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IgG subclass response to *Helicobacter pylori* and CagA antigens in children

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SUMMARY

Specific serum IgG subclass antibodies against *Helicobacter pylori* antigens and recombinant CagA were analysed in 75 symptomatic children with histologically confirmed *H. pylori* infection. *H. pylori* stimulated an IgG1 predominant response, and IgG3 titres showed a positive association with peptic ulcer disease, chronicity of antral inflammation and density of *H. pylori* colonization. Two methods used for assessing serum IgG CagA antibody status, i.e. Western blotting and enzyme-linked immunosorbent assay (ELISA), were concordant. CagA stimulated an IgG1 and IgG3 predominant humoral response. Total CagA IgG titres were higher in children with active and more severe chronic antral inflammation. These findings suggest that in children the systemic humoral immune response to *H. pylori* infection may reflect gastroduodenal pathology.

Keywords CagA antigens children *Helicobacter pylori* IgG subclass response

INTRODUCTION

Helicobacter pylori infection, which affects approximately half the world's population, is now accepted as an important pathogenic factor in chronic gastritis, peptic ulcer disease, gastric carcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [1]. Despite such a high prevalence only a small proportion of infected individuals present clinical manifestations of the disease, and conditions influencing the course of infection have not been determined fully.

Bacterial virulence factors such as the *cag* pathogenicity island (*cag* PAI) [2,3], the vacuolating cytotoxin [4] and BabA2 adhesin [5] have been associated in several studies to more severe clinical outcome (reviewed in [6]). Infection with *cag* positive strains was initially linked with peptic ulcer disease and active gastritis [7]. However, more recently a strong association between infection with *cag* positive *H. pylori* strains and increased risk of gastric atrophy [8,9] and gastric cancer [10–13] has become evident. Infection with *cag* positive strains is associated with increased gastric mucosal C-X-C chemokines [14,15], which are likely to contribute to enhanced neutrophilic responses associated with active gastritis. Previous studies in both children [16] and adults [17,18] have shown that the *cag* genotype can influence the colonization density.

Accumulating evidence suggests that other factors, such as the host response to the *H. pylori* infection, are important in the pathogenesis of *H. pylori*-induced mucosal changes. Genetic polymorphisms in proinflammatory and immunoregulatory cytokines have been linked to an increased risk of developing gastric atrophy and/or gastric cancer [19–21]. These studies provide the most convincing evidence that clinically, host immune responses contribute to clinical outcome. Cytokines such as interleukin (IL)-12 and IL-18, which are increased with *H. pylori* infection [22,23], are important in polarizing the Th1 mucosal responses [24–26]. The role of the immune response in the outcome of gastric *Helicobacter* infection has been demonstrated clearly in mice lacking T cells [27].

In many infections the IgG subclass response to infecting pathogens has been associated with severity of clinical symptoms and inflammatory response [28–30]. Although the specific subclass response to *H. pylori* in adult populations has been analysed in a few studies [31–35], the relationship between *H. pylori* IgG subclasses and gastric inflammation in children has not been investigated fully. In addition, the IgG subclass antibodies against the immunogenic *H. pylori* CagA protein have not been studied. The aim of this study was to assess the association between IgG subclass response to *H. pylori* antigens and recombinant CagA and gastric histology in symptomatic children.

MATERIALS AND METHODS

Patients

Symptomatic children presenting for endoscopy at the Children's Memorial Health Institute, Warsaw, Poland, were eligible for inclusion. From each child, 2 ml of blood was taken for serology

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and antral biopsies were obtained for histology and culture during routine upper gastrointestinal endoscopy. The study was undertaken with ethics committee approval of the Children's Memorial Health Institute and informed consent was obtained from all patients/parents.

Gastric histology

Antral biopsy specimens were fixed in formalin. Sections stained with haematoxilin–eosin and modified Giemsa stain were assessed histologically according to the updated Sydney System by a single experienced pathologist (M. G.-D), who was unaware of the clinical diagnosis. Sections were graded for the presence and extent of chronic lymphocytic infiltration, active neutrophilic infiltration, atrophy, intestinal metaplasia and the density of *H. pylori* colonization on a scale of 0–3.

Serological assays

Serum samples were assayed for the presence of total IgG antibodies to CagA and specific IgG subclasses to *H. pylori* and CagA. Total CagA IgG antibodies were measured by enzyme-linked immunosorbent assay (ELISA), as described previously [12], and by Western blotting (Helicoblot 2·0; Genelabs Diagnostic, Singapore) according to the manufacturer's instructions. Positivity in the CagA IgG ELISA was determined by reference to a standard curve of positive control serum assayed on each plate as previously described [9,12]. Cut-off values were validated with paediatric sera from *H. pylori*-negative children with histologically normal mucosa as described previously [36].

For the *H. pylori* and CagA IgG subclasses ELISAs an ultracentrifuged sonicated whole cell preparation of *H. pylori* [36,37] and a recombinant fragment of CagA [38] (kindly provided by Dr G. del Guidice, Chiron Vaccines, Siena, Italy) respectively, were used as antigens. Flat-bottomed 96-well microtitre plates were coated with 125 ng/well CagA or 500 ng/well *H. pylori* antigen in 0·1 M bicarbonate buffer (pH 9·6) for 24 h at 4°C. Plates were washed with phosphate buffered saline (PBS) containing 0·1% Tween 20, and blocked with 1% bovine serum albumin (BSA) in PBS-Tween for 1 h at 26°C. Serum samples diluted 1/200 (*H. pylori* ELISA) or 1/50 (CagA ELISA) in 1% BSA/PBS-Tween were incubated in duplicate for 90 min at 26°C. Following further washing and incubation with biotin-conjugated monoclonal anti-human IgG1, IgG2, IgG3 and IgG4 antibodies (Sigma Chemicals, Poole, UK) the plates were incubated with avidin alkaline phosphatase (Sigma Chemicals), and bound antibodies were detected with p-nitrophenyl phosphate substrate (Sigma Chemicals) solution at 1 mg/ml in diethanolamine-$MgCl_2$ buffer pH 9·8. On each plate positive control sera diluted 1/200–1/12800 (*H. pylori*) or 1/50–1/3200 (CagA) were used to generate standard curves of arbitrary units. The cut-off for positivity in the IgG subclass ELISAs was the mean ±2 s.d. of 37 patients who were *H. pylori* and CagA seronegative by Western blotting and histologically negative for *H. pylori*.

Culture

Primary isolation was performed on Wilkins Chalgren agar with 7% horse blood and Dent's selective supplement SR 147 (Oxoid, UK). The plates were incubated under microaerophilic conditions (CampyPak Plus, BBL generators) at 37°C for up to 7 days. *H. pylori* was identified by colony morphology, Gram staining and urease, catalase and oxidase activities.

Statistical analysis

The relation between antibody titres (CagA IgG, *H. pylori* IgG subclasses, CagA IgG subclasses), histological parameters and the presence of peptic ulcer disease (PUD) were analysed by non-parametric statistics: the Mann–Whitney *U*-test for comparison of two groups and the Kruskal–Wallis ANOVA for groups greater than two. The Spearman correlation coefficient was used to determine the relation between histological parameters and *H. pylori* IgG subclass titres and to assess the relation between *H. pylori* IgG subclass titres and CagA IgG subclass titres. Associations between disease manifestations [PUD *versus* non-ulcer dyspepsia (NUD)] and specific *H. pylori* and CagA IgG subclass responses were analysed by Fisher's exact test. The same test was used to assess the relationship between positivity of specific *H. pylori* IgG subclasses and CagA status. The associations between the ratio of IgG1/IgG2 *H. pylori* antibodies, age and disease manifestations were analysed by χ^2 test.

RESULTS

A total of 75 children with histologically confirmed *H. pylori* infection were included in the study. Histologically, all the children had gastritis. The children were all ethnically Polish; the mean age was 13·2 years (range 7–18 years), 34 were female and 41 male. Twenty-four (32%) children had duodenal ulcers (DU). *H. pylori* cultures were performed in 68 children, 62 of whom were positive. CagA IgG antibodies were detected by ELISA in 60 of 75 (80%) of the children. To determine the sensitivity of the CagA ELISA in the population, Western blotting for CagA antibodies was undertaken in a subgroup of 57 children. Forty-six of 57 (81%) children were CagA positive by Western blotting and the two methods showed a 100% concordance for detecting CagA IgG antibodies. CagA IgG titres were higher in patients with active ($n = 67$) compared to inactive gastritis ($n = 8$) ($P < 0{\cdot}05$; Fig. 1) and in children with more severe chronic antral inflammation ($P < 0{\cdot}05$). Higher (although not significant) levels of CagA IgG antibodies were observed in children with DU (mean 74 units) compared to a

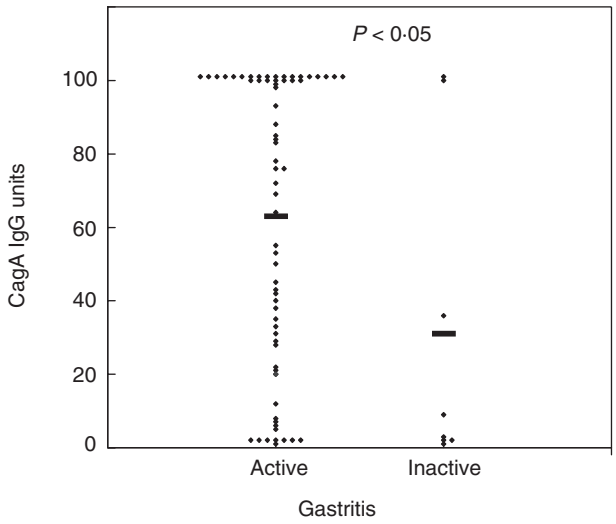

Fig. 1. CagA IgG Units in *H. pylori* infected children with active ($n = 67$) *versus* inactive ($n = 8$) gastritis (Mann–Whitney *U*-test, $P < 0{\cdot}05$). The bars represent the mean values.

group with non-ulcer dyspepsia (NUD) (mean 53 units) ($P = 0{\cdot}06$).

H. pylori *IgG subclass response*

The specific IgG subclass positive responses to *H. pylori* in the infected children consisted of all four subclasses, with 84% of children having IgG1 subclass antibodies, 68% IgG2 and IgG3 subclasses and 53% IgG4 (Table 1). Levels of all IgG subclasses (expressed as units) to *H. pylori* were moderately correlated. There was no significant difference in the IgG1, IgG2, IgG3 and IgG4 subclass positivity to *H. pylori* between DU and NUD groups (Table 1). There was also no difference in the IgG subclass response to *H. pylori* between CagA seropositive and CagA seronegative groups. The *H. pylori* IgG1, IgG2 and IgG4 titres did not differ between DU and NUD patients; however, the *H. pylori* IgG3 titres were significantly higher in children with DU compared to NUD group ($P < 0{\cdot}05$; Fig. 2). There was no association between the levels of *H. pylori* IgG subclasses and activity of antral inflammation. However, titres of specific IgG1 and IgG3 antibodies correlated moderately with chronicity of antral inflammation (Spearman correlation coefficient, $r = 0{\cdot}27$, $P < 0{\cdot}05$ and $r = 0{\cdot}37$, $P < 0{\cdot}01$, respectively), and IgG3 titres correlated moderately with the density of *H. pylori* colonization ($r = 0{\cdot}31$, $P < 0{\cdot}01$). Comparison of the ratio of IgG1/IgG2 *H. pylori* antibodies showed no difference between DU and NUD patients and no age-related differences in subclass ratios were observed.

Table 1. *H. pylori* IgG subclass positivity in relation to disease status in 75 children

	H. pylori IgG subclass response (no. and % positive)			
Diagnosis	IgG1	IgG2	IgG3	IgG4
Duodenal ulcer ($n = 24$)	23 (96%)	20 (83%)	20 (83%)	14 (58%)
Non-ulcer dyspepsia ($n = 51$)	40 (78%)	31 (61%)	31 (61%)	26 (51%)
Total (75)	63 (84%)	51 (68%)	51 (68%)	40 (53%)

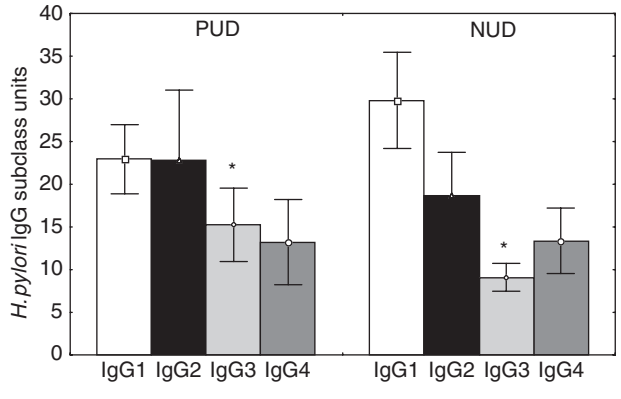

Fig. 2. IgG1, IgG2, IgG3 and IgG4 *H. pylori* subclass antibodies in *H. pylori* infected children with duodenal ulcer (DU; $n = 24$) and non-ulcer dyspepsia (NUD; $n = 51$). *IgG3 titres were significantly higher in patients with DU compared to NUD (Mann–Whitney *U*-test, $P < 0{\cdot}05$) The squares represent mean ± s.e.m. values.

CagA IgG subclass response

CagA IgG subclasses were analysed in 60 children who were positive for CagA IgG antibodies by ELISA. Preliminary studies showed that in subjects infected with CagA-positive *H. pylori* strains there is virtually no specific IgG4 response to the CagA protein; therefore, in the present study only CagA IgG1, IgG2 and IgG3 subclass antibodies were assayed. The specific CagA IgG subclass response involved predominantly IgG1 and IgG3 subclasses (Table 2), and titres of the two subclasses showed moderate correlation ($r = 0{\cdot}43$; $P < 0{\cdot}001$). No significant difference was found in the distribution and levels of CagA IgG subclass response between DU and NUD patients. There was also no association between CagA IgG subclass response and histological features of antral inflammation and density of *H. pylori* colonization.

DISCUSSION

There are relatively few data on the IgG subclass response to *H. pylori* and how it may relate to gastroduodenal disease. Studies to date have largely focused on adult populations [31,32,34,35] and the results have varied depending on the population studied. Mitchell *et al*. found IgG2 predominant response in Australian and German adults and IgG1 predominant response in Sowetan children and adults, and suggested that the difference could result from simultaneous helminthic infections in Sowetan subjects modulating immune response against *H. pylori* [35]. In the present study an IgG1 predominant response to *H. pylori* in Polish children was observed; however, because the prevalence of intestinal parasitic infection in this population has been shown to be relatively low (22%) [39], it seems unlikely that parasitic infection would influence anti-*H.pylori* response markedly. It is possible, however, that the difference in IgG subclass response to *H. pylori* could be related to antigenic variation of infecting strains. A similar phenomenon has already been observed in mice vaccinated with different serotypes of *Streptococcus pneumoniae* capsular polysaccharide [40]. Similarly to the Sowetan population, where over 90% of *H. pylori* infections are caused by *cagA* positive strains [41], the vast majority of children (80%) analysed in the present study had *cagA*$^+$ infections. CagA is a strongly immunogenic protein which, as shown in this study, elicits an IgG1 predominant response. Therefore, it could be speculated that IgG1 predominant response in Sowetan and Polish patients could result potentially from a predominance of CagA IgG1 antibodies in the total pool of anti-*H. pylori* antibodies. The composition of the antigen preparations used to determine the *H. pylori* subclass IgG response in earlier studies [31–35] may influence strongly the ratio *H. pylori* IgG subclass responses. In the current study the

Table 2. CagA specific subclass positivity in 60 children positive for CagA IgG antibodies

	H. pylori IgG subclass response (no. and % positive)		
Diagnosis	IgG1	IgG2	IgG3
Duodenal ulcer ($n = 21$)	20 (95%)	4 (19%)	10 (48%)
Non-ulcer dyspepsia ($n = 39$)	35 (90%)	3 (8%)	14 (36%)
Total (60)	55 (92%)	7 (12%)	24 (40%)

antigen preparation used for the *H. pylori* IgG subclasses ELISA did not contain CagA.

There is some evidence to suggest *H. pylori cag* positive strains could modulate the immune response to *H. pylori*, shifting it towards IgG1 predominance. Previous studies have indicated that mucosal IL-12p40 transcript levels are greater in patients with peptic ulcers and those infected with *cagA* positive strains than *cagA* negative strains [22]. Additionally, Th1 cell clones generated from the gastric mucosa of *H. pylori* positive patients frequently recognize CagA [42]. Apart from environmental and bacterial factors, the immune response against *H. pylori* can be also influenced by host factors, such as age. Andersen *et al*. reported that *H. pylori* IgG2 was the IgG subclass which increased the most with age [31]. A similar observation has also been made by Lottenbach *et al.*, who noted that children vaccinated with pneumococcal vaccines responded predominantly with IgG1 antibodies, whereas adults had mainly an IgG2 antibody response [43]. In the present study no age-related differences between IgG1/IgG2 ratios of *H. pylori* antibodies was observed, but the age range of our patients was relatively narrow (7–18 years). To verify this hypothesis it would be worthwhile to analyse *H. pylori* subclass response in a large ethnically homogeneous population of adults and children free from diseases known to influence immune response.

The present study has shown significantly higher levels of *H. pylori* IgG3 antibodies in children with PUD comparing to NUD group. This observation differs from previous studies, which reported either higher IgG2 subclass response in adults with duodenal ulcers compared to NUD patients, or lack of an association between *H. pylori* subclass response and disease manifestations [31,32,34]. However, Valnes *et al*. who analysed the distribution of gastric IgG-producing immunocytes in various types of gastritis, found a significantly higher proportion of IgG3 plasma cells in duodenal ulcer patients subjected previously to Billroth II resection than in patients with simple gastritis [44]. Although the latter study did not examine the presence of *H. pylori* infection, its involvement in chronic gastritis and peptic ulcer disease is now well documented. Valnes *et al*. [44] suggested that because IgG3 has a strong capacity for complement activation and binding to Fcγ receptors on mononuclear cells, it can be involved in gastroduodenal pathology.

In the current study the titres of *H. pylori* IgG1 and IgG3 antibodies correlated moderately with chronicity of antral inflammation, and IgG3 titres correlated moderately with the density of antral *H. pylori* colonization. In adults an association between *H. pylori* IgG3 levels and gastric inflammation was also observed by Mitchell *et al.*, who noted a positive relationship between IgG3 levels and grade of chronic inflammation in the fundus and active inflammation in the transitional zone [34]. A similar phenomenon has also been found in other bacterial infections. In patients with cystic fibrosis increased levels of IgG3 to *Pseudomonas aeruginosa* were associated with enhanced inflammation and poorer pulmonary status [45]. Additionally, in patients with leprosy progression of the disease correlated significantly with a selective increase of IgG1 and IgG3 antibodies, and levels of these antibodies correlated with bacterial load within lesions [28].

This study has also demonstrated that in children infected with *H. pylori* there is a positive association between CagA antibody titres and active and chronic inflammatory response in the antral mucosa. Two methods were used to determine CagA IgG seropositivity, Western blotting and ELISA. The methods showed 100% correlation demonstrating that both are equally reliable for assessing serum IgG CagA antibody status in this paediatric population. The specific IgG subclass response to CagA has not been examined previously. In this study it was found that CagA elicits predominantly an IgG1 and IgG3 antibody response and virtually no IgG4 antibodies. This is in agreement with previous observations indicating that protein antigens stimulate mainly IgG1 and IgG3 synthesis [46]. No association was observed between the distribution and levels of CagA IgG subclass response and disease manifestation or antral inflammation. Because there are geographical allelic variations in *cagA* [47], it would be interesting to investigate the relation between CagA IgG subclass response and gastric pathology in different populations.

In summary, this study has demonstrated a positive association between *H. pylori* IgG3 titres and peptic ulcer disease, chronicity of antral inflammation and density of *H. pylori* colonization in children. CagA elicits predominantly an IgG1 and IgG3 antibody response and there is no relationship between CagA subclass response and gastroduodenal pathology in paediatric patients.

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