Production and Application of New Monoclonal Antibodies Specific for a Fecal Helicobacter pylori Antigen

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The aim of the present study was to establish monoclonal antibodies that could be used to produce a diagnostic test composed of one kind of monoclonal antibody recognizing a fecal Helicobacter pylori antigen. The need to develop such a test arose from disadvantages of the diagnostic test that uses a polyclonal antibody or plural kinds of monoclonal antibodies, such as the lower specificity for H. pylori antigen and the difficulty of reproduction with consistent quality. Mice were immunized with sonicated cells of the coccoid form of H. pylori, and fecal samples from H. pylori-positive subjects were screened by a direct sandwich enzyme immunoassay (EIA) for antibody production from 32 hybridoma clones. The three stable clones produced antibodies (21G2, 41A5, and 82B9) that reacted with the same soluble antigen. Gel filtration chromatography showed that the molecular masses of the cellular antigen and the fecal antigen were the same, 260 kDa. The antigen was labile in response to sodium dodecyl sulfate and heat treatments. A single-step direct sandwich EIA using a single monoclonal antibody, 21G2, was developed. The EIA could detect the antigen in 41 H. pylori clinical isolates and in fecal samples from seven H. pylori-positive subjects. Several kinds of Helicobacter species (Helicobacter felis, Helicobacter hepaticus, Helicobacter mustelae, and Helicobacter cinaedi) except H. pylori, major bacteria in feces (Campylobacter jejuni, Bacteroides vulgatus, Bifidobacterium breve, Bifidobacterium infantis, and Escherichia coli), and fecal samples from six H. pylori-negative subjects showed negative results. These results indicate that the new monoclonal antibodies and the new specific EIA would be useful as a noninvasive method of diagnosis of H. pylori infection.

Helicobacter pylori causes gastritis and peptic ulcers, and its association with stomach cancer has been studied recently. H. pylori infection can be diagnosed by tests requiring endoscopic biopsy of the gastric mucosa (culture, histology, and the rapid urease test) and by noninvasive tests (serology and the urea breath test) (17). H. pylori is difficult to culture from a fecal sample, because H. pylori outside the stomach probably converts to the nonculturable coccoid form (3, 9).

Recently, enzyme immunoassays (EIAs) for the direct detection of the H. pylori antigens in feces have been developed. These assays include an EIA that uses polyclonal rabbit antibody (Premier Platinum HpSA; Meridian Diagnostics Inc., Cincinnati, Ohio) and an EIA that uses plural kinds of monoclonal antibodies (MAbs) (FemtoLab H. pylori; Connex GmbH, Martinsried, Germany). The EIAs have been shown to be reliable tools for noninvasive diagnosis of H. pylori infection (2, 11, 12, 16). However, the lower specificity of the Premier Platinum HpSA assay has been reported in several articles (5, 6, 15). Moreover, the H. pylori antigen profile in feces that is recognized by the polyclonal antibody or the plural kinds of MAb remains uncertain and would be of interest to elucidate.

Therefore, our interest was to establish MAb recognizing a fecal H. pylori antigen with a higher specificity so that a more efficient diagnostic test using one kind of MAb could be developed and a more profound study of the H. pylori antigen profile in feces could be performed.

To develop a diagnostic test for H. pylori infection with a higher specificity, we produced new MAb recognizing the fecal H. pylori antigen and developed a new single-step EIA that used one kind of MAb for the detection of fecal H. pylori antigen.

MATERIALS AND METHODS

Fecal samples. Fecal samples were obtained from 13 healthy Japanese male subjects (average age, 48 years) and stored at −35°C before use. Seven subjects were H. pylori positive and six subjects were negative for H. pylori by the urea breath test and serology. Consent was obtained from all participants in the study.

Bacterial strains, culture conditions, and preparation of disrupted cells. The following type cultures were used: H. pylori ATCC 43504, H. felis ATCC 49179, H. hepaticus ATCC 51448, H. mustelae ATCC 43772, H. cinaedi ATCC 35683, C. jejuni ATCC 29428, E. coli ATCC 25922, B. vulgatus IFO14291, B. breve JCM1192, and B. infantis JCM1222. Forty-one H. pylori strains isolated from gastric biopsy samples from Japanese patients with gastric ulcer, duodenal ulcer, gastric cancer, gastric mucosa-associated lymphoid tissue lymphoma, or atrophic gastritis were used. Helicobacter species and C. jejuni were cultured on brain heart infusion agar (BHI) plates containing 5% horse blood in a microaerophilic environment (Anaero Pack Helico System; Mitsubishi Gas Chemical Co.,...
Inc.) for 4 days. For transformation of _H. pylori_ to the coccoid form, the culture plates were incubated for a further 7 days in an anaerobic environment (Anaero Pack Anseron System; Mitsubishi Gas Chemical Co., Inc.) (18). _B. vulgatus_ and _Bifidobacterium_ species were cultured anaerobically on glucose blood liver agar (Nissui Pharmaceutical Co., Ltd.) plates containing 5% horse blood for 4 days. _E. coli_ was cultured aerobically on brain heart infusion agar plates for 3 days. All cultures were incubated at 37°C.

Bacterial cells were harvested, washed in phosphate-buffered saline (PBS), suspended in PBS containing 0.5% formalin, and then incubated overnight at 4°C. The bacterial cells were washed three times in PBS and disrupted by sonication (output 3, 50% duty cycle for 10 min) (Biom’C Model 7250; Seiko Instruments & Electronics, Ltd.).

**Production of MAbs.** The immunogen used to immunize mice consisted of sonicated cells of the coccoid form of _H. pylori_ ATCC 43504. Six BALB/c mice (female, 6 weeks old) were immunized by subcutaneous injection of the immunogen mixed with the same volume of Freund’s complete adjuvant (Difco). On day 27, a final injection of the immunogen without adjuvant was administered intraperitoneally. On day 30, spleen cells and _PSX6_8.4g6.53 myeloma cells (10:1) were fused with 50% polyethylene glycol (PEG 4000). Hybridomas were selected in a hypoxanthine-aminopterin-thymidine medium.

Culture supernatants of hybridoma cells were screened for antibody production by an indirect EIA. Plastic 96-well EIA microtiter plates (Costar) were coated with 200 μl of the immunogen (10 μg of protein/ml in PBS) and incubated overnight at 4°C. After nonspecific binding sites were blocked with 250 μl of PBS containing 1% skim milk (Dilco) (blocking buffer) for 1 h at 4°C, 200 μl of each hybridoma clone culture supernatant was added to each well in duplicate. The plates were incubated for 1 h at 37°C, washed five times with PBS containing 0.05% Tween 20 (washing buffer), and further incubated with 200 μl of PBS containing 0.1% skim milk (dilution buffer) and peroxidase-conjugated anti-mouse immunoglobulin G (Cappel; dilution, 1:20,000) for 1 h at 37°C. After additional washings, 200 μl of substrate solution (8 mM o-phenylenediamine and 3 mM _H₂O₂_ in 90 mM phosphate buffer [pH 6.0]) was added. The reaction was stopped after 15 min by the addition of 50 μl of 3.5 _N_ H₂SO₄. All assay results were measured with a microplate reader (model 550; Bio-Rad Laboratories) at dual wavelengths (492 and 630 nm).

**Screening of MAbs recognizing cecal antigen by direct sandwich EIA.** The precipitate containing MAbs was obtained by adding ammonium sulfate to a solution containing 0.1% ammonium sulfate to a concentration of 50% of its saturation. For labeling, MAbs were diluted from ascitic fluid to a concentration of 50% of its saturation. For labeling, MAbs were biotinylated with biotin-N-hydroxysuccinimide as described in the manufacturer’s instructions (Zymed Laboratories). The fecal samples diluted threefold with dilution buffer were centrifuged at 2,000 × _g_ for 10 min, and the resulting supernatants were used for the EIA. Plastic 96-well EIA microtiter plates were coated with a MAbs (5 μg of protein/ml in PBS) and incubated overnight at 4°C. After nonspecific binding sites were blocked as described above, 200 μl of immunogen or fecal supernatant was added to each well in duplicate. The plates were incubated for 1 h at 37°C, washed five times with washing buffer, and incubated with 200 μl of biotinylated MAbs (dilution, 1:1,000) for 1 h at 37°C. After five washings, the plates were incubated with 200 μl of peroxidase-conjugated streptavidin (Zymed; dilution, 1:10,000) for 1 h at 37°C. The subsequent peroxidase reaction steps were performed as described above.

**Isotyping and isolectric points of MAbs.** MAbs were isotyped with the mouse immunoglobulin typing kit (Wako Pure Chemical Industries, Ltd.). The isoelectric points of the MAbs were determined by isoelectric focusing on a polyacrylamide gel at pH 3 to 10 with a broad pl marker kit (Amersham Pharmacia Biotech).

**Characterization of antigen.** The sonicated cells of the helical form of _H. pylori_ ATCC 43504 were fractionated by centrifugation. A fecal sample (24 g) from _H. pylori_ ATCC 43504 helical forms was similar to that of coccoid forms (data not shown). The result indicated that antigens recognized by the MAbs are conserved during morphological changes.

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**TABLE 1. Reactivity of MAbs in direct sandwich EIA with fecal samples**

| MAbs | Coating Labeling | Positive subject no. | Negative subject no. |
|------|-----------------|----------------------|----------------------|
| 21G2 | 21G2            | 3.3                   | 0.043                |
| 21G2 | 41A5            | 1.2                   | 0.028                |
| 21G2 | 82B9            | >3.5                  | 0.052                |
| 41A5 | 41A5            | 3.3                   | 0.017                |
| 41A5 | 21G2            | >3.5                  | 0.073                |
| 41A5 | 82B9            | >3.5                  | 0.056                |
| 82B9 | 82B9            | >3.5                  | 0.086                |
| 82B9 | 21G2            | 3.3                   | 0.063                |
| 82B9 | 41A5            | >3.5                  | 0.077                |

Data are mean values in duplicate.
version. Thus, we used the helical form of *H. pylori* ATCC 43504 for antigen characterization. The reactivity of the sonicated *H. pylori* helical forms was about 100 times higher than that of intact cells in the two sandwich EIAs (data not shown).

To locate antigens in *H. pylori* cells, we measured the antigenicity in the cellular fractions (Fig. 1A). Because the antigenicity per protein was the highest in the soluble fraction, it could be assumed that the antigen was present mainly in the soluble fraction in the cytoplasm, not exposed to the cellular surface. In a fecal sample from an *H. pylori*-positive subject as well, most of the antigenicity was detected in the ultracentrifugal supernatant (Fig. 1B).

Figure 2 shows the elution profile of the antigen on a Sephacryl S-300 RH gel filtration column. The elution patterns of the soluble fraction of *H. pylori* and the fecal supernatant were the same, and the molecular masses of both were estimated to be 260 kDa, suggesting the equality of the two antigens. The same results were obtained with two fecal supernatants from two different *H. pylori*-positive subjects.

Immunoblotting using the three MAbs resulted in no detectable bands in either the *H. pylori*-soluble fraction or the fecal supernatants. Therefore, we examined the effect of sample pretreatments with SDS-polyacrylamide gel electrophoresis (SDS, 2-mercaptopetoanol, and heat treatments) on antigenicity by dot blotting. We could not detect any dot signals after 1% SDS and/or heat treatment (100°C, 5 min) but could detect a dot signal at the same intensity after treatment with 2.5% 2-mercaptopetoanol (data not shown). The results show that the antigens are labile in response to SDS and heat treatment.

**Development of single-step direct sandwich EIA with one MAb.** For clinical application, we developed a single-step direct sandwich EIA using one kind of MAb. We selected MAb 21G2 because of its stable and high productivity in ascitic fluid.

In the EIA, the lower limit for detection of the *H. pylori* soluble fraction was 37.5 ng of protein/ml and the minimal number of bacteria required for detection was estimated to be 10⁵ per ml. To show the sensitivity and specificity of the EIA, we tested the reactivity of the MAb with 41 *H. pylori* clinical isolates, different kinds of *Helicobacter* species (*H. felis, H. hepaticus, H. mustelae, and H. cinaedi*), and major bacteria in feces (*C. jejuni, B. vulgatus, E. coli, B. breve, and B. infantis*) (Table 2). The EIA reacted with all *H. pylori* clinical isolates at 1 μg of protein/ml but did not react with the other kinds of bacterial species at 200 μg of protein/ml. To evaluate the clinical application, we measured 13 fecal samples by the EIA. The mean absorbance values of the fecal samples were 1.56 (range, 0.284 to 3.5; all positive) and 0.032 (range, 0.019 to 0.067; all negative) in seven *H. pylori*-positive and six *H. pylori*-negative subjects, respectively (P < 0.01, Mann-Whitney U test).

**DISCUSSION**

The first screening of hybridoma clones with the new MAb was performed, and 32 stable clones were obtained by an indirect EIA that uses the sonicated cells of the coccoid form.
of \textit{H. pylori} ATCC 43504 as the antigen. As for the 32 clones, the 1,024 sandwich combinations were screened for the purpose of obtaining MAbs for a sensitive sandwich EIA by a direct sandwich EIA using the same antigen, and 91 combinations that could react with the antigen were found. The 91 combinations were further tested by a direct sandwich EIA using fecal samples as the antigen. As a result, nine combinations that could react with fecal samples were found, and three hybridoma clones that produced hitherto-undescribed MAbs, designated 21G2, 41A5, and 82B9, were established. The remarkably lower number of MAb combinations that could react with fecal antigen, 9 compared with 91 that reacted with the hybridoma clones, showed that most of the antigenicities from \textit{H. pylori} disappeared when the bacterium passed through the gut and that some antigenicities could resist the denaturing process in the gut.

The first goal of our screening was to find antibodies that were specific for the coccoid form of \textit{H. pylori}, because it is well known that \textit{H. pylori} outside the stomach probably converts from the helical form to the coccoid form. Therefore, we used the \textit{H. pylori} coccoid form as an immunogen in our first screening. However, the MAbs finally obtained, 21G2, 41A5, and 82B9, were the antibodies that reacted with both the coccoid and the helical forms.

The two direct sandwich EIAs [(i) coating MAb, 21G2; labeling MAb, 41A5; (ii) coating MAb, 41A5; labeling MAb, 82B9] with different combinations of the three MAbs could react with the soluble fraction in the \textit{H. pylori} cells and with fecal samples (Fig. 1 and 2). Therefore, it was clear that 21G2, 41A5, and 82B9 recognized the same single antigen, although they are different MAbs because of their different isoelectric points. Furthermore, the structure of the antigen in both \textit{H. pylori} and feces was considered to consist of more than one epitope, like a homodimer, because the sandwich EIA consisting of one kind of coating MAb and a different labeling MAb could detect the antigen. To compare the antigen of \textit{H. pylori} and that in feces, gel filtration chromatography was performed. The elution patterns of both antigens were almost the same, and the molecular masses of both antigens were estimated to be 260 kDa. Therefore, it was assumed that the fecal antigen recognized by the MAbs was an antigen of \textit{H. pylori} which was excreted in intact form without being denatured in the gut. Although several papers have reported the production and application of a MAb specific for \textit{H. pylori} antigen (1, 4, 7, 13), there has been no report on a MAb specific for a distinct fecal \textit{H. pylori} antigen. To elucidate the \textit{H. pylori} antigen profile in feces and to guarantee \textit{H. pylori} diagnosis when the \textit{H. pylori} antigen in feces is used, identification of the fecal \textit{H. pylori} antigen is important.

The high sensitivity and specificity of the single-step direct sandwich EIA developed for \textit{H. pylori} and fecal samples have been elucidated by comparative studies of the assay’s reactivity with some \textit{Helicobacter} species and with major bacteria in feces. Therefore, the EIA developed in the present study, which is characterized by the application of one kind of MAb, would be useful as a diagnostic tool for \textit{H. pylori} infectious diseases.

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