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Isolation and Preliminary Evaluation of a Low-Molecular-Mass Antigen Preparation for Improved Detection of *Helicobacter pylori* Immunoglobulin G Antibodies

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Previously, immunoglobulin G (IgG) antibodies to five antigens with a relative molecular mass of between 15 and 30 kDa from *Helicobacter pylori* were found to be significantly more frequent in *H. pylori*-infected patients than in noninfected patients. In this study, these specific low-molecular-mass (LMW) antigens were separated by ultrafiltration of whole-cell sonicates. The LMW antigen preparation was evaluated by enzyme-linked immunosorbent assay with serum samples from 76 children with abdominal symptoms and 151 adults with dyspeptic symptoms. *H. pylori* was cultured or seen in 40 (53%) children and 83 (55%) adults. Increased antibody levels to *H. pylori* were found in serum from 35 (46%) children and 88 (58%) adults. Values for sensitivity, specificity, and predictive value of positive and negative results of the test were higher with LMW antigens than with the heat-stable antigen previously described. The low specificity and predictive value of a positive result were due to seropositive results for 21 persons with a negative culture for *H. pylori* and negative microscopy results for *Helicobacter*-like organisms in biopsies from gastric mucosa. Histologically, chronic gastritis was demonstrated in 43% of these persons, and 19% had peptic ulcer, indicating that they have or have had *H. pylori* infection. Specific antibodies to *H. pylori* were confirmed in all 21 patients by the Western immunoblot technique. Use of the LMW antigen improved the IgG antibody detection in patients with *H. pylori* infection, even though the results reflect the difficulties in establishing a true gold standard for diagnosis of *H. pylori* infection.

Helicobacter pylori has been established as the major cause of chronic gastritis and peptic ulcer (2, 18) and may play an important role in the pathogenesis of gastric cancer (9, 17). Patients with *H. pylori* infection do not always have specific symptoms or specific endoscopic findings which establish the diagnosis (1, 26). The laboratory findings are therefore important for a reliable diagnosis of *H. pylori* infections. One of the cheapest and simplest tests is the detection of antibodies to *H. pylori*. Detection of antibodies may, in contrast to most other tests, also reveal "hidden" infections caused by coccoidal forms (15) or intracellular survival of *H. pylori* in phagocytes (4), which may obscure the diagnosis, especially after treatment. Serological studies by different techniques and with different antigen preparations have shown that patients with *H. pylori* infection have increased immunoglobulin G (IgG) antibody levels compared with subjects without *H. pylori* infection (5, 7, 13, 22), and IgG antibody levels decrease significantly after successful treatment of the infection (13). Most serological tests developed for diagnostic use have sensitivities and specificities of between 70 and 90% (8, 11, 16).

The serological cross-reactions between *H. pylori* and other bacteria that may cause false-positive results are still incompletely investigated (6, 12, 21). It has been possible to pinpoint several antigens that were specific for *H. pylori* by immunoblot analysis (3). Of these antigens, five bands had a relative molecular mass in the range of 15 to 30 kDa (3). The purpose of this study was to purify these low-molecular-mass (LMW) antigens of *H. pylori* by simple ultrafiltration, to evaluate this antigen preparation in an enzyme-linked immunosorbent assay (ELISA) for routine detection of IgG antibodies to *H. pylori*, and to compare it with a previously used heat-stable (HS) antigen preparation in sera from consecutive children with abdominal symptoms and consecutive adult patients with dyspeptic symptoms.

MATERIALS AND METHODS

Patients and serum samples. Serum was obtained from (i) 76 consecutive Czech children and young adults (median age, 13 years; range, 5 to 20 years) with abdominal pain as their major symptom, admitted to hospital for endoscopy, and (ii) 151 consecutive adult Danish patients (median age, 52 years; range, 21 to 85) admitted to hospital for endoscopy because of dyspepsia. The majority of the adult patients were treated with H_2 blockers prior to endoscopy.

Endoscopy, histology, and growth of *H. pylori*. All patients had an upper endoscopy, and at least four biopsies were obtained from the antral part of the stomach during endoscopy. Tissue sections of formalin-fixed biopsies were stained with hematoxylin-eosin to evaluate the morphology. Additional silver staining was done to evaluate the presence of *Helicobacter*-like organisms (HLO). Biopsies from adults were examined by two pathologists, and biopsies from children were examined by a third pathologist. Biopsies from areas corresponding to biopsies taken for histology were cultured under microaerobic conditions on 7% lysed horse blood agar plates at 37°C for up to 6 days. *H. pylori* was identified according to conventional laboratory methods (23).

Bacterial strain. A clinical isolate of *H. pylori* (CH-20429) from an adult male with duodenal ulcer has previously been tested (3, 5) and was used for the antigen preparations.

Whole-cell preparations. The bacteria were cultured under microaerobic conditions on lysed horse blood agar plates for 24 to 48 h, harvested, and washed two times in phosphate-buffered saline (PBS, pH 7.4). The preparations were centrifuged at 7,000 × *g* for 10 min, and the pellet was stored at −20°C. The pellets were resuspended in PBS (pH 7.4) to a concentration of 0.5 g (wet weight) per ml of PBS when used.

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Sonicated cell preparations. Whole-cell preparations (0.5 g [wet weight] per ml of PBS) were broken by sonication at 20,000 Hz for 45 s, which was repeated five times with a Rapidis 300 19-mm probe with a 9.5-mm tip. The preparations were cooled during sonication by immersion in ice water. The sonicated bacteria were centrifuged at 11,000 × *g* for 30 min, and the supernatants were stored at −20°C.

LMW antigen preparation. The supernatants of the sonicated cell preparations were filtered through a filter with a pore size of 0.2 μm, then through a filter with a cutoff at 100,000 kDa (Millipore catalog no. UFP2THK24), and finally through a filter with a cutoff at 30,000 kDa (Millipore catalog no. UFP2TTK24) mounted on a syringe. The filtered preparations were dialyzed against PBS (pH 7.4) in a dialysis bag with a cutoff at 12,000 kDa for 2 days with one exchange of PBS.

HS antigen preparation. The preparation of the HS antigen has been described previously (22). The bacteria were boiled for 2 h in PBS (pH 7.2) containing 1% Triton X-100, sonicated five times for 45 s each with 60-s intervals, and centrifuged at 11,000 × *g* for 30 min.

SDS-PAGE. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method has previously been described in detail (3). The whole-cell preparation, the ultrasonicated cell preparation, and the filtered preparations were diluted 1:30 in PBS (pH 7.4) and mixed with equal volumes of sample buffer containing 0.4% SDS and 4.8% (wt/vol) DL-dithiothreitol. The suspensions were boiled for 5 min in a waterbath. Electrophoresis was carried out as described by Laemmli (14) with a 15% separation gel and a 5% stacking gel. Relative molecular weight kit; Pharmacia Fine Chemical). Coomassie brilliant blue staining was performed as described by Weber and Osborne (28). Silver staining was performed with silver nitrate after fixation with 10% glutaraldehyde and developed with 75 μl of 24.5% formalin in 100 ml of 3% sodium carbonate.

Immunoblot analysis. Immunoblot analysis was done by a modification of the technique described by Towbin et al. (24) carried out as described previously (3).

Indirect ELISA. The ELISA method has for HS antigen preparation has been described previously (5, 27). The same method was adapted for the LMW antigen preparation (protein concentration, 0.53 g/liter). The microtiter plates were coated overnight at room temperature with the LMW antigen preparation diluted 1:100. The antigen coating was optimized by checkerboard titration. Serum samples from adults were diluted 1:800 and those from children were diluted 1:100 because of the much higher IgG antibody levels to *H. pylori* in adults than in children. The assays were done in triplicate with an uncoated well as a control for nonspecific binding of IgG or conjugated anti-human IgG to the plastic matrix. After incubation for 1 h at room temperature, the plates were washed, and horseradish peroxidase-conjugated rabbit antibodies to human serum IgG antibodies were added to each well. The plates were incubated for 1 h at room temperature and washed, and enzyme activity was detected by using the ortho-phenylenediamine dihydrochloride-H_2O_2 system. The chromogenic reaction was stopped with H_2SO_4 after 15 to 30 min, and the optical density (OD) was read in a photometer at 492 nm. The antibody amount was expressed as ELISA units (EU), which are OD values corrected for day-to-day and plate-to-plate variation. The predictive values, sensitivities, and specificities were calculated by standard methods.

RESULTS

LMW antigen preparation. Figure 1 demonstrates the SDS-PAGE pattern of the LMW antigen preparation compared with the sonicated preparation and the preparation after filtration through different filters and dialysis. It can be seen that several antigens with molecular masses above 30 kDa and below 15 kDa are still present in the LMW antigen preparation, but in clearly reduced amounts compared with those antigens with molecular masses of between 15 and 30 kDa.

***H. pylori* status.** All patients were considered *H. pylori* positive if either *H. pylori* was cultured or HLO were seen in tissue sections of the biopsies. Forty (53%) of 76 children and 83 (55%) of 151 adults were found to be *H. pylori* positive (Table 1).

IgG antibody response to *H. pylori* in children. The distributions of the antibody levels to both HS and LMW antigen according to *H. pylori* status are shown in Table 1. The best IgG antibody discrimination between *H. pylori*-positive and *H. pylori*-negative subjects was found when the cutoff for negative results was <100 EU and the cutoff for positive results was ≥200 EU, with a gray area between 100 and 199 EU for the LMW antigen (Table 1). With these cutoff levels, none of 36 *H. pylori*-negative children were seropositive and only 1 (2.8%) of them was borderline seropositive. Samples from 2 (5%) of 40 *H. pylori*-positive children did not respond to the LMW antigen, and 3 (7.5%) were borderline seropositive (Table 1). By using these cutoff levels for detection of antibodies to LMW antigens in children, the sensitivity, specificity, predictive value for a positive result (PVP), and predictive value for a negative result (PVN) were 95, 100, 100, and 95%, respectively. The best distribution for the HS antigen was obtained when the gray zone was set between 400 and 799 EU. The sensitivity, specificity, PVP, and PVN were 82, 80, 85, and 77%, respectively, for the HS antigen.

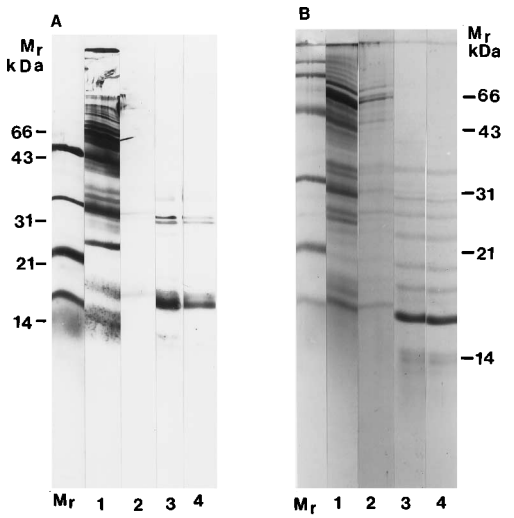

FIG. 1. Commassie stained (A) and silver-stained (B) SDS-PAGE of whole-cell sonicate of *H. pylori* (lane 1), fraction below 100 kDa (lane 2), and fraction below 30 kDa, before (lane 3) and after (lane 4) dialysis through a membrane with a cutoff at 12 kDa. M_r, relative molecular mass.

IgG antibody response to *H. pylori* in adults. By using identical cutoff levels for adults and for children, the sensitivity, specificity, PVP, and PVN were 99, 45, 72, and 96% for the LMW antigen and 96, 36, 70, and 85% for the HS antigen, respectively. By changing the lower cutoff level to 200 and the upper cutoff level to 599 for adults for the LMW antigen, the sensitivity, specificity, PVP, and PVN were optimized to 95, 73, 80, and 93%, respectively. By changing the upper cutoff level to 799 and keeping an unchanged lower cutoff level for adults for the HS antigen, the sensitivity, specificity, PVP, and PVN were optimized to 96, 35, 70, and 85%, respectively. Both the PVPs and the specificities seem to be rather low, because of a great proportion of seropositive patients among the *H. pylori*-negative patients (Table 1).

Analysis of the patients according to *H. pylori* status. Of all *H. pylori*- or HLO-positive adult patients, 94% were detected by culture and only 6% were detected by microscopy of histological sections alone.

Analysis of the false-positive adult patients. All 21 adult patients were admitted for dyspeptic symptoms, and 9 (43%) had histological chronic gastritis. Four (19%) had peptic ulcer, four (19%) had endoscopic gastritis, and two (9.5%) had gastric cancer. Only five (24%) patients had normal endoscopic findings and normal morphology of the stomach biopsies. Two of these five patients had very high antibody levels to *H. pylori* LMW and HS antigens. Antibodies to *H. pylori*-specific antigens were confirmed in all 21 seropositive patients by Western blot analysis.

DISCUSSION

A high prevalence of antibodies against five LMW antigens in the range from 15 to 30 kDa (bands H to L) in patients with

TABLE 1. IgG antibody response to *H. pylori* LMW and HS antigens in children with abdominal pain and adults with dyspeptic symptoms[a]

ELISA result (EU)	*H. pylori* status results (no. of patients)							
	Children				Adults			
	LMW antigen		HS antigen		LMW antigen		HS antigen	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
>1,000	14	0	24	3	41	9	63	27
800–1,000	2	0	4	2	5	3	8	4
600–800	3	0	2	6	10	2	6	10
400–600	6	0	4	5	11	7	3	10
200–400	10	0	1	9	13	10	3	10
100–200	3	1	3	5	2	12	0	4
<100	2	35	2	6	1	25	0	3
Total	40	36	40	36	83	68	83	68

[a] *H. pylori* infection was confirmed by culture and/or microscopy.

H. pylori infection compared with subjects without *H. pylori* infection was demonstrated in a previous study (3). In this study, a filtration method was used to purify these antigens. The protein bands with molecular masses greater than 40 kDa were diminished compared with the five low-molecular-mass bands, as shown in Fig. 1. Minor amounts of a broad range of proteins were, however, still present in the antigen preparation. It is suggested that these additional proteins are of less importance than the predominant low-molecular-mass protein fraction for the detection of the antibody response to *H. pylori*, even though small amounts of antigens which cross-react with other bacteria may be present in the preparation (3).

This antigen preparation was found to be very reliable for detection of *H. pylori* infections in children by ELISA, with predictive values, sensitivities, and specificities of 95 to 100% and with a rather narrow gray zone of 100 to 199 EU. This is a great improvement over the HS antigen and may be an advantage over other antigen preparations used for the serological detection of *H. pylori* infections in children (28, 29). *H. pylori* or HLO were detected by both microscopy of histological sections and culture in all children, which is in accord with other studies (25), whereas *H. pylori* or HLO were detected in 6% of the adult patients by microscopy alone. The PVP and the specificity were rather low for adult dyspeptic patients compared with children, even with an extended gray zone of between 100 and 399 EU (Table 1). The results obtained with ELISA, however, were better for adults with the LMW antigen than with the HS antigen. The low PVPs and specificities were due to 21 seropositive patients with negative culture and without HLO by microscopy. Thirteen (62%) had very high antibody levels to *H. pylori* LMW antigen, and 16 (76%) of the 21 patients had either morphological or endoscopic abnormalities in the stomach. IgG antibodies to *H. pylori*-specific antigens were confirmed in all 21 patients by Western immunoblotting.

It is impossible to establish whether the disagreement between culture/microscopy and antibody detection is caused by an underestimation of the *H. pylori* infection by culture/microscopy or caused by low antibody levels in patients with an eradicated or suppressed *H. pylori* infection. It is therefore difficult to establish a satisfactory gold standard. This is in agreement with a previous study in which the adult population was used to evaluate commercial kits, and low PVPs and specificities of the test results were found (10). An important reason for these low values was probably the transportation time from the clinic to the laboratory, which might diminish the number of *H. pylori*, as a previous study of another population with a shorter transportation time showed higher PVPs of the test results (11). Another reason for this might be the number of biopsies taken from each patient, which was only half the number recommended in the Sydney classification (20). Alternatively, a majority of these patients could have had an *H. pylori* infection previously, even though four of these patients had peptic ulcer, which occurs 16% of the total number of patients with peptic ulcer included in this study, and in addition three (12%) patients with peptic ulcer were borderline seropositive but *H. pylori* infection negative. This is an unusually high number of *H. pylori*-negative patients with peptic ulcer compared with that found in other studies. This study reflects the difficulties in using consecutive unselected patients for evaluation of serological tests for detection of infections with fastidious microorganisms.

The *H. pylori* LMW antigen has improved IgG antibody detection as a predictive factor for *H. pylori* infection over use of the HS antigen and other first-generation tests. Detection of IgG antibodies to LMW antigens appears to be reliable for detection of *H. pylori* infection in children, who are difficult to endoscopy, whereas use of the LMW antigen seems to be of similar benefit as in most other commercial kits for adults.

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