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GASTROENTEROLOGY

Selective reduction of anti-*Helicobacter pylori* IgG subclass antibody in gastric carcinoma

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Abstract

Background: Epidemiological studies have demonstrated strong links between *Helicobacter pylori* infection and gastric adenocarcinoma. Recent studies suggest that cell-mediated immunity influences the outcome of infection, including the development of gastric adenocarcinoma. The T-cell response can be characterized in terms of the secreted cytokine profile, which in turn influences the B-cell response including the balance of IgG subclass antibody.

Methods: Serum anti-*H. pylori* IgG, IgG1 and IgG2 antibodies were studied by ELISA in subjects with benign gastric diseases, gastric dysplasia and gastric adenocarcinoma.

Results: The distribution patterns of IgG subclass anti-*H. pylori* antibody varies significantly between *H. pylori*-linked benign and malignant disease in subjects infected with *H. pylori*. Significantly lower IgG2 levels were found in subjects with gastric adenocarcinoma compared with those with reflux esophagitis, chronic gastritis, gastric ulcer, and peptic ulcer, while IgG1 antibody remained at similar levels in both benign and malignant disease. A limited study of seropositive subjects with premalignant change was consistent with the fall in IgG2 antibody pre-dating malignant change, although pre-eradication results are needed to validate these data.

Conclusions: These studies indicate that subjects with low levels of IgG2 anti-*H. pylori* antibody are at risk of gastric adenocarcinoma, and that the previously described linkage between gastric adenocarcinoma and low total IgG antibody does not simply reflect reduced gastric colonization. The diagnostic value of this assay for pre-endoscopy screening is attractive.

Key words: dysplasia, gastric adenocarcinoma, *Helicobacter pylori*, IgG subclass.

INTRODUCTION

The diagnostic value of serology for *Helicobacter pylori* infection has been extensively studied, with the better assays having sensitivity and specificity for infection at around 90%.[1,2] Despite this good clinical correlation, antibodies do not appear to influence eradication of the bacteria from the stomach.[3] Epidemiological studies in the early 1990s showed that seropositivity was associated with an increased risk for the development of gastric cancer (GC),[4,5] leading to the International Agency for Research on Cancer to classify *H. pylori* as a class 1 carcinogen in 1994.[6] Subsequent study conclusions were more ambivalent although a meta-analysis by Forman showed that a longer time interval between blood sampling and cancer diagnosis strengthened the association.[7,8] These observations were all consistent with the hypothesis that *H. pylori* produces GC by inducing gastric atrophy that is linked to a reduction in colonization and thus antigen stimulus.[9] This concept was supported

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by a prospective study of more than 10 000 Japanese subjects having endoscopy, where 'weakly positive antibody tests' and an age over 60 were a high risk for GC.[10] In this latter study, elderly subjects (≥70 years) with weakly positive antibody assays had three times the prevalence of GC compared to those with strongly positive antibody assays.

We have shown that subjects with GC 'switch' mucosal cytokine secretion, with a marked reduction of interferon (IFN)-γ and an increase in interleukin (IL)-4 secretion from gastric mucosal tissue cultures.[11] Because the cytokine milieu is known to influence the balance of the IgG subclass antibody response,[12] we have tested total IgG and IgG subclass anti-*H. pylori* antibody in subjects infected with *H. pylori*, to determine whether particular IgG subclass antibody assays may have value in detecting those with GC.

METHODS

Sample collection

Venous blood was drawn from each patient and transferred to the laboratory in plain tubes. Serum was separated and aliquots were stored at −70°C until assayed. Gastric biopsies were collected during upper gastrointestinal endoscopy, for histological examination and the rapid urease test (CLO test, Delta West, WA, Australia). The study was approved by the Hunter Area Human Ethics Committee and informed consent was obtained from each patient. A total number of 103 subjects with dyspepsia were recruited for the study. Studies were performed at the time of referral for diagnostic gastroscopy. Eleven subjects with dysplasia of the gastric epithelium (treated for *H. pylori* infection 1–4 years before study), and 11 subjects with GC (nine Chinese subjects and two Caucasian subjects) were included. Gastric carcinoma was classified as intestinal type in eight subjects and diffuse type in three subjects, according to Lauren's classification.[13] Four subjects with GC had localization in the antrum, four had localization in the corpus, two in the cardia and one in the corpus/cardia. Three subjects with GC were in stage 1–2, three in stage 3, and five in stage 4. One Caucasian subject with gastric dysplasia has been followed up for 4 years.

Helicobacter pylori antigen preparation

Acid glycine-extracted proteins (AGE) of *H. pylori* National Collection of Type Cultures (NCTC) 11637 strain were used for *H. pylori* antigen preparation according to modified methods described by Goodwin *et al.*[14] This preparation contained antigens that were reactive to all IgG subclass antibodies in *H. pylori*-positive but not *H. pylori*-negative serum using an ELISA assay. Protein concentration in the extract was measured using a Bio-Rad Kit (Bio-Rad Laboratories, Australia). Aliquots were stored at −70°C until assayed.

IgG antibody detection by ELISA

Serum anti-*H. pylori* IgG antibody was measured according to the method described previously.[15] The results were expressed as ELISA units according to the standard curve, which was generated using known pooled *H. pylori*-positive sera. Positive and negative quality control sera were included in each run to control for intra- and inter-assay variation.

Detection of IgG subclass antibody by ELISA

To maximize IgG subclass antibody detection, a checkerboard titration was performed with serial twofold dilution of serum antibody for each IgG subclass against a range of *H. pylori* antigen concentrations. Based on the results obtained, the following conditions were used. A 96-well flat-bottomed Polysorb plate (NUNC, Denmark) was coated with *H. pylori* AGE at 5 μg/mL at 4°C for overnight. After washing and then blocking the wells with 5% milk in phosphate-buffered saline (PBS)/Tween-20 for 60 min, 100 μL of serum samples at 1:300 dilution (for detection of IgG1 and IgG2 antibody) or at 1:50 dilution (for detection of IgG3 and IgG4 antibody) were added to individual wells in triplicate. After 60 min incubation, the wells were washed and biotinylated mouse monoclonal anti-human IgG1 or IgG2 or IgG3 or IgG4 (Sigma, USA) antibodies were added (at 1:1000 dilution for IgG1, 1:3000 dilution for IgG2, and 1:2000 dilution for IgG3 and IgG4, respectively). Plates were washed after 60 min incubation, and horseradish peroxidase-conjugated streptavidin (Silenus, Australia) was added to each well at 1:2000 dilution. Following a further 60 min incubation, the plates were washed and then TMB substrate (Sigma) was added to each well. The reaction was stopped using 1 moL/L H_2SO_4 and absorbance was read at 450 nm in an ELISA plate reader (Bio-Rad, 450, CA, USA). Pooled *H. pylori*-positive sera were used as the standard for each plate. The ELISA unit of each sample was calculated according to the known standard using Softmax software (Version 2.3 FPU, USA).

Statistical analysis

Data are expressed as the mean ± SEM. Differences in means among patient groups were analyzed by a single-factor ANOVA followed by an unpaired *t*-test. $P < 0.05$ were considered significant. All calculations were performed using a statistical software program (StatView 4.5, Abacus Concepts, CA, USA).

RESULTS

Diagnosis was made by endoscopy and histological examination of gastric biopsies. The *H. pylori* infection was determined by demonstration of bacteria in Giemsa-stained biopsies and/or detection of urease

using the CLO test. All patients selected for this study were infected with *H. pylori*. There were 43 patients diagnosed as having chronic gastritis (CG), 11 with GC, 10 with gastric ulcer (GU), 22 with duodenal ulcer (DU) and 28 with reflux esophagitis (RO). Additionally 11 patients with dysplasia of the gastric mucosa were investigated. These patients had received eradication antibiotic therapy 12–48 months before the study, and successful bacterial eradication was achieved in all but one by the time of the study. The mean age of patients in each group is shown in Table 1.

Serum-specific anti-*H. pylori* antibody detection

Serum IgG antibody levels were similar in all groups, although there was a trend toward low antibody levels in subjects with GC and GU. Higher IgG levels were measured in those with RO, but this was not statistically significant (Fig. 1).

Serum anti-*H. pylori* IgG subclass antibody detection

IgG1 and IgG2 subclass antibodies were detected in all patient groups, while only very low levels of IgG3 were measured. IgG4 antibody was undetectable in most subjects. Consequently only IgG1 and IgG2 antibody could be meaningfully compared. Similar levels of IgG1 were obtained from subjects with RO, gastritis, GU, DU and cancer subjects, although the IgG1 level in subjects with reflux was lower than in the other four tested groups (Fig. 2). In contrast IgG2 levels were different among the groups. In particular IgG2 levels were significantly lower in GC subjects, compared to those with CG, DU and RO. Although higher IgG2 levels were seen in those with benign GU compared to those with GC, this difference was just short of significance ($P = 0.068$). Furthermore, similar levels of *H. pylori* IgG1 and IgG2 antibody were observed in both Chinese and Caucasian subjects with GC (Table 2).

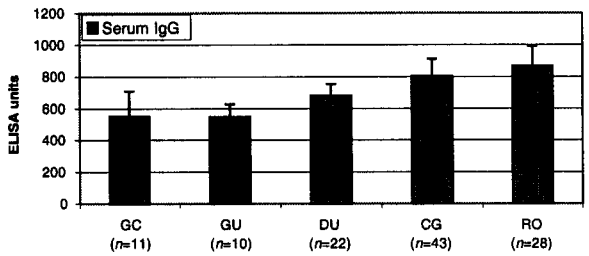

Figure 1 Serum *Helicobacter pylori*-specific IgG antibody in subjects with gastric cancer (GC), gastric ulcer (GU), duodenal ulcer (DU), chronic gastritis (CG) and reflux esophagitis (RO). Results shown are presented as mean ± SEM.

Serum-specific antibodies in subjects with dysplasia

Because lower antibody IgG2 was found in GC, we measured subclass IgG1 and IgG2 in the 11 subjects

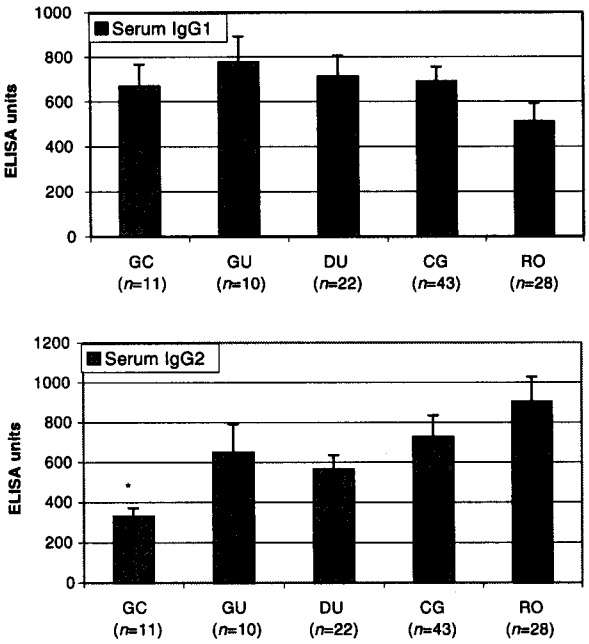

Figure 2 Serum *Helicobacter pylori*-specific IgG1 and IgG2 antibody in subjects with gastric cancer (GC), gastric ulcer (GU), duodenal ulcer (DU), chronic gastritis (CG) and reflux esophagitis (RO). Results shown are presented as mean ± SEM. $*P < 0.05$ compared with subjects with DU, CG and RO.

Table 1 Age and gender in different disease groups

	n	Age (years)	Median ± SD	M/F
Gastric carcinoma	11	27–75	54.1 ± 14.9	7/4
Gastric ulcer	10	38–77	56.2 ± 14.1	4/6
Duodenal ulcer	22	27–85	50.7 ± 17.1	13/9
Chronic gastritis	43	25–85	50.0 ± 15.1	22/21
Reflux esophagitis	28	30–80	54.6 ± 16.5	16/12
Gastric dysplasia	11	50–78	60.0 ± 10.0	5/6

Table 2 Serum *Helicobacter pylori* antibody levels in Chinese and Caucasian subjects with gastric carcinoma

Subgroup	*n*	*H. pylori* IgG ELISA units (Mean ± SEM)	*H. pylori* IgG1 ELISA units (Mean ± SEM)	*H. pylori* IgG2 ELISA units (Mean ± SEM)
Chinese subjects with cancer	9	557.0 ± 194.6	644.0 ± 114.3	317.0 ± 48.9
Caucasian subjects with cancer	2	517.6 ± 141.8	786.6 ± 151.4	390.5 ± 6.5

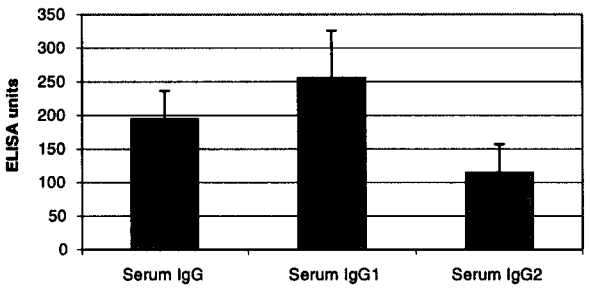

Figure 3 Serum *Helicobacter pylori*-specific IgG, IgG1 and IgG2 antibody in subjects with gastric dysplasia. Results shown are presented as mean ± SEM from 11 subjects with gastric dysplasia.

with gastric dysplasia who had received eradication therapy 12–48 months prior to the present study (Fig. 3). These serum IgG antibodies, however, were significantly lower in the dysplasia group compared with other tested groups. This was expected given the eradication therapy. The IgG1 antibody appeared to be retained compared to IgG2 antibody, although ELISA units were used (not absolute amounts).

Reduction of endoscopy by referring cut-off value of IgG2

Because significantly low levels of IgG2 were observed in subjects with GC, we established a threshold to decide a cut-off value for IgG2 that could screen subjects with a high risk of GC. The mean of IgG2 plus various SD were calculated from 11 subjects with cancer, and then was applied to 103 subjects with benign gastric disease. Results are summarized in Table 3. At a conservative level of 3 SD, approximately 40% of subjects could be considered according these data as being of low risk for GC, and could thus be given eradication therapy as a first-up management strategy without endoscopy.

DISCUSSION

The present study of seropositive subjects infected with *H. pylori*, compared total IgG with IgG subclass antibody assays as diagnostic tools in those with gastritis and those with GC. No significant differences were detected between the different diagnostic categories

Table 3 Cut-off values of IgG2 to exclude low risk for gastric cancer

Cut-off values (ELISA unit)†	No. above the cut-off	Low risk for cancer (%)
Mean + 1 SD (464.9)	61	59.2
Mean + 2 SD (599.5)	53	51.5
Mean + 3 SD (734.1)	43	41.7

†The cut-off values were based on ELISA results from 11 subjects with gastric cancer.

with the IgG and IgG1 antibody assays, but seropositive subjects with GC had significantly lower IgG2 antibody levels compared to those with a benign diagnosis. The IgG3 and IgG4 antibody levels were either very low or not detected, and thus comparison was unhelpful.

The relationship between seropositivity and GC has been established and remains the scientific basis of linkage between infection and cancer. Attention has focused on falling levels of IgG antibody as being particularly predictive of cancer in a 15-year study in Iceland.[16] This was thought to reflect premalignant gastric atrophy with loss of colonization and antigen stimulus.[9] The results in the current study do not show a significant reduction in total IgG antibody in GC, although the observed levels do tend to be lower than those found in subjects with benign gastritis. An elevated level of IgG2 antibody in DU described by others[17,18] was not seen in the present study.

The selective reduction in IgG2 antibody in cancer could reflect either different kinetics of antibody response to gastric colonization, or an altered cytokine milieu affecting the balance of IgG antibody subclass secretion. Persistent colonization status was confirmed at endoscopy, indicating contemporary antigenic stimulation. We have examined short-term clearance of IgG and IgG subclass antibody by following respective levels over 6 months following successful eradication, and demonstrated no difference in the relative rates of fall (data not shown). Most of the GC subjects were studied in China. We have shown, however, that no significant differences in IgG2 antibody levels exist between Western and Chinese populations with this assay.

We have previously shown a marked shift in cytokine secretion in gastric mucosal organ cultures from subjects with GC, and that mucosal secretion rates correlate with those from blood culture.[11,15] Cytokine patterns appear to influence B-lymphocyte differentiation and antibody secretion patterns.[19] Subjects with GC in the present

study were also used in a previous study that aimed to correlate cytokine secretion from gastric mucosal cultures, where IFN-γ was the dominant cytokine secreted in subjects with CG.[11] This compared to the demonstration of significant levels of IL-4 secreted from the gastric mucosa from subjects with dysplasia and carcinoma. The latter group also had undetectable amounts of IFN-γ in most subjects. Those with dysplasia, however, had retained secretion of IFN-γ.[11] The IgG2 deficiency has been linked to reduced secretion of IFN-γ.[20] Thus, reduced secretion of mucosal IFN-γ in GC subjects may determine lower levels of IgG2 antibody. It is possible that the increased amount of mucosal IL-4 is relevant, because IL-4 downregulates the T-helper 1 (Th1) response and thus indirectly reduces IgG2 subclass secretion.[21] The relative reduction in levels of IgG2 antibody in subjects with GC may therefore reflect a profound shift in local cytokine balance.

It has also been suggested that the malignant process itself could interfere with the biosynthesis of IgG subclasses at the B-lymphocyte level.[22] An IgG1/G2 subclass shift has been reported in subjects with squamous cell carcinoma of the head and neck, breast cancer, colorectal cancer and with carcinomas of various locations of the female reproductive tract.[22-24] This effect of malignant tumors may be mediated by modulation of the secretion of certain cytokines and/or their soluble receptors.

The present demonstration of selective reduction in anti-*H. pylori* IgG2 antibody differs from recent studies claiming a linkage between low levels of total IgG antibody and falling levels of total IgG antibody and GC.[10,16] In those latter situations a reduction in antigenic drive through reduction of colonization due to altered binding circumstances, is likely to be responsible for lower levels of all IgG subclass. However, it has been recently reported that *H. pylori* strains isolated from tumors of patients with GC had significantly lower immunogenicity with respect to IgG antibody response than strains isolated from patients with CG and DU.[25] The dominant subclass of IgG that reacted with a polysaccharide determinant on *H. pylori* lipopolysaccharide (LPS) was IgG2 in all *H. pylori*-infected subjects tested including those with GC.[26] Thus a diminished IgG2 subclass antibody response due to low immunogenicity of *H. pylori* LPS antigen epitope or to the loss of *H. pylori* in some subjects in evolving GC could reflect the premalignant phase of gastric atrophy. Eleven subjects with premalignant gastric dysplasia were shown to have particularly low levels of IgG2 antibody. This cohort had been shown to have high levels of secretion of IL-4 from gastric organ mucosal cultures, while retaining secretion of IFN-γ. In all but one of these subjects, however, *H. pylori* had been eradicated 1–4 years earlier. Antibody was quantified in ELISA units rather than absolute amounts, and the relative half-lifes of IgG subclass antibody over these longer times, is not known. Although of interest, the significance of these results remains uncertain, requiring a pre-eradication study of subjects with premalignant histology.

The biological significance of these observations is unclear, because circulating anti-*H. pylori* antibody has not been shown to have a role in eradication.[3] However, enhancement of the diagnostic value of serology in the management of *H. pylori* disease is of potential clinical significance. Medical practice is governed by the risk of cancer. In areas of low cancer incidence, primary endoscopy in subjects without 'change' symptoms is restricted to those over the age of 45 years,[27] whereas serology had little clinical value in populations with a high incidence of GC.[28] The current results challenge these rules, by showing that above certain levels of antibody, irrespective of age, the risk of cancer is low and that primary endoscopy could be restricted to those with antibody values below this level. Using the current data, and establishing a cut-off of 3 SD (734 ELISA units), endoscopy could be avoided as the initial investigation in 41.7% of dyspeptic subjects. To substantiate a clinical value for an IgG2 anti-*H. pylori* test, predictive diagnostic values for *H. pylori* colonization in populations at high risk of cancer need to be done, as do larger prospective studies of subjects with dyspepsia in high-risk areas for GC, to validate the current data and to determine safe cut-off levels for low cancer risk.

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