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 Helicobacter pylori antigen and the difficulty of reproduction with consistent quality. Mice were immunized with sonicated cells of the coccoid form of Helicobacter pylori, and fecal samples from Helicobacter 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 Helicobacter pylori clinical isolates and in fecal samples from seven Helicobacter pylori-positive subjects. Several kinds of Helicobacter species (Helicobacter felis, Helicobacter hepaticus, Helicobacter mustelae, and Helicobacter cinaedi) except Helicobacter pylori, major bacteria in feces (Campylobacter jejuni, Bacteroides vulgatus, Bifidobacterium breve, Bifidobacterium infantis, and Escherichia coli), and fecal samples from six Helicobacter 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 Helicobacter pylori infection.

Helicobacter pylori causes gastritis and peptic ulcers, and its association with stomach cancer has been studied recently. Helicobacter 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). Helicobacter pylori is difficult to culture from a fecal sample, because Helicobacter pylori outside the stomach probably converts to the nonculturable coccoid form (3, 9).

Recently, enzyme immunoassays (EIAs) for the direct detection of the Helicobacter pylori antigens in feces have been developed. These assays include an EIA that uses polyclonal rabbit antibodies (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 Helicobacter 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 Helicobacter pylori antigen profile in feces that is recognized by the polyclonal antibody or the plural kinds of MAbs remains uncertain and would be of interest to elucidate. Therefore, our interest was to establish MAbs recognizing a fecal Helicobacter 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 Helicobacter pylori antigen profile in feces could be performed.

To develop a diagnostic test for Helicobacter pylori infection with a higher specificity, we produced new MAbs recognizing the fecal Helicobacter pylori antigen and developed a new single-step EIA that used one kind of MAb for the detection of fecal Helicobacter 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 Helicobacter pylori positive and six subjects were negative for Helicobacter 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 culture types were used: Helicobacter pylori ATCC 43504, Helicobacter felis ATCC 49179, Helicobacter hepaticus ATCC 51448, Helicobacter mustelae ATCC 43772, Helicobacter cinaedi ATCC 35683, Campylobacter jejuni ATCC 29428, Escherichia coli ATCC 25922, Bacteroides vulgatus IFO14291, B. breve JCM1192, and B. infantis JCM1222. Forty-one Helicobacter 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 Campylobacter jejuni were cultured on brain heart infusion agar (BIFco) 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 coccolid form, the culture plates were incubated for a further 7 days in an anaerobic environment (Anaero Pack Anaero System; Mitsubishi Gas Chemical Co., Inc.) (18). *B. subtilis* and *Bifidobacterium* species were cultured anaerobically on glucose blood agar (Nissui Pharmaceutical Co., Ltd.) plates containing 5% horse blood for 4 days. *E. coli* was cultured aerobically on a brain heart infusion agar plate 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) (Biorad Model 7250; Seiko Instruments & Electronics, Ltd.).

**Production of MAbs.** The immunogen used to immunize mice consisted of sonicated cells of the coccolid 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 zero. On days 10 and 20, mice were boosted with the immunogen mixed with Freund's incomplete adjuvant (Difco). On day 27, a final injection of the immunogen without adjuvant was administered intraperitoneally. On day 30, spleen cells and PSX65.Ag8.653 myeloma cells (1:10) 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 (Difco) (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; diluted 1:800 in Block Ace) at room temperature for 1 h, and the peroxidase activity was developed in a substrate solution (0.6 mM 3,3'-diaminobenzidine and 4 mM H₂O₂ in PBS).

**Direct sandwich EIA using MAB 21G2.** MAB 21G2 was purified from ascitic fluid using Affi-Gel protein A (Bio-Rad) column chromatography. For labeling, MAB 21G2 was conjugated with peroxidase by the maleimide method of Ishikawa (8). Microtiter plates were coated with MAB 21G2 and blocked as described above. About 100 mg (~0.1 ml) of each fecal sample was diluted in 0.4 ml of dilution buffer. Fifty microliters of diluted fecal sample or sonicated bacterial cells and 50 μl of peroxidase-conjugated MAB 21G2 were added to each well of the plates, and the plates were incubated for 1 h at 25°C. After the plates were washed five times with 250 μl of washing buffer, 100 μl of substrate solution (TMB 1 component microwell peroxidase substrate containing 3,3',5,5'-tetramethylbenzidine and H₂O₂; BioFX Laboratories) was added. The reaction was stopped after 10 min by adding 50 μl of 1 N H₂SO₄, and the absorbance at dual wavelengths (450 nm and 630 nm) was measured on a microplate reader. The cutoff values used were ≤0.100 for a negative result and ≥0.100 for a positive result.

**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 each *H. pylori*-positive subject was suspended in 100 ml of PBS, and a supernatant was obtained by centrifugation 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 MAB (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.

**Screening of MAbs recognizing fecal antigen by direct sandwich EIA.** The precipitate containing MAbs was obtained by adding ammonium sulfate to a precipitate containing MAbs was obtained by adding ammonium sulfate to as much as 75% of the total protein. The precipitate was redissolved in PBS containing 0.5% formalin and then incubated overnight at 4°C. The precipitate was then dialyzed against PBS containing 0.1% Tween 20, and the resulting 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 PBS containing 0.1% skim milk (dilution buffer) and peroxidase-conjugated anti-mouse immunoglobulin (G; Cappel; diluted 1:800 in Block Ace) at room temperature for 1 h, and the peroxidase activity was developed in a substrate solution (0.6 mM 3,3'-diaminobenzidine and 4 mM H₂O₂ in PBS).

<table>
<thead>
<tr>
<th>MAB</th>
<th>Coating</th>
<th>Labeling</th>
<th>Positive subject no.</th>
<th>Negative subject no.</th>
<th>Aavgpos, Aavgneg:</th>
</tr>
</thead>
<tbody>
<tr>
<td>21G2</td>
<td>21G2</td>
<td>21G2</td>
<td>3.3</td>
<td>2.8</td>
<td>2.1</td>
</tr>
<tr>
<td>21G2</td>
<td>41A5</td>
<td>21G2</td>
<td>1.2</td>
<td>0.85</td>
<td>0.59</td>
</tr>
<tr>
<td>21G2</td>
<td>82B9</td>
<td>&gt;3.5</td>
<td>3.5</td>
<td>3.1</td>
<td>2.7</td>
</tr>
<tr>
<td>41A5</td>
<td>41A5</td>
<td>41A5</td>
<td>3.3</td>
<td>&gt;3.5</td>
<td>0.4</td>
</tr>
<tr>
<td>41A5</td>
<td>21G2</td>
<td>&gt;3.5</td>
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<td>2.2</td>
<td>0.68</td>
</tr>
<tr>
<td>41A5</td>
<td>82B9</td>
<td>&gt;3.5</td>
<td>3.5</td>
<td>2.3</td>
<td>1.5</td>
</tr>
<tr>
<td>82B9</td>
<td>82B9</td>
<td>82B9</td>
<td>3.5</td>
<td>&gt;3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>82B9</td>
<td>21G2</td>
<td>21G2</td>
<td>3.3</td>
<td>&gt;3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>82B9</td>
<td>41A5</td>
<td>41A5</td>
<td>&gt;3.5</td>
<td>&gt;3.5</td>
<td>&gt;3.5</td>
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</tbody>
</table>

* Data are mean values in duplicate.

**RESULTS**

**Screening of MAbs.** Thirty-two stable hybridoma clones that reacted with the sonicated *H. pylori* ATCC 43504 coccolid cells by indirect EIA were obtained. To select MAbs for sensitive sandwich EIA, we screened coating and labeling MAbs combinations of 32 clones for reactivity against the immunogen in direct sandwich EIA. Of 1,024 sandwich MAB combinations, 91 could react with the immunogen. Moreover, we tested 91 sandwich MAB combinations for reactivity with fecal samples. As shown in Table I, in nine sandwich EIA with three MAbs (21G2, 41A5, and 82B9), the absorbance values of fecal supernatants from four *H. pylori*-positive subjects were markedly higher than those from three negative subjects.

All MAbs belonged to the IgG1 subclass, and the isoelectric points of 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 each *H. pylori*-positive subject was suspended in 100 ml of PBS, and a supernatant was obtained by centrifugation 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 MAB (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.

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All MAbs belonged to the IgG1 subclass, and the isoelectric points of MAbs 21G2, 41A5, and 82B9 were 5.1, 4.7, and 5.6, respectively.

**Characterization of antigen.** In two sandwich EIA using the three MAbs ([i] coating, 21G2; labeling, 41A5; [ii] coating, 41A5; labeling, 82B9), the reactivity of the sonicated *H. pylori* ATCC 43504 helical forms was similar to that of coccolid forms (data not shown). The result indicated that antigens recognized by the MAbs were conserved during morphological con-
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 MAb 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-mercaptoethanol, 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-mercaptoethanol (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 fluids. We measured the lower limit for detection was estimated to be 10^5 per ml.

<table>
<thead>
<tr>
<th>Bacterial species and strain(s)</th>
<th><em>A</em>&lt;sub&gt;500/400&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pylori</em> ATCC 43504</td>
<td>2.6</td>
</tr>
<tr>
<td><em>H. pylori</em> clinical isolates</td>
<td>1.6 (0.58–3.5)</td>
</tr>
<tr>
<td><em>H. hepaticus</em> ATCC 51448</td>
<td>0.014</td>
</tr>
<tr>
<td><em>H. felis</em> ATCC 49179</td>
<td>0.015</td>
</tr>
<tr>
<td><em>H. mustelae</em> ATCC 43772</td>
<td>0.017</td>
</tr>
<tr>
<td><em>H. cinædi</em> ATCC 35083</td>
<td>0.015</td>
</tr>
<tr>
<td><em>C. jejuni</em> ATCC 29428</td>
<td>0.015</td>
</tr>
<tr>
<td><em>B. vulgatus</em> IFO14291</td>
<td>0.016</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>0.015</td>
</tr>
<tr>
<td><em>B. breve</em> JCM1192</td>
<td>0.014</td>
</tr>
<tr>
<td><em>B. infantis</em> JCM1222</td>
<td>0.015</td>
</tr>
</tbody>
</table>

* Sonicated cells were used. Protein concentrations of sonicated cells were 1 and 200 μg/ml for *H. pylori* strains and for bacterial species other than *H. pylori*, respectively.

* Data are mean values in duplicate.

* This value is the mean (range) of 41 isolates.

**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 coccoïd form.
of 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 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 H. pylori, because it is well known that H. pylori outside the stomach probably converts from the helical form to the coccoid form. Therefore, we used the 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 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 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 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 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 H. pylori antigen (1, 4, 7, 13, 14), there has been no report on a MAb specific for a distinct fecal H. pylori antigen. To elucidate the H. pylori antigen profile in feces and to guarantee H. pylori diagnosis when the H. pylori antigen in feces is used, identification of the fecal H. pylori antigen is important.

The high sensitivity and specificity of the single-step direct sandwich EIA developed for H. pylori and fecal samples have been elucidated by comparative studies of the assay’s reactivity with some 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 H. pylori infectious diseases.

REFERENCES