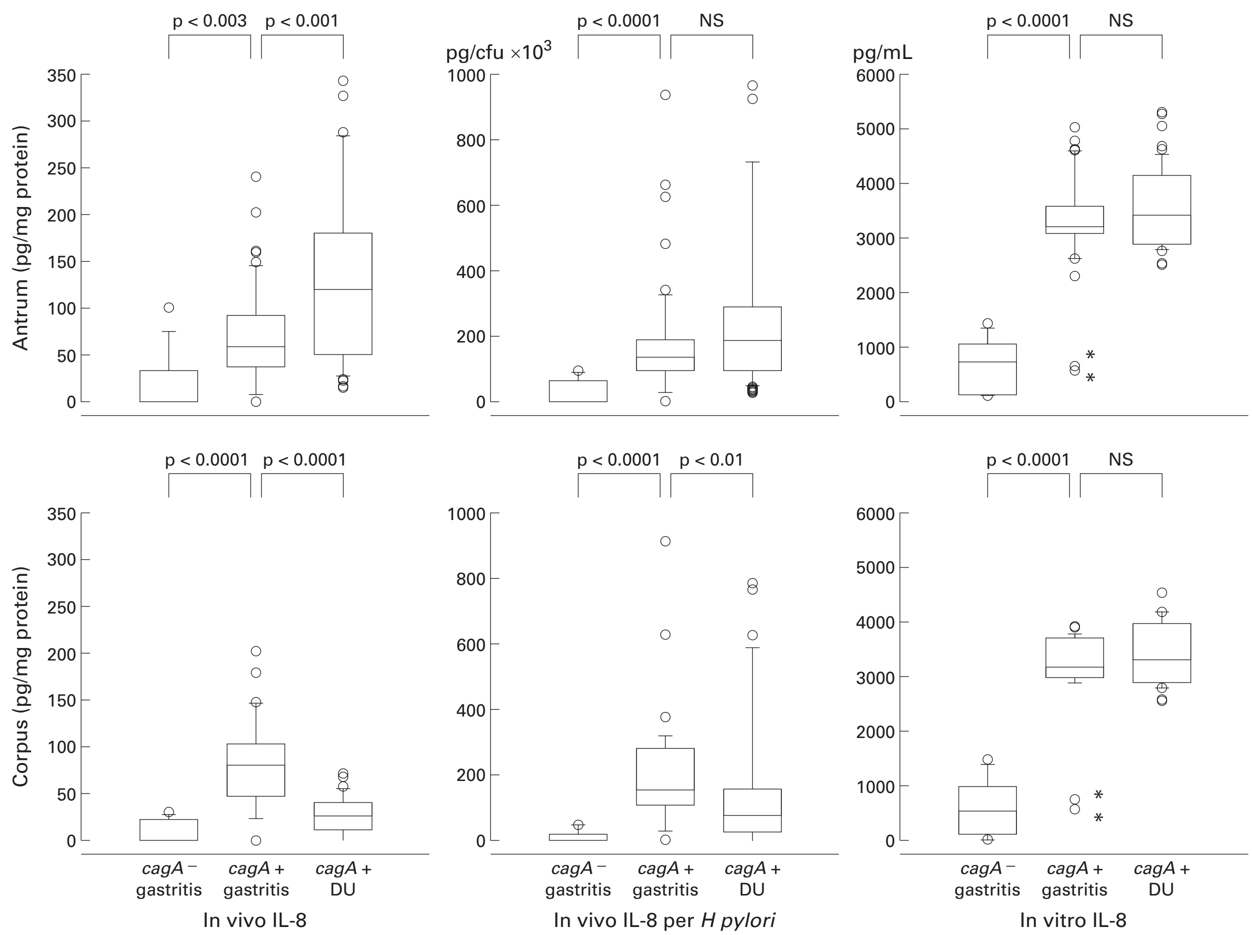

*Figure 3 Mucosal interleukin (IL) 8 production. The end of the bars indicates the 25th and 75th percentiles. The 50th percentile (median) is indicated with a line in the box and the 10th and 90th percentiles are indicated with error bars. *Two cagA+ gastritis cases with extremely low in vitro IL-8 production, which indicate cagA positive, cagE, cagG negative strains. DU, duodenal ulcer.*

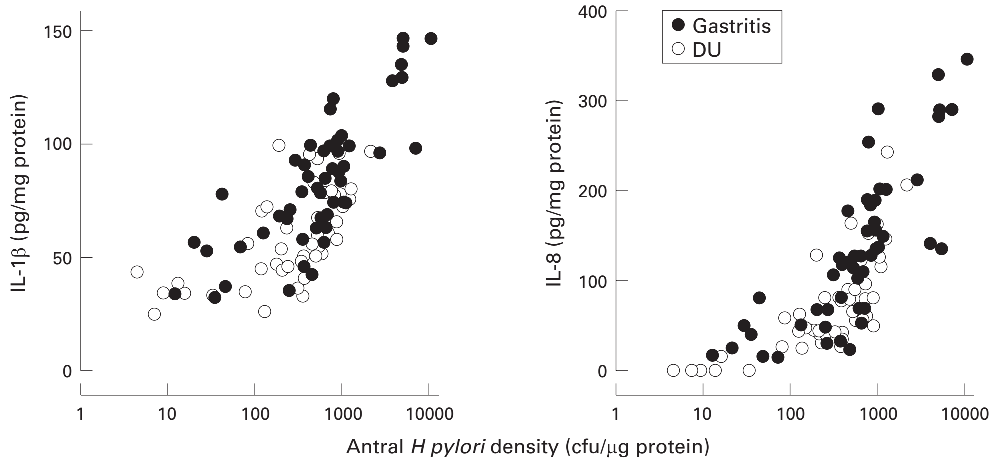

Figure 4 Relation between mucosal interleukin (IL) 1β and IL-8 production and Helicobacter pylori density of cagA+ strains in the antrum.

average level from patients without *H pylori* infection (IL-1β: median = 35.3 and 31.2 pg/mg protein; IL-8: median = 0 and 0 pg/mg protein, in antrum and corpus, respectively). Importantly, the overall interpretation was unchanged whether or not these adjustments were made.

Antral IL-1β and IL-8 production per *H pylori* was similar in *cagA*+ infection irrespective of presentation (IL-1β = 3.8 versus 5.6 × 10^5 pg/cfu, p=0.11; IL-8 = 13.7 versus 18.8 × 10^5 pg/cfu; p=0.12, for gastritis or DU patients, respectively; table 2, fig 3). On the contrary, the corporal IL-1β and IL-8 production per *H pylori* was significantly lower in DU patients than in gastritis patients (IL-1β = 1.3 versus 10.4 × 10^5 pg/cfu, p<0.0001; IL-8 = 7.3 versus 15.1 × 10^5 pg/cfu, p<0.01, for gastritis or DU patients, respectively). In *cagA*− gastritis cases, normalised IL-1β and IL-8 levels were also significantly lower than in *cagA*+ gastritis cases (p<0.0001; table 2).

IN VITRO IL-1β AND IL-8 PRODUCTION FROM GASTRIC CANCER CELL LINE (MKN-45)

IL-8 production from supernatants of medium cocultured with *cagA*+ *H pylori* and MKN-45 cells was approximately equal to *cagA*+ control strain (60190). In this experiment the number of *H pylori* was kept constant and the in vitro IL-8 production did not differ between strains from *cagA*+ DU patients and from *cagA*+ gastritis patients, regardless of whether the isolate was from the antrum or corpus (antrum: median production = 3471 pg/ml versus 3278 pg/ml; corpus: 3420 pg/ml versus 3468 pg/ml, for DU and gastritis, respectively; table 2, fig 3).

The two *cagA*+ gastritis cases which were *cagE* and *cagG* negative by PCR had very low in vitro IL-8 production (JK47A (antrum): 635 pg/ml; JK47B (corpus): 853 pg/ml; JK72A (antrum): 570 pg/ml; JK72B (corpus): 689 pg/ml) and in vivo IL-8 production (JK47: 15.0 (antrum) and 0 (corpus) pg/mg protein; JK72: 0 (antrum) and 23.2 (corpus) pg/mg protein), and cellular infiltration.

The *cagA*− control strain 93–68 did not induce IL-8 (142 pg/ml) whereas the other *cagA*− control strain (Tx30) produced small amounts (860 pg/ml). In vitro IL-8 production was extremely low in *cagA*− cases (median production = 724 pg/ml (antrum) and 622 pg/ml (corpus); table 2 and fig 3). In four patients with zero IL-8 production in vivo in either the antrum or corpus, IL-8 production in vitro was similar to that of the medium control without *H pylori* (median production = 123

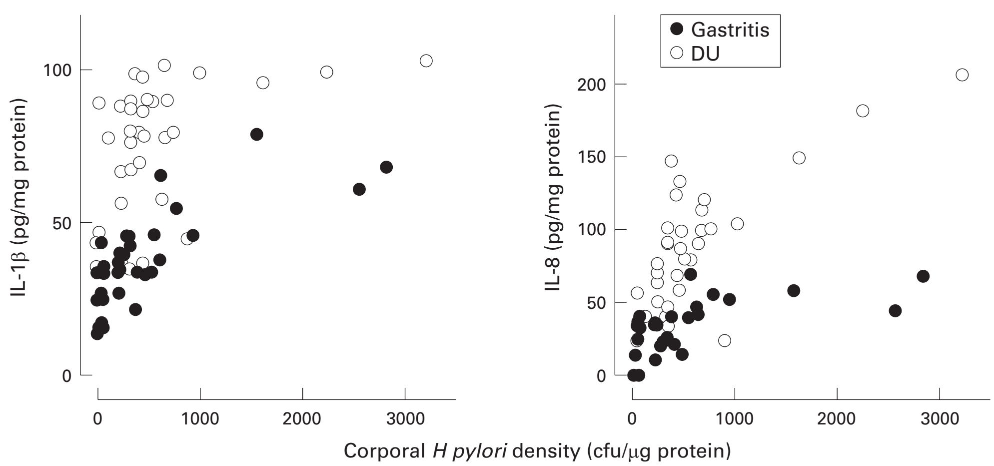

Figure 5 Relation between mucosal interleukin (IL) 1β and IL-8 production and Helicobacter pylori density of cagA+ strains in the corpus.

pg/ml versus 176 pg/ml (control)). The remaining seven *cagA*− cases induced small amounts of IL-8 in vitro (622–1458 pg/ml).

The level of IL-1β protein from supernatants of medium cocultured with *H pylori* and MKN-45 cells was below the threshold of sensitivity in all strains.

IL-1β AND IL-8 PRODUCTION AND HISTOLOGICAL FINDINGS

IL-1β and IL-8 production in biopsy specimens was closely related to cellular infiltration both in the antrum and corpus (IL-1β: MNC, r=0.75, PMN, r=0.74, $p<0.0001$ for each (antrum) and MNC, r=0.45, PMN, r=0.44, $p<0.0005$ for each (corpus); IL-8: MNC, r=0.81, PMN, r=0.83, $p<0.0001$ for each (antrum) and MNC, r=0.41, $p<0.001$, PMN, r=0.49, $p<0.0001$ (corpus)). IL-1β and IL-8 production in biopsy specimens was weakly related to atrophy only in the corpus (IL-1β: r=0.31, $p<0.05$, IL-8: r=0.33, $p<0.01$).

Discussion

This study confirms and extends previous histological observations that duodenal ulcer disease is associated with an antral predominant (or corpus sparing) pattern of gastritis. The average density of *H pylori* in the antrum of DU patients was greater than in the antrum of gastritis patients; the converse was also true (*H pylori* density was lower in the corpus of DU patients than in gastritis patients). We were unable to confirm the hypothesis of Khulusi *et al* regarding the possibility that a threshold density of antral *H pylori* is required for development of DU.[14] We do not believe that the differences between studies are methodological as we prepared the mucosal homogenates immediately after biopsy with aliquots being plated within 10 minutes. It is also unlikely that our results reflect differences in *H pylori* density among specimens as we examined IL-1β and IL-8 production in vivo (from biopsy specimens) and in vitro (coculture of *H pylori* with MKN-45 cells), as well as cytokine production and *H pylori* density from the same biopsy specimens. To our knowledge this study is the first to investigate the relation between in vitro and in vivo cytokine production or *H pylori* density and cytokine secretion on the same tissue.

We also could not confirm the observation by Atherton *et al* that the mean antral density of *cagA*+ strains was significantly higher than that of *cagA*− strains.[4] The majority of their *cagA*+ isolates were from DU patients. The predominance of DU patients precluded them from identifying that the increase in *H pylori* density was related to the clinical presentation (DU) rather than *cagA* status. It is now clear that studies attempting to relate putative virulence factors to outcome must control for both disease and virulence factor (for example, *cagA* status). Our findings that *H pylori* density was independent of *cagA* status have also been confirmed by Warburton *et al*[15] who evaluated *H pylori* density by histology.

In the antrum, IL-1β and IL-8 production were closely related to the *H pylori* density and cellular infiltration. Both IL-1β and IL-8 production were significantly greater in the antrum of DU cases than gastritis cases. Conversely, in the corpus, IL-1β and IL-8 production were significantly lower in DU cases than in gastritis cases. In the corpus both IL-1β and IL-8 production were closely related to the *H pylori* density but not to cellular infiltration. The finding that corporal IL-8 production could not be directly correlated to cellular infiltration is consistent with previous studies[6 16] and with numerous histological studies showing high levels of *H pylori* and low levels of inflammation in DU (antral predominant gastritis).[17] The fact that corpus inflammation increases rapidly after vagotomy or high level antisecretory therapy suggests that the host factor, especially acid secretion, is largely responsible for the disconnection between cellular inflammation, *H pylori* density, and cytokine production in the gastric corpus.[18 19]

The amount of antral mucosal IL-1β and IL-8 produced per viable *H pylori* did not differ between patients with *cagA*+ DU or gastritis. Nevertheless, duodenal ulcer patients exhibited a higher range of both *H pylori* density and the corresponding cytokine levels (fig 4). In both the antrum and the corpus, the relation between *H pylori* density and IL-8/IL-1β levels has the appearance of a typical dose-response curve with a threshold, a rapid increase, and a plateau (figs 4 and 5). In the antrum very low levels of in vivo IL-8 production in *cagA*+ infection were related to the presence of very low *H pylori* density or to the presence of *H pylori* without a functional *cag* pathogenicity island (*cagE* negative). Recently, Maeda *et al* also reported the existence of a *cag* pathogenicity island of some Japanese isolates that was partially deleted and had low ability to induce IL-8 in vitro.[20] Our findings that in vivo IL-8 levels were also lower in *cagA*+, *cagE* negative cases compared with those in *cagA*+, *cagE*+ cases confirm and extend their observations.

The ability of *cagA*+ *H pylori* to induce IL-8 in vitro was similar and independent of whether the strain was obtained from a patient with DU or gastritis, or was obtained from the antrum or the corpus. In those in vitro experiments the quantity of *H pylori* added was the same and the results are consistent with the notion that the amount of IL-8 per functional *cag* pathogenicity island in the antrum is similar in both DU and gastritis. In the corpus, the plateau levels of IL-1β and IL-8 were greater for gastritis patients compared with DU patients and, as a result, in vivo cytokine production per *H pylori* density was also higher in gastritis than in DU. As noted above, because in vitro IL-8 production did not differ between strains from *cagA*+ DU patients and from *cagA*+ gastritis patients, we speculate that the higher acid secretion in DU patients may have been responsible for the different response of cytokine production between DU and gastritis seen in vivo. This hypothesis is currently being tested.

Recent studies have shown that acid secretion is reversibly inhibited in the presence of corpus inflammation and a number of different factors may be responsible for the inhibition of acid secretion, including inflammation related loss of M3 muscarine receptors, and increased concentration of growth factors, such as hepatocyte growth factor, transforming growth factor β1, and gastric juice epithelial growth factor.[18 21 22] In addition, IL-1β is considered to be a possible mediator of this inhibition.[23–25] It is important to note that the method of cytokine measurement used in this study included all forms of mucosal cytokine protein; not all of these may be functional. It is also not known whether there are differences in the number of IL-1β and IL-8 receptors between the antrum and corpus, which could also explain why there might be regional differences in the response to cytokines. While both IL-8 and IL-1β levels were related to *H pylori* density, there was no corresponding increase in inflammation in the corpus. Thus, histological evaluation of corpus inflammation may underestimate the degree of inhibition of parietal secretion present in an individual case.

Infection with *cagA*− strains generally resulted in low to absent vivo IL-1β and IL-8 production, regardless of the density of *H pylori.* Those with low IL-8 production in vivo also had low to absent IL-8 production in vitro and mild cellular infiltration. Of interest, some *cagA*− strains produced no IL-8 in vitro or in vivo. Other *cagA*− strains produced IL-8 in vitro at a level between one third and one half of the *cagA*+ in controls and produced IL-8 in vivo in relation to *H pylori* density. For example, one *cagA*− case had an in vivo IL-8 production of 101 pg/mg protein, which was higher than median IL-8 values of *cagA*+ cases, along with a high density of *H pylori* and severe cellular infiltration. It appears that a virulence factor(s) other than the *cag* pathogenicity island may also be involved in IL-8 production.[26 27] Recently, we found that the 34 K outer membrane protein, HP638 (omp13) plays an important role in IL-8 production from gastric cancer cell lines, AGS cells.[28] Further studies will be necessary to investigate the relation between IL-8 production and virulence factors.

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LETTERS TO THE EDITOR

Towards immunotherapy for pancreatic cancer

EDITOR,—McKenzie and Apostolopoulos' recent article on immunotherapy for pancreatic carcinoma (*Gut* 1999;**44**:767–769) gave an excellent overview. We agree that the poor prognosis of this disease makes it imperative that new agents and novel therapeutic strategies are investigated. However, although this paper discusses classical immunotherapy (where immune competent cells are stimulated to attack pancreatic cancer cells directly), the induction of antibodies directed against growth factors by immunisation (where the immunogen stimulates the immune system to inhibit the growth of tumour cells indirectly) is not mentioned. We are currently undertaking a phase II clinical trial for inoperable pancreatic cancer using one such immunogen, Gastrimmune, which induces neutralising antibodies against amidated gastrin-17 and its precursor glycine extended gastrin-17 (this immunogen is also undergoing a phase II trial for gastric cancer at the University Department of Surgery, Nottingham, UK).

Gastrin has been shown to be a growth factor in a variety of malignancies including colorectal, gastric, and pancreatic cancers in both in vitro and in vivo studies[1]; precursor forms such as progastrin and glycine extended gastrin also have a trophic effect.[2] More recently the autocrine/paracrine pathway, in which tumour cells produce and respond to gastrin, has been shown to be increasingly important.[3] In vitro and in vivo studies have also shown the trophic effect of gastrin and the inhibitory effect of both gastrin receptor antagonists and anti-gastrin antibodies,[1 4 5] and further studies have confirmed gastrin expression in human pancreatic cancer cell lines and resection specimens.[4 6] Thus, there is good evidence to suggest that immunisation against gastrin may be beneficial in the prevention of pancreatic cancer.

We have shown that Gastrimmune induced antibodies inhibit the growth of human pancreatic cancer cell lines,[7] and they have previously been shown to inhibit the growth of gastric, colonic, and hepatocellular cancer cell lines in vitro and in vivo.[8] Over 150 patients have now received Gastrimmune in several trials. The side effect profile has been extremely good and the early efficacy data in colorectal cancer has been encouraging[9]; phase III studies are currently being designed for both pancreatic and colorectal cancer.

Pancreatic cancer has an appalling prognosis. New molecular insights provide encouragement that novel therapeutic strategies may improve the outlook. The immune system can be employed directly and indirectly to target pancreatic cancer cells, and we hope the promise of these new strategies is fulfilled in the next decade.

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Reply

EDITOR,—Brett and Caplin have highlighted that our paper was biased towards cellular immunotherapy. We specifically excluded reference to antibodies, but welcome the opportunity to mention these in the context of immunotherapy of pancreatic cancer.

In the early 1980s, murine monoclonal antibodies offered great hope for the diagnosis and cure of cancer, but by the end of the decade, the outlook was pessimistic. Used alone, murine monoclonal antibodies had little effect, mostly because of the occurrence of HAMA (human anti-mouse response) which curtailed the life of the monoclonal antibody in patients by forming immune complexes; furthermore, the murine Fc pieces of immunoglobulin are particularly poor at marshalling human defence mechanisms to cause inflammation and tumour eradication and thus, these antibodies proved ineffective in the treatment of most types of human cancer. More recently, however, an effort has been made to "humanise" the antibodies, either by making them as chimeras (essentially murine Fab to bind the antigen, coupled with human Fc), or by retaining only the critical murine amino acids so that the rest of the molecule is human. These techniques are achieved by genetic engineering and sophisticated computer modelling. Principally, such humanised antibodies are less immunogenic than their murine counterpart, but this is not always so, and they should be more active with the human Fc piece, by activating complement and macrophages. However, at present such antibodies and are in phase I/II trials, and with several exceptions (see later), trials have not been particularly rewarding. We are particularly pessimistic about the use of antibodies, be they humanised or not, against solid tumours in humans, as we are experienced in rejecting grafts and with tumour grafts using antibody and complement.[2] For mucin 1, we have not been able to cause rejection of human MUC1+ tumours in mice by using large amounts of monoclonal antibodies and additional complement, under circumstances which lead to rapid destruction of lymphoid cells.

However, total pessimism now seems unwarranted. Firstly, murine antibodies in lymphoma and leukaemia have been found to be particularly useful in the treatment of these diseases. The antibodies need to be "armed", especially with isotopes, and using antibodies to CD19 and CD20 ^{131}I labelled antibodies have been effective in patients who are resistant to other forms of treatment.[3]

Furthermore, it takes a long time for appropriate clinical trials to be completed and more patience is needed before abandoning potentially effective treatment. A blanket statement that monoclonal antibodies are not effective in cancer is too broad and this is proved by the use of such antibodies in the treatment of lymphomas; they are particularly sensitive to irradiation and because the patients were immunosuppressed by the disease, they made little HAMA and therefore could be treated successfully.

Antibodies that do not act primarily via their Fc piece by activating complement but have a direct effect on cell surface molecules are another exception. A well known antibody, Her2/neu, reacts with molecules present in 20–30% of patients with breast cancer. Her2/neu molecules have a growth effector function and if they can be blocked on the cell surface by the antibody, which inhibits the binding of the ligand (growth factor), the cells die.[4] This has been further illustrated by the use of Gastrimmune against amidated gastrin-17 by Brett and Caplin. Antibodies to other growth factor receptors have also been described, which are proving to be extremely useful in early clinical trials.[4] Thus, selective antibodies against growth factor receptors may be useful in the treatment of diseases like pancreatic cancer. It is possible that the antibodies will be immunogenic and a HAMA or HAHA (human anti-human antibody) response will occur, but this can only be shown by a clinical trial. There may also be problems with antibodies obtaining access to tumours, or tumours not expressing the appropriate molecules as they de-differentiate. However, these are problems of any cellular or humoral immune response, and are no longer regarded as being peculiar to antibodies. Thus, it is appropriate to consider special antibodies and growth factors to be part of immunotherapy for pancreatic cancer.

Finally, the colon cancer trial in which patients with Dukes's C disease had improved prognoses after receiving 171A antibody, is of interest,[6] although it has still to be established whether the specific or non-specific nature of the antibody was responsible for the improvements. Nevertheless, phase III trials are now in progress to assess this. It is easier to treat disease by immunotherapy if treatment starts at an early stage. Unfortunately, early diagnosis of pancreatic cancer remains difficult—how can a disease with a relatively low frequency be diagnosed in the absence of symptoms? When the symptoms finally appear it may already be too late for immunotherapy.

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Ring-like elevations in the large bowel: endoscopic signs to distinguish the artefact from true neoplastic lesions

EDITOR,—We read with great interest the article by Martin *et al* (*Gut* 1999;**45**:147) on normal histological findings in small depressed lesions of the large bowel. They described three patients with 7–8 mm depressed rectal lesions, similar to small flat or depressed neoplastic lesions, during sigmoidoscopy (two cases) and total colonoscopy (one case). Specimens taken by extensive biopsy or removed by endoscopic mucosectomy were histologically normal with no evidence of neoplasia. Two weeks later, colonoscopy with chromoscopy in one patient failed to locate the lesion. In contrast to true flat adenomas characterised by rough, reddened central mucosa and an irregular outline, the lesions had normal central mucosa and a regular circular elevation. The authors therefore concluded that flat lesions with a regular circular shape and normal central mucosa are likely to be of little significance, and recommended diagnostic cold biopsy in these cases.

Despite their claim of the first report of normal histological findings in small depressed lesions, we had described similar lesions as ring-like elevations.[1] Histological assessment of biopsy and endoscopic mucosectomy specimens of the elevations revealed slight oedema within the lamina propria. Whereas we observed such elevations predominantly in the ascending colon, their lesions were seen in the rectum. Because cleansing preparation fluid tends to be retained often in the ascending colon and rectum, requiring frequent aspiration, we suspect that their lesions are also pseudolesions caused by suction of the mucosa into a colonoscope forming ring-like elevations.[1] Although they mentioned that suction had not been applied to the mucosa, experienced endoscopists usually aspirate retained fluid unconsciously during colonoscopy. Although histological findings of their lesions showed normal mucosa, we suspect that slight oedema was probably present within the lamina propria of their ring-like lesions. We believe their lesions are the same as ring-like elevations.

Some diminutive polyps disappear during maximal colonoscopic insufflation. This phenomenon is invariably associated with hyperplastic polyps, but not with adenomas.[2] Dye-spraying techniques readily visualise the innumerable fine grooves, the so-called innominate grooves, which remain visible in non-neoplastic lesions and in normal colonic mucosa, but not in neoplastic lesions.[3] Because ring-like elevations usually disappear after vigorous air insufflation and the innominate grooves are always visualised in the elevations, the disappearing phenomenon and the presence of innominate grooves in the lesions serve to differentiate this artefact from true neoplastic lesions.[1] With these endoscopic signs, we can avoid biopsy or removal, and thus the cost of pathological examinations.

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Reply

EDITOR,—We value the comments made by Matsushita *et al* and are grateful to them for bringing to our attention their earlier letter which reports ring-like elevations of the colon thought to be due to suction artefacts.[1] Although we agree with their points regarding the use of suction by experienced colonoscopists, we believe it is unlikely that the lesions we reported were suction related. All of our lesions were initially visualised in the distance, away from the colonoscope tip, and showed no signs of mucosal trauma such as spotted haemorrhage, on close inspection. In addition, there was no histological evidence of increased mucosal oedema to suggest suction trauma.

Maximum air insufflation and observation of these lesions over several minutes was performed routinely, and a striking feature noted was their "fixed nature". We agree that if a normal groove pattern is seen following dye spray and the lesion disappears after air insufflation, then biopsy is unnecessary. However, if lesions fail to disappear, as in the cases we reported, some doubt must remain regarding their nature. In this situation biopsy seems a safe precaution, particularly given the relatively high incidence of advanced neoplasia in true flat adenomas.

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Liver biopsy under ultrasound control: implications for training

EDITOR,—As a gastroenterologist/hepatologist, I appreciate the anxiety expressed by Shah *et al* (*Gut* 1999;**45**:628–629) about having to surrender liver biopsy samples to radiologists as a result of reduced training opportunities. The desire of an overwhelming number of gastroenterology trainees to become proficient in ultrasound techniques[1] should send a loud and clear message to the relevant regulatory training bodies. Once ultrasound has been introduced into gastroenterology training programmes in the United Kingdom, such as in Europe[2] or the USA,[3] the questions raised by Shah *et al* would have been partially solved, albeit indirectly.

The purpose of ultrasound guidance in liver biopsy is threefold: (i) to target the liver, (ii) to target the lesion, and (iii) to avoid the gall bladder.[4] In this day and age, it would be unthinkable to perform a blind liver biopsy on a patient who has a discrete liver lesion. However when it comes to diffuse or generalised disease, "X marks the spot" or ultrasound assisted technique should suffice. Although the liver is a large and superficial organ, targeting it, even for diffuse or generalised disease, should not be left completely to chance. In the ultrasound assisted technique, the procedural aspect of liver biopsy is essentially blind subsequent to the initial ultrasound to mark the spot. Therefore becoming proficient in this technique would prevent loss of expertise in the blind approach, and yet the medicolegal position remains sound.

Inadvertent biopsy of the gall bladder can be minimised by allowing the patient to have a light breakfast, as the gall bladder becomes contracted following a meal.[4] However, the more cautious would prefer patients to be fasted, in case they develop a complication requiring operative intervention.

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Reply

EDITOR,—Dr Chuah makes some valuable points about the problems of training in gastroenterology in relation to ultrasound. One of the key messages of our article was to differentiate the "X marks the spot" ultrasound technique from the real time ultrasound guided method, which is in standard use within our hospitals. Using this technique, the needle is continuously visualised throughout its time within the liver and therefore there is minimal risk of biopsy of gall bladder or intrahepatic vessels. We consider this to be the safest technique to use but it is also the most difficult method in which to become proficient. Most training schemes for specialist registrars in gastroenterology will have difficulty in accommodating the additional time required to learn this technique.

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BOOK REVIEW

Vitamin D. Molecular Biology, Physiology and Clinical Applications. Edited by Holick MF. (Pp 458; illustrated; $145.00.) US: Humana Press, 1999. ISBN 0 896 03467 4.

A comprehensive study of vitamin D, this book starts with a brief consideration of the evolutionary aspects of vitamin D and the essential role of photosynthesis of the vitamin in the conservation of calcium in aquatic and land animals. Cutaneous synthesis is the principal source of vitamin D for most healthy people but dietary intake becomes increasingly important in the very young and the elderly. Adequate intakes (formerly called recommended daily allowances) for all age groups, and for pregnant or lactating women, are provided and the central question of how to define vitamin D deficiency is revisited; based on serum parathyroid hormone responses to vitamin D supplementation, a threshold level of intake of 20 ng/ml (50 nmol/l) is suggested.

Vitamin D deficiency is a common side effect of hepatic and gastrointestinal diseases and often results in bone disease; gastroenterologists should, therefore, have some knowledge of the causes, consequences, and treatment of vitamin D related bone disorders. It also has a wide range of actions which are unrelated to its effects on calcium metabolism; receptors for its active metabolite, 1,25-dihydroxyvitamin D, are found in many places including the stomach, thymus, immune system, gonads, and some cancer cells. The antiproliferative and prodifferentiation effects of vitamin D have already been exploited in the development of treatment for psoriasis and other skin disorders and the exciting potential applications of vitamin D in some malignant diseases are discussed towards the end of the book.

Since the pivotal research in the 1960s on the metabolism of vitamin D there has been intense research activity in a number of related areas, including the synthesis and metabolism of vitamin D metabolites and analogues, the molecular biology of the vitamin D receptor, and the mechanisms by which 1,25-dihydroxyvitamin D affects the renal, intestinal, and skeletal transport of calcium. These aspects are covered in considerable detail and occupy about one half of the book; there is also a detailed chapter on the methodology for assays of vitamin D, although the authors do not discuss the usefulness of these assays in clinical practice. The latter part of the book is devoted to clinical issues—for example, rickets and osteomalacia, osteoporosis, inherited defects of vitamin D metabolism, and the pathophysiology of hypercalcaemia associated with the extrarenal production of 1,25-dihydroxyvitamin D, which occurs in conditions such as sarcoidosis and lymphoproliferative disorders. There is also an interesting chapter on the epidemiology of cancer risk and vitamin D. Disappointingly, at least for the gastroenterologist, there is very little coverage of vitamin D deficiency associated with hepatic and gastrointestinal disorders.

The book is well produced and has many illustrations and diagrams; it provides an excellent and comprehensive account of the substantial advances occurring in this area. Furthermore, the chapters are well referenced, many containing over 100 references. This book is not for the gastroenterologist who wishes to extract information about the diagnosis and management of vitamin D deficiency in clinical practice, but will be highly valued by those with a close interest in following the fascinating progress of this hormone.

J E COMPSTON

CORRECTIONS

An error occurred in the keys to figures 4 and 5 of the paper by Yamaoka et al (Gut 1999;45:804–11). Gastritis should be represented by open circles and duodenal ulcer by closed circles. We apologise for any confusion this error may have caused.

The authors of Nardone et al (Gut 1999;44:789–99) have conceded an error. Figure 3(B) was an inverted image of figure 3(A) at a different magnification. The correct figure is published below. The authors regret any confusion this may have caused.

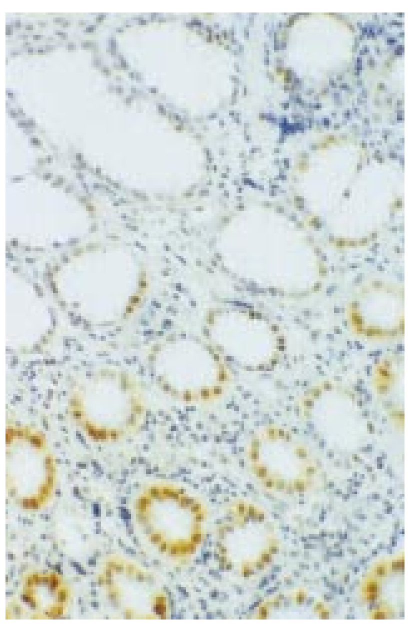

Figure 3(B) H pylori positive chronic gastritis. Original magnification × 250.

NOTES

The Wellcome Institute for the History of Medicine with the 20th Century History of Medicine Group present A Witness Seminar—Peptic Ulcers: rise and fall in the twentieth century

This seminar will be held on 12 May 2000 in London. Registration is £15 (Students/Friends £10) and the closing date is 5 May 2000. For registration/further information: Ms Frieda Houser, The Wellcome Institute for the History of Medicine, 183 Euston Road, London NW1 2BE, UK. Tel: +44 (0)20 7611 8619/8888.

Falk Symposia and Workshops

The Symposium on Hepatology 2000 will be held in Munich, Germany, on 4 and 5 May 2000.

The Symposium on Hiking and Health will be held in Titisee, Germany, on 19 and 20 May 2000.

The Workshop on Hepatobiliary Diseases: Cholestasis and Gallstones will be held in Cluj Napoca, Romania, on 9 and 10 June 2000.

The Symposium on Non-Neoplastic Diseases of the Anorectum—An Interdisciplinary Approach on 1 and 2 October 2000, and the Symposium on Immunosuppression in Inflammatory Bowel Diseases—Standards, News, Future Trends on 3 and 4 October 2000 will be held at Gastroenterology 2000 in Freiburg, Germany.

The Symposium on Biology of Bile Acids in Health and Disease will be held at the XVI International Bile Acid Meeting in Den Haag, The Netherlands on 12 and 13 October, 2000.

The Symposium on Steatohepatitis (NASH and ASH) will be held in Den Haag, The Netherlands, on 14 and 15 October.

The Symposium on Chronic Inflammatory Bowel Diseases—Progress and Controversies at the End of the Century will be held in Bucharest, Romania, on 4 November 2000.

For further information on any of these symposia or workshops, please contact: Falk Foundation e.V.—Congress Division, Leinenweberstr. 5, PO Box 6529, D-79041 Freiburg, Germany. Tel: +49 761 15140; fax: +49 761 1514359; email: symposia@falkfoundation.de

Digestive Disease Week

The Digestive Disease Week will be held at the San Diego Convention Centre, San Diego, California, USA, on 21–24 May 2000. Further information from: DDW Administration, 7910 Woodmont Avenue, 7th Floor, Bethesda, Maryland 20814, USA. Tel: +1 301 272 0022; fax: +1 301 654 3978; website: www.ddw.org