Systemic and mucosal humoral responses to *Helicobacter pylori* in gastric cancer

J E Crabtree, J I Wyatt, G M Sobala, G Miller, D S Tompkins, J N Primrose, A G Morgan

Abstract

The systemic IgG response to *Helicobacter pylori* was examined in 70 patients with gastric cancer. *H. pylori* IgG antibodies were assayed by enzyme linked immunosorbent assay (ELISA), and serological recognition of *H. pylori* antigens was characterised by western blotting. A percentage of 78.5 were seropositive by ELISA. Two of five patients under age 50 were seronegative. Positivity was unrelated to age, sex, tumour type, or site. Ninety one per cent of ELISA positive cancer patients recognised the *H. pylori* cytotoxin associated 120 kilodalton (kD) protein, significantly more than a control group of 47 ELISA positive patients with non-ulcer dyspepsia (72%). Four of 15 ELISA negative cancer patients also showed recognition of this protein in western blots. Mucosal IgA responses to *H. pylori* were examined by immunoblotting supernatants of in vitro cultured resected antral mucosa in an overlapping group of 19 gastric cancer patients. Eighteen had a positive response, including 10 of 11 negative for *H. pylori* by biopsy urease testing. The systemic and local immunoblotting results show that the high seroprevalence of *H. pylori* antibodies detected by ELISA is nevertheless an underestimate of past infection. Dyspepsia screening policies based solely on *H. pylori* ELISA would miss some young patients with gastric cancer. Further study of the relation of the *H. pylori* cytotoxin to gastric precancerous lesions is warranted.

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Helicobacter pylori is a causative agent of chronic gastritis¹⁻³ and infection is strongly associated with peptic ulcer disease.¹ There is a strong systemic⁴⁻⁷ and mucosal^{8,9} antibody response to the bacterium. Patients whose gastritis has progressed to severe atrophy have a lower incidence of gastric *H. pylori* infection than non-atrophic subjects,¹⁰⁻¹² but the former are often *H. pylori* seropositive, indicative of previous infection.^{10,11}

Atrophic gastritis is a precursor condition for gastric carcinoma¹³ and therefore the question has been raised as to whether *H. pylori* is a causative factor in gastric carcinogenesis. Three longterm prospective studies have established a positive association between *H. pylori* seropositivity and subsequent development of gastric carcinoma.¹⁴⁻¹⁶ Additionally, in regions with a high incidence of gastric cancer, there is a high level of *H. pylori* seropositivity^{17,18} particularly in children.¹⁸ Histological studies, however, which have examined the relation between gastric cancer and *H. pylori* infection¹⁹⁻²² have found a great variability in the histological fre-

quency of *H. pylori*, ranging from 19 to 80%.²³ This probably reflects problems associated with obtaining adequate non-tumour tissue and in detecting the organism in resection material. Additionally, extensive intestinal metaplasia¹¹ or changed gastric microflora associated with hypochlorhydria may militate against continued infection with *H. pylori*.¹⁰ *H. pylori* serology in patients presenting with gastric cancer should give a more accurate indication of both current and past¹⁰ infection. There are currently, however, only limited serological data available from the United States²⁴ and Finland.²¹

The relations between *H. pylori* positivity and gastric cancer type – (that is, intestinal *v* diffuse) and tumour site – (that is, cardia *v* non-cardia) are still unclear. Furthermore, the possibility of using *H. pylori* serology to screen dyspeptic patients before endoscopy,²⁵ makes it imperative that we know the prevalence of *H. pylori* seropositivity in patients with gastric cancer. The aims of this study were firstly to examine *H. pylori* seropositivity in a United Kingdom population with gastric cancer and its relation to age, tumour type, and site.

Secondly, as there is increasing evidence of strain heterogeneity in *H. pylori*^{26,27} and recent studies from an area with a high incidence of gastric cancer have shown that cytotoxic strains of *H. pylori*²⁶ are more frequently associated with atrophic gastritis,²⁸ we characterised the specific *H. pylori* antigens recognised serologically by western blotting with particular respect to the cytotoxin associated 120 kilodalton (kD) protein.²⁷

Finally, as severe atrophic gastritis can be associated with histological negativity for the organism but serological positivity,¹⁰ we examined the mucosal IgA antibody response to *H. pylori* in patients with gastric cancer and its relation with bacterial positivity and systemic IgG responses.

Methods

GASTRIC CANCER PATIENTS

Serum samples were obtained from 70 patients with gastric cancer (mean age 66.7, 43 men, 27 women) diagnosed between 1989 and 1991. Samples were taken either at the time of endoscopy or before gastric surgery. Patients who had received recent blood transfusions and those who had had previous gastric surgery were excluded. Serum was stored at -20°C until assayed.

The gastric carcinomas were classified histologically according to the Lauren system²⁹ by one histopathologist without knowledge of the experimental results.

Freshly resected non-neoplastic antral mucosa

Departments of Clinical Medicine

J E Crabtree
G M Sobala
G Miller
J N Primrose

and Pathology, St James's University Hospital, Leeds
J I Wyatt

Public Health Service Laboratory, Leeds
D S Tompkins

Gastroenterology Unit, Airedale General Hospital, Keighley
A G Morgan

Correspondence to: Dr J E Crabtree, Department of Clinical Medicine, St James's Hospital, Leeds LS9 7TF

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was obtained from an overlapping group of 19 patients (mean (SEM)) age 68.2 (9.8) with gastric adenocarcinoma (2 cardia, 17 non-cardia). Serum was available from seven of these subjects. Five biopsy specimens were taken for examination of urease activity using the CLO test (Delta West Ltd, Bentley, Australia). The remaining antral mucosa was cultured in vitro.

As resected gastric mucosa from patients without neoplasia was not available, antral endoscopic biopsy specimens obtained from 25 patients with histologically normal and *H pylori* negative antral mucosa (mean age 46 (16.7)) were used as controls for western blotting.

To determine the prevalence of serum IgG antibodies to the 120 kD cytotoxin associated protein of *H pylori* in ELISA positive patients without gastric cancer, 47 patients with non-ulcer dyspepsia (24 men, 23 women, mean age 52.2 (15.9)) from the same regional area were examined.

IN VITRO CULTURE

Using aseptic techniques, freshly resected gastric mucosa was sectioned into biopsy sized pieces and cultured in vitro for three days in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 40 µg/ml gentamicin as previously described.³⁰ The medium was changed daily and culture supernatants stored at -70°C until western blotting was done. Endoscopic antral specimens were cultured using similar techniques.

ELISA FOR *H PYLORI*

Serum *H pylori* IgG antibodies were assayed by ELISA as previously described.^{7,8} This assay uses an ultracentrifuged sonicated antigen preparation of *H pylori*. The cut off for positivity was determined using serum from 116 patients of known histological *H pylori* status. The sensitivity and specificity of the ELISA were 97% and 95% respectively.

SDS-PAGE AND WESTERN BLOTTING

Whole cell preparations of *H pylori* (NCTC 11637), grown for 72 hours on Columbia agar (Oxoid, Basingstoke) with 5% defibrinated horse blood, were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis as previously described.^{7,8} After electrophoresis (10% separating gel, 5% stacking gel), proteins were transferred to nitrocellulose paper (Schleicher and Schuell Co) by semi dry blotting (80 minutes at 145 mA in an LKB NovaBlotter). Serum samples were immunoblotted for *H pylori* IgG antibodies at a dilution of 1 in 50 in a miniblitter apparatus (Biometra, Manchester) as previously described.⁷ Positive and negative control serum samples were included in each assay.

Gastric culture supernatants from cancer patients and controls were immunoblotted for *H pylori* IgA antibodies.^{8,9} Immunoblots were considered positive if five or more bands were present. To confirm the specificity of the local IgA response to *H pylori*, positive culture supernatants were preabsorbed for two hours at 26°C

with whole cell preparations of *H pylori* as previously described⁸ and immunoblotted together with unabsorbed supernatants. Culture supernatants were also immunoblotted against whole cell preparations of *Campylobacter jejuni* (NCTC 11168), *C fetus* subsp *fetus* (NCTC 10348) and *C sputorum* subsp *sputorum* (NCTC 11528).

STATISTICAL ANALYSIS

The association of positivity with age was tested for by a χ^2 test for linear trend. Differences in proportions between groups were tested by Yates's corrected χ^2 or Fisher's exact test as appropriate.

Results

H PYLORI SEROLOGY

Fifty five of 70 gastric cancer patients (78.6%, 95% CI: 67.1 to 87.5%) were seropositive for *H pylori* IgG antibodies by ELISA. The percentage of seropositives was similar in men (79%) and women (78%).

Table I shows the relation between *H pylori* seropositivity and age of presentation with gastric cancer. Three of five patients under 50 years were seropositive (aged 38, 41, and 47). The two *H pylori* seronegative patients were 44 and 37 years. There was no significant trend for seropositivity with age ($\chi^2=0.047$, $p>0.80$) (Table I).

Eighteen of 70 patients had adenocarcinoma of the cardia, 47 had non-cardia cancer, and five gastric tumours were of unclassified origin because of extensive tumour infiltration. *H pylori* seropositivity in patients with non-cardia cancer (83%) was not significantly different from those with cancer of the cardia (72%) (Fisher's exact test 2p >0.04).

Table II shows the relation between the histological type of cancer and *H pylori* serology. There was a trend for increased seropositivity in intestinal type cancer compared with diffuse type but this did not reach significance (Yates's corrected $\chi^2=1.99$, $p>0.15$).

WESTERN BLOTTING ANALYSIS OF SERUM IgG AND GASTRIC IgA RESPONSES TO *H PYLORI*

Figure 1 shows the serum IgG recognition of *H pylori* whole cell preparations in western blots. In the gastric cancer patients antigen recognition patterns by the systemic IgG response were comparatively homogeneous particularly with respect to antigens between 60 to 120 kD. Fifty of 55 ELISA positive patients with gastric cancer (91%, 95% CI: 80 to 97%) showed serum IgG recognition of the cytotoxin associated 120 kD protein. Interestingly, four of 15 ELISA negative cancer patients (27%) also recognised this protein (Fig 1). The prevalence of recognition of the 120 kD protein in the ELISA positive cancer patients (91%) was significantly higher than in the ELISA positive controls with non-ulcer dyspepsia (72%) (Yates's corrected $\chi^2=4.80$, $p=0.028$).

The biopsy urease test was positive at 24 hours

TABLE I Relation between *H pylori* ELISA positivity and age in patients with gastric cancer

Age	No	Positive	%
Under 50	5	3	60
50-59	13	11	85
60-69	21	17	81
Over 70	31	24	77

TABLE II Relation between *H pylori* seropositivity and histopathology

Histology	No	Positive	%
Diffuse	33	23	70
Intestinal	26	23	88
Mixed	6	5	83
Unclassified	5	4	80

Figure 1: Western blot of serum IgG antibodies to *H pylori* in patients with gastric cancer. Track 1 = negative second layer only control, tracks 2–13 *H pylori* ELISA positive patients, tracks 14–18 ELISA negative patients. Left hand figures refer to molecular weight standards in kilodaltons. Tracks 2–13 and 16–18 show recognition of the 120 kD protein.

in eight of 19 patients (42%) from whom fresh non-neoplastic resected gastric mucosa was obtained. Western blotting of the gastric culture supernatants showed a positive mucosal IgA response to *H pylori* in 18 of 19 subjects (95%) (Fig 2). Ten of 11 patients who were negative by the biopsy urease test nevertheless had a strong IgA response to the bacterium (Fig 2). The patient in track 1 was judged as negative, lacking strong recognition of five or more bands. By contrast with the systemic IgG responses, the mucosal IgA recognition of *H pylori* showed considerable heterogeneity.

Preabsorption with *H pylori* removed immunoreactivity in western blotting (Fig 3). Immunoblotting of gastric culture supernatants for IgA

Figure 2: Western blot of IgA antibodies to *H pylori* in gastric culture supernatants of cancer patients. C = *H pylori* negative control with histologically normal antral mucosa. Tracks 1–11 urease negative patients, tracks 12–19 urease positive patients. Left hand figures refer to molecular weight standards in kilodaltons.

antibodies against *C jejuni*, *C fetus*, and *C sputorum* showed minimal cross reactivity. By contrast with the gastric cancer patients, only one of 25 culture supernatants of histologically normal endoscopic and *H pylori* negative antral specimens showed a positive mucosal IgA reactivity to *H pylori* by western blotting.

Comparative analysis of the local IgA and systemic IgG responses to *H pylori* in patients with gastric cancer (Fig 4) showed some variability in antigen recognition patterns between mucosal and systemic sites. Local IgA positivity to *H pylori* was evident in three ELISA IgG seronegative subjects. These three patients showed minimal IgG response by western blotting recognising less than five bands, but one (track 5) recognised the 120 kD protein.

Discussion

The 78.5% *H pylori* seropositivity in patients with gastric cancer seen in this study confirms reports from the United States²⁴ and Finland²¹ of a high prevalence of *H pylori* infection in gastric cancer. In these latter studies, *H pylori* seropositivity was 52% (both cardia and non-cardia)²⁴ and 70% (non-cardia only).²¹ Although local data exist for the seroprevalence of *H pylori* infection in dyspeptic patients³¹ and blood donors,³² neither of these are an adequate control group to contrast against the cancer patients and therefore such an analysis was not performed. An ideal control group would be age, sex, social class, and geographically matched, for example, patients drawn from general practitioner patient registers. This was not practicable within the constraints of this study.

We found no significant trend for seropositivity with age in gastric cancer patients in this study, confirming earlier findings of Sipponen *et al.*²¹

No significant difference in *H pylori* seropositivity was found between patients presenting with cardia *v* non-cardia cancer. This contrasts with the results of Talley *et al.*,²⁴ who found a significantly lower seropositivity in cancer of the cardia.²⁴ Two longterm prospective studies from the United States also suggest a lack of association between *H pylori* infection and cancer of the cardia.^{14,16} Our findings with respect to cardia cancers may reflect high background prevalence of *H pylori* seropositivity locally; the incidence of which in local dyspeptic patients over 70 years of age is as high as 70%.

H pylori seropositivity was similar in both intestinal and diffuse types of gastric cancer. Although one histological study suggested *H pylori* prevalence in the intestinal type was greater than diffuse gastric cancer,²⁰ both long-term prospective studies^{14,16} and this and other recent serological studies^{21,24} in patients presenting with gastric cancer show equal prevalences of *H pylori* infection with both histological types of gastric cancer.

Two of the subjects under 45 years of age with gastric cancer were *H pylori* seronegative. Both had diffuse type gastric cancer and their non-neoplastic gastric mucosa was histologically normal. About 25% of gastric cancers are considered not to be associated with *H pylori* infec-

Figure 3: Western blot of IgA antibodies to *H pylori* in three gastric cancer culture supernatants before (tracks 1, 3, and 5) and after (tracks 2, 4, and 6) absorption with *H pylori*. C=*H pylori* negative control with histologically normal antral mucosa. Left hand figures refer to molecular weight standards in kilodaltons.

tion.²¹ If *H pylori* serology had been used for screening dyspeptic patients,²⁵ these two subjects would have been excluded from endoscopy unless other clinical parameters pointed to neoplasia, suggesting a need for caution in adopting such screening methods.

The patients with gastric cancer showed a high percentage of serological recognition of the cytotoxin associated 120 kD protein of *H pylori* compared with those with non-ulcer dyspepsia. Although the two groups were not age matched, there is no *a priori* reason why 120 kD protein recognition should vary with age in ELISA positive subjects, nor was any such age related trend evident in either group of patients. Worldwide studies show that the incidence of cytotoxicity in *H pylori* strains is about 55%.³³ The recent findings of Fox *et al.*,²⁸ showing a high prevalence of cytotoxin positive strains in chronic atrophic gastritis in New Orleans, suggest that the cytotoxin may have a role in cancer precursor lesions as well as being associated with peptic ulceration.^{9 26 27 34} Treatment studies have shown that patients whose antibody concentrations fall to below the threshold for ELISA seropositivity retain western blot positivity for the 120–130 kD protein up to 24 months after eradication.³⁵ The 120 kD protein is not enriched in the antigen preparation used in the ELISA and addition of purified 120 kD protein to standard ELISA antigen preparations improves serological detection of *H pylori* infection in dyspeptic patients.³⁶ Twenty seven per cent of the ELISA negative subjects with gastric cancer in this study recognised this protein by western blotting pointing to previous *H pylori* infection. Thus the true prevalence of current and previous *H pylori* infection in patients developing gastric cancer could be greater than that detected serologically by ELISA at the time of presentation. The presence of a gastric IgA response to *H pylori* in

three ELISA negative patients supports this viewpoint.

This study has shown a strong mucosal IgA response to *H pylori* in non-neoplastic antral mucosa of gastric cancer patients irrespective of the biopsy urease results. Although patients received perioperative intravenous antibiotics, these should not have immediately affected bacterial urease activity. Perhaps more sensitive detection techniques such as polymerase chain reaction^{37 38} would have shown bacterial colonisation in the urease negative patients. It is now well established, however, that seropositivity for *H pylori* can be associated with histologically *H pylori* negative atrophic gastritis in patients with²¹ or without gastric cancer.^{10 11} The maintenance of a specific local immune response in the absence of *H pylori* could result from a number of causes. Lipopolysaccharide from non-*Helicobacter* gram negative organisms, which colonise the gastric environment after the development of hypochlorhydria, could stimulate the local production of cytokines such as interleukin-6. Interleukin-6 is important in terminal maturation of B cells³⁹ and could non-specifically stimulate gastric mucosal B cells thus maintaining a local *H pylori* specific IgA response after eradication of infection. Our recent studies have shown very high secretion of antral interleukin-6 in patients with extensive intestinal metaplasia⁴⁰ and epithelial cells are becoming increasingly recognised as a potential source of proinflammatory cytokines.^{41 42} Alternatively, the presence of cross reacting epitopes between *H pylori* and the gastric mucosa^{43 44} could be responsible for the maintenance of bacterial specific responses after the loss of *H pylori* infection with gastric atrophy.

In conclusion, this study shows a high prevalence of *H pylori* seropositivity in patients in the United Kingdom with gastric cancer, which did not vary significantly with age, site, or type of tumour. Western blotting data suggest that the ELISA results are an underestimate. Nevertheless, dyspepsia screening policies based on *H pylori* ELISAs would miss a few young patients with gastric cancer. The high percentage recognition of the cytotoxin associated 120 kD protein in gastric cancer patients warrants further investigations into the possible role of the cytotoxin in pathogenicity.

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Figure 4: Western blot of gastric IgA (A) and serum IgG (G) antibodies to *H pylori* in patients with gastric cancer. C=control with histologically normal antral mucosa. Tracks 1–4 *H pylori* ELISA positive patients, tracks 5–7 ELISA negative patients. Left hand figures refer to molecular weight standards in kilodaltons.

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