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Immune Responses to Differentiated Forms of *Helicobacter pylori* in Children with Epigastric Pain

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***Helicobacter pylori* infection affects human populations of all ages. This gastric bacterium exists in spiral form and the reported viable but nonculturable coccoid form. The present study aims to examine the probable role of the coccoid form in *H. pylori* infection by comparing the seroprevalences of the spiral and the coccoid forms in children with epigastric pain. Four hundred eighty-nine children (mean age, 8.5 years) with epigastric pain formed the basis of this study. Five hundred ninety-nine schoolchildren of comparable ages and with no record of dyspepsia served as controls. The seroprevalence of antigens prepared from both morphological forms was examined by enzyme-linked immunosorbent assay. The results showed that 65 (13.3%) and 273 (55.8%) of 489 symptomatic children were seropositive for antigens of the *H. pylori* spiral and coccoid forms, respectively. In contrast, only 7.0% of the control group had elevated levels of immunoglobulin G antibodies against the spiral form, while 26.5% were positive for antibodies against the coccoid form. There were no significant differences between genders or among ethnic groups. The study showed a rise in seroprevalence corresponding with age: 7.1% for those ≤5 years to 21.4% for those ≥11 years. The seroprevalence of antigens of the *H. pylori* spiral and coccoid forms in children with epigastric pain was twofold higher than that in the control subjects. Interestingly, there was a fourfold increase in seropositivity for coccoid-form antigen compared to that for the spiral-form antigen among the symptomatic pediatric patients as well as the control group, indicating a possible infective role of the coccoid form of *H. pylori* in the pediatric patients with epigastric pain.**

Since the discovery of *Helicobacter pylori* by Warren and Marshall (32) two decades ago, it has been found that the organism infects approximately 50% of the world's population (30).

H. pylori exists in two morphological forms: the spiral form and the coccoid form. The spiral form has been implicated as the causative agent of type B antral gastritis and peptic ulcer disease (8). Spiral-shaped *H. pylori* converts into the coccoid form under unfavorable conditions such as nutrient deprivation, exposure to antibiotics, and extended incubation (2, 19, 33). Unlike the spiral form, the role of the coccoid form in *H. pylori* infection has not been established. Experimental data from Kusters et al. (16) suggested that the coccoid form is a morphological manifestation of cellular degeneration or cell death. However, several reports have shown that although the coccoid form is nonculturable, it may be viable, as findings support the notion that transcription and translation may actively take place in coccoid cells (1, 20, 28).

H. pylori DNA has been detected in saliva (29) and stool (21) specimens by PCR. However, isolation of *H. pylori* from saliva and stool specimens by culture has had limited success (14, 24). This had led to the hypothesis that the coccoid form, which is believed to be the survival form in the extragastric environment, may play an important role in the *H. pylori* transmission cycle.

Infection with *H. pylori* is likely acquired during childhood (6, 9). A recent study on *H. pylori* infection status from infancy to adulthood concluded that most newly acquired infections occur before age 10 years (18). Although *H. pylori* infects the younger population, its association with the development of upper gastrointestinal symptoms such as epigastric pain and the pathogenesis of recurrent abdominal pain has been inconsistent (17, 26, 27).

The present study aims to examine the hypothesis that the coccoid form may play an essential role in the transmission cycle of *H. pylori* infection by determining the seroprevalence of *H. pylori* infection in children with epigastric pain by using an enzyme-linked immunosorbent assay (ELISA) with antigens prepared from cells of the spiral and the coccoid forms.

MATERIALS AND METHODS

Study population. A total of 489 consecutive children (mean age, 8.5 ± 3.3 years) with epigastric pain formed the study population. They were outpatient referrals to the pediatric gastroenterology clinic at the National University Hospital, which is a tertiary referral center in Singapore. All children had been having epigastric pain for at least 3 months and had consulted their own general practitioners or pediatricians prior to referral to the specialist gastroenterology clinic. The patient population consisted of 263 girls and 226 boys. None of the symptomatic children had received any antibiotics within 4 weeks of the study. The control group comprised 599 schoolchildren (mean age, 9 ± 0.5 years) who participated in a seroepidemiological survey of dengue in the eastern part of Singapore. A blood sample was collected from each individual. Serum was separated, aliquoted, and kept frozen at −20°C until it was analyzed. Informed consent was obtained from the parents of the children.

Determination of *H. pylori* antibody status. The presence of immunoglobulin G (IgG) antibodies against the spiral and the coccoid forms of *H. pylori* was determined by an in-house ELISA with antigen prepared from *H. pylori* RH54, which had been isolated from a local patient with a healed duodenal ulcer (22).

Antigens of the spiral and the coccoid forms were prepared by acid glycine extraction (11) of 3- and 150-day-old cultures, respectively. ELISA was per-

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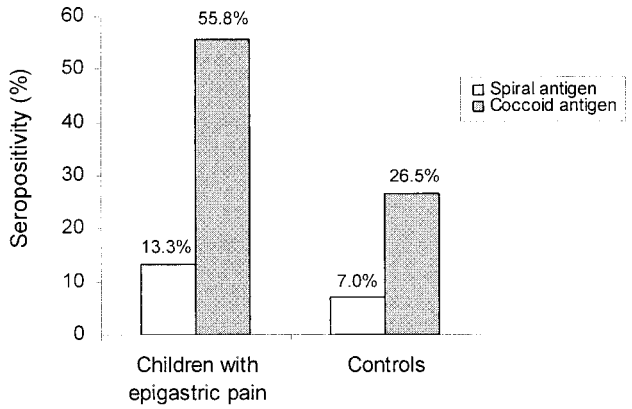

FIG. 1. Comparison of seropositivity between control subjects and children with epigastric pain. A total of 489 children with epigastric pain and 599 control schoolchildren formed the study cohort.

formed by the method described by Ng et al. (22). In brief, the test sera were diluted (1:100) and allowed to react with the antigen bound to flat-bottom microtiter plates (Maxisorp; Nunc). Peroxidase-labeled rabbit anti-human IgG (Dako) was used as the conjugate, and the substrate was 0.04% *o*-phenylenediamine dihydrochloride (Sigma). The enzymatic reaction was terminated with 2.5 M sulfuric acid. The optical density at 492 nm was determined with a Multiskan Ascent microtiter plate reader (Labsystems, Vantaa, Finland). Samples were tested in triplicate. The cutoff value used for the ELISA was based on a comparative study of 114 local pediatric patients evaluated previously. On the basis of a cutoff value of 2 standard deviations above the value for histology-confirmed negative sera, the sensitivity and specificity of our in-house ELISA were 100 and 97%, respectively.

As the coccoid form is viable but nonculturable (23), the histology and culture results were based on observation or culture of the spiral form. Hence, the cutoff value for the ELISA was obtained by using the spiral-form antigen.

Statistical evaluation. Statistical analysis was performed with SPSS (version 10) statistical software (Scientific Package for Social Sciences, Chicago, Ill.). The χ^2 test and Fisher's exact test were used to compare the proportions between groups. The level of significance was set at a P value of <0.05.

RESULTS

The serology results showed that 65 of 489 (13.3%) of the children with epigastric pain had increased levels of IgG against the spiral form of *H. pylori*, while 273 of 489 (55.8%) were seropositive for the antigen prepared from the coccoid form (Fig. 1). Of the 65 samples seropositive for the spiral-form antigen, 36 were from girls and 29 were from boys. A relative proportional distribution of seropositivity for the coccoid-form antigen was also observed among the groups by gender, with 149 girls and 124 boys being seropositive for the coccoid-form antigen. The distributions by gender for the groups seropositive for the *H. pylori* spiral and coccoid forms did not differ significantly ($P > 0.05$).

The children who participated in this study were of various ethnic backgrounds. Of these, 360 were Chinese, 43 were Malay, 46 were Indian, and 40 were of other ethnic groups. The prevalence of seropositivity among the different races and age groups is illustrated in Table 1. Although the result was not statistically significant ($P > 0.05$), children of the Indian race were observed to have the highest rate of seropositivity (21.7%) for antibodies against the spiral-form antigen. Malay children appeared to have a slightly higher prevalence (14.0%) than Chinese children (12.5%) and children of other races (10%). The study also showed a gradual increase in the rate of acquisition of *H. pylori* infection with age (Table 1). Children aged 5 years and younger had a prevalence of seropositivity of 7.1%, with the prevalence increasing to 21.4% for those aged 11 years and older. The serology results for antibodies against the coccoid form (Table 2) showed that 195 of 360 (54.2%), 26 of 43 (60.5%), 28 of 46 (60.9%), and 24 of 40 (60%) Chinese, Malay, Indian, and children of other ethnic groups were seropositive, respectively. It was noted that 188 of 358 (52.5%) children aged 10 years and younger were seropositive for the coccoid-form antigens, whereas 85 of 131 (64.9%) children aged 10 years and older were seropositive for the coccoid-form antigen (Table 2).

Among the 65 children with epigastric pain and increased levels of IgG antibodies against the spiral-form antigen, 58 were also found to have antibodies against the coccoid form of *H. pylori*. The remaining seven samples seropositive for the spiral-form antigen were, however, found to have no significantly increased levels of IgG antibodies against the coccoid-form antigen. In total, 215 children who were seronegative for the spiral-form antigen were seropositive for antibodies against the coccoid-form antigen. A total of 209 of 489 (42.7%) children in this study showed no antibodies against either form.

Of the 599 asymptomatic schoolchildren tested, 7.0% had elevated levels of IgG antibodies against the spiral-form antigen and 26.5% had elevated levels of antibodies against the coccoid-form antigen (Fig. 1).

It should be noted that for validation of the use of coccoid-form antigen for serologic testing, the sensitivity and the specificity of the test were calculated by using the spiral-form antigen as the standard for comparison. The spiral-form antigen was chosen as the standard for comparison because the spiral form is routinely used for the serodiagnosis of *H. pylori* infection (5, 22). Furthermore, histology results have been based on tests with this actively dividing spiral form (25). When spiral-

TABLE 1. Seropositivity for *H. pylori* spiral-form antigen among children with epigastric pain

Age range (yr)	No. of children seropositive/total no. (%)				
	Chinese (n = 360)	Malay (n = 43)	Indian (n = 46)	Other[a] (n = 40)	Total (n = 489)
0–5	5/78 (6.4)	1/6 (16.7)	1/7 (14.3)	0/7 (0)	7/98 (7.1)
6–10	19/188 (10.1)	2/22 (9.1)	5/26 (19.2)	4/24 (16.7)	30/260 (11.5)
11–15	21/94 (22.3)	3/15 (20)	4/13 (30.7)	0/9 (0)	28/131 (21.4)
Total	45/360 (12.5)	6/43 (14.0)	10/46 (21.7)	4/40 (10.0)	65/489 (13.3)

[a] Other ethnic groups.

TABLE 2. Seropositivity for *H. pylori* coccoid-form antigen among children with epigastric pain

Age range (yr)	No. of children seropositive/total no. (%)				
	Chinese (n = 360)	Malay (n = 43)	Indian (n = 46)	Other[a] (n = 40)	Total (n = 489)
0–5	43/78 (55.1)	3/6 (50)	4/7 (57.1)	4/7 (57.1)	54/98 (55.1)
6–10	91/188 (48.4)	11/22 (50)	17/26 (65.4)	15/24 (62.5)	134/260 (51.5)
11–15	61/94 (64.9)	12/15 (80)	7/13 (53.8)	5/9 (55.6)	85/131 (64.9)
Total	195/360 (54.2)	26/43 (60.5)	28/46 (60.9)	24/40 (60.0)	273/489 (55.8)

[a] Other ethnic groups.

form antigen was used as the standard for comparison, the sensitivity and the specificity were 89.2% (58 of 65 children) and 49.3% (209 of 424 children), respectively, with positive and negative predictive values of 21.2% (58 of 273 children) and 96.8% (209 of 216 children), respectively.

DISCUSSION

Recurrent abdominal pain is common in children. It can be due to peptic ulcer disease, gastritis, gastroesophageal reflux, and other organic and psychogenic disorders. The results from the present study have demonstrated a strong association of *H. pylori* infection and epigastric pain in local pediatric patients. Of the 489 symptomatic children, 13.3% were seropositive for antibodies against the *H. pylori* spiral-form antigen, whereas only 7.0% (42 of 599) of the schoolchildren in the control group were found to have antibodies against the spiral-form antigen. There was an approximately twofold increase in the seroprevalence of *H. pylori* infection in children with epigastric pain ($P < 0.001$) when the spiral-form antigen was used. The rates of seropositivity for antibodies against the coccoid form for the children with epigastric pain and the control children were 55.8 and 26.5%, respectively. Therefore, when either form of the antigen was used in the ELISA, there were twofold differences in the rates of seropositivity for the two groups of subjects. The differences in the proportions of children within each study group positive for the spiral form and the coccoid form were fourfold. The results for our control group agree with those from an earlier study based on 2,626 subjects in various age groups (6 months to older than 65 years), in which 6.7% (48 of 717) of children younger than age 15 years tested seropositive (5).

The present study with children having epigastric pain showed that there is no preponderance of seroprevalence by gender. However, there was an increased seroprevalence with age, and the highest seropositivity was among Indian children. This is in agreement with the findings of Boey et al. (3) for asymptomatic Malaysian children: the rate of seropositivity increased with age, and seropositivity was most common among the Indian children. A study by Kang et al. (15) demonstrated that racial differences in the seroprevalence of *H. pylori* were more pronounced in the adult population. The study also showed that Indians had the highest prevalence of infection, followed by Chinese and Malay, but the Indian subjects had a lower frequency of peptic ulcer disease than the Chinese subjects (15).

The present study has demonstrated that symptomatic pediatric patients have increased levels of IgG antibodies against both the spiral form and the coccoid form of *H. pylori*. Interestingly, there was a fourfold increase in the rate of seropositivity for antibodies against the coccoid-form antigen (55.8%) compared to that for antibodies against the spiral-form antigen (13.3%) ($P < 0.001$). However, a study by Hua et al. (12) reported that in the adult symptomatic population, the rates of seropositivity for antibodies against either form were found to be comparable, with 50.7% seropositive for antibodies against the spiral-form antigen and 49.6% seropositive for antibodies against the coccoid-form antigen. More recently, a publication by Figueroa et al. (7) also revealed that the sera of 295 infected individuals were highly reactive to both the spiral and the coccoid forms by ELISA. The contrast may be due to the possibility that the coccoid form is involved in the initial stage of *H. pylori* infectivity and that there is a relative increase in the rate of seropositivity for antibodies against the spiral form with age. This may also explain why we detected a fourfold increase in the rate of seropositivity for the coccoid form compared to that for the spiral form in asymptomatic children. Furthermore, the observed increased prevalence of seropositivity for the coccoid form of *H. pylori* in children with epigastric pain could suggest a possible infective role of the coccoid form.

It was also observed that the coccoid form is strongly associated with the spiral form in the infection process, as reported by Chan et al. (4). In this study, it was shown that when IgG antibodies against the spiral form were present, antibodies against the coccoid form were always found except in the sera of seven children who tested seropositive for the spiral form but who were found to be seronegative for the coccoid form. This observation contributes to the reduced sensitivity (89.2%) of using the coccoid-form antigen for serologic testing when the spiral-form antigen is used as the standard for comparison. However, detailed analysis of the data showed that six of these seven individuals had borderline titers of IgG antibodies against the coccoid-form antigen. The low specificity (49.3%) may mean that there are numerous false-positive results. This is expected because, as illustrated in Table 2, more than 50% of the test subjects harbored the coccoid form. In view of this observation, the possibility of underreporting of *H. pylori* infection in the population of young symptomatic subjects must be given due consideration, as the tests used at present are designed with reference to the spiral form and not the coccoid form.

The coccoid form is nonculturable in vitro, and its viability is still subject to debate. However, in a recent study by Nilsson et al. (23), ATP was detectable for at least 25 days after the morphological conversion from the spiral form to the coccoid form, and they found that the mRNAs of several genes were present in aged cultures. This supports the notion that the

coccoid form is probably viable. In addition, a study by Vijayakumari et al. (31) showed that the coccoid form of *H. pylori* adhered to Kato III cells in vitro, which is similar to the observed interaction of the spiral form with the gastric epithelium in vivo (10). Furthermore, a study by electron microscopy with epithelial cells showed the presence of both spiral and coccoid forms in three of eight *H. pylori*-positive children (13). It is therefore probable that the coccoid form, like the spiral form, is viable and infective, even though it is nonculturable in vitro.

In the present study with young symptomatic children (mean age, 8.5 years) with epigastric pain, the *H. pylori* coccoid-form antigen was used for serologic testing, and an increased seroprevalence of antibodies against the coccoid form was noted. We postulate that the infection may well begin with colonization by the coccoid form. It is therefore not surprising that most of the symptomatic children were not diagnosed as *H. pylori* positive because the usual serologic tests used for diagnosis and histological examination are based on the spiral form.

Whether the coccoid form plays an infective role is not definite at this stage, as the coccoid form is reported to be viable but nonculturable (23). Our findings have described another facet of the role of the coccoid form of *H. pylori*. Future studies will have to focus on looking for the coccoid-form-specific antigen(s) and using it as a specific diagnostic marker. The present study demonstrates that the effect that the coccoid form has on the immune response in infected individuals, especially children, cannot be underestimated.

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