Helicobacter pylori infection can be diagnosed by tests requiring endoscopic biopsy of the gastric mucosa (culture, histology, and a rapid urease test) and by noninvasive tests (serology and urea breath test) (20). Recently, two enzyme immunoassays (EIAs) for the direct detection of the fecal H. pylori antigens have been developed. One uses polyclonal rabbit antibody (Premier Platinum HpSA; Meridian Diagnostics, Cincinnati, Ohio), and the other uses plural kinds of MAbs (FemtoLab Inc., Cincinnati, Ohio), and the other uses plural kinds of MAbs. The cellular and fecal antigen, and development of a new single-step EIA that uses one kind of MAb, 21G2, for the detection of fecal H. pylori antigens (17). The developed EIA was able to detect 41 H. pylori isolates and fecal samples from seven H. pylori-positive human subjects. Other members of the Helicobacter species, major bacteria in feces, and fecal samples from six H. pylori-negative human subjects gave negative results. The antigen was characterized as follows. (i) The molecular masses of the cellular antigen and the fecal antigen were the same, 260 kDa. (ii) The antigen was labile to sodium dodecyl sulfate (SDS) and heat treatment. (iii) The structure of the antigen was considered to be composed of more than one epitope, such as a homodimer or homotetramer, because the sandwich EIA consisting of one kind of MAb could detect the antigen. For the purpose of contributing to the elucidation of the H. pylori antigen profile in human feces and establishing the basic aspects of the newly developed EIA that uses one kind of MAb, we intended to clarify one of the human fecal antigens originating from H. pylori.

In the present paper, we describe both the identification of the antigen purified by immunoaffinity chromatography with the MAb 21G2 as a ligand and the specificity of the MAbs for the antigen.

MATERIALS AND METHODS

MAbs and human fecal samples. The MAbs 21G2, 41A5, and 82B9 were produced as described previously (17). The precipitates containing MAbs were obtained by addition of (NH₄)₂SO₄ to mouse ascitic fluid to a concentration of 50% saturation and then centrifugation at 2,000 × g for 20 min. The MAbs were further purified with Affi-Gel according to the procedures indicated by the manufacturer (Bio-Rad Laboratories). Human fecal samples were obtained from two healthy Japanese male subjects (subject A, 56 years old; subject B, 53 years old) and stored at −35°C before use. The subjects were determined to be H. pylori positive by the urea breath test and serology. Consent was obtained from the participants in the study.

Antigen extraction from H. pylori cells. H. pylori ATCC 43504 was cultured on brain heart infusion (BHI) agar (Difco) plates containing 5% horse blood in a

Catalase, a Specific Antigen in the Feces of Human Subjects Infected with Helicobacter pylori

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Recently, we reported the production of three new monoclonal antibodies with high specificity for a Helicobacter pylori antigen suitable for diagnosis of H. pylori infection. The aim of the present study was to identify the antigen recognized by these monoclonal antibodies concerning both H. pylori and the feces of human subjects infected with H. pylori. The cellular antigen was purified from an H. pylori cell extract by immunoaffinity column chromatography with the monoclonal antibody as a ligand. The amino-terminal amino acid sequences (eight residues) of the purified antigen and H. pylori catalase were the same. The molecular weights of native and subunit, specific catalase activity, and UV and visible spectra of the purified antigen were in good agreement with those of H. pylori catalase. The human fecal antigens were purified from two fecal samples of two H. pylori-positive subjects by ammonium sulfate precipitation, CM-Sephadex C₅₀ chromatography, and the same immunoaffinity chromatography used for the H. pylori cellular antigen. The fecal antigens had catalase activity. The amino-terminal amino acid sequences (five residues) of the human fecal antigen and H. pylori catalase were the same. The monoclonal antibodies reacted with the native cellular antigen, but did not react with the denatured antigen, human catalase, and bovine catalase. The results show that the target antigen of the monoclonal antibodies is native H. pylori catalase and that the monoclonal antibodies are able to specifically detect the antigen, which exists in an intact form, retaining the catalase activity in human feces.
microaerobic environment (Anaero Pack Helico system; Mitsubishi Gas Chemical Co., Ltd.) for 4 days at 37°C. The bacterial cells were harvested, washed in phosphate-buffered saline (PBS), suspended in PBS containing 0.5% formalin, and then stored overnight at 4°C. The bacterial cells were washed three times in PBS and disrupted by sonication with a Bioron’s model 7250 (output 3, 50% duty cycle for 10 min; Seiko Instruments and Electronics, Ltd.). The soluble fraction containing the antigen was obtained by ultracentrifugation at 90,000 × g for 30 min.

Antigen extraction and partial purification from human fecal samples. Two human fecal samples (165 g from subject A and 92 g from subject B) were used. A fecal sample was suspended in fourfold volumes of PBS, and the supernatant was obtained by centrifugation at 10,000 × g for 30 min and then ultracentrifugation at 90,000 × g for 30 min. (NH₄)₂SO₄ was added to the supernatant to a concentration of 40% saturation, and the mixture was centrifuged at 8,000 × g for 30 min. (NH₄)₂SO₄ was further added to the resultant supernatant to a concentration of 80% saturation, and the mixture was centrifuged at 8,000 × g for 30 min. The precipitate containing the antigen was dissolved in 10 mM potassium phosphate buffer (pH 7.0) and dialyzed against the same buffer. The dialysate was applied to a column of CM-Sephadex C₁₀ (1 by 2.5 cm) equilibrated with the same buffer. The column was washed with the buffer, and the antigen was eluted with PBS. The antigenicity-positive fractions in the eluate were pooled (partially purified antigen).

Affinity chromatography on MAb 21G2 column. Fifty milligrams of MAb 21G2 was immobilized on 3 g of CNBr-activated Sepharose 4B according to the procedures indicated by the manufacturer (Amersham Pharmacia Biotech). The soluble fraction from _H. pylori_ (5 ml, 4 mg of protein/ml) or the partially purified antigen from human feces was applied to a column of MAb 21G2-immobilized Sepharose 4B (2 by 3 cm) equilibrated with PBS for 2 h at room temperature. The column was washed with PBS, and the antigen was eluted with 0.2 M glycine-HCl buffer (pH 3.0). Each fraction was neutralized with 1 M Tris, and the fractions containing the antigen were pooled and concentrated by dehydration with polyethylene glycol 20000 (Wako Pure Chemical Co.).

Identification of antigen. The purity and subunit molecular weight of the antigen were estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 20% gradient) (9). A low-molecular-mass-SDS calibration kit (Amersham Pharmacia Biotech) was used for molecular mass standards. Proteins were visualized by staining the gel with silver stain (Kanto Chemicals Co., Inc.). The molecular weight of the native antigen was estimated by Sephacyr-S300HR gel filtration column chromatography (1.5 by 140 cm, 0.1 M phosphate buffer [pH 6.5]) with molecular mass calibration kits from Amersham Pharmacia Biotech. The amino-terminal amino acid sequence was determined by automated Edman sequencing with the HP G1005A protein sequencing system (Hewlett-Packard Company). UV and visible spectra (220 to 600 nm) were recorded by a spectrophotometer (U-3210; Hitachi).

Assays. Antigenicity determination by a direct sandwich ELISA with MAb 21G2 was performed as described previously (17). Briefly, plastic 96-well ELISA microtiter plates (Coster) were coated with MAb 21G2 (5 μg of protein/ml in PBS) overnight at 4°C. After blocking nonspecific binding sites with 250 μl of PBS containing 1% skim milk (Difco) (blocking buffer) for 1 h at 4°C, 50 μl of samples 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 PBS containing 0.05% Tween 20 (washing buffer), 100 μl of substrate solution containing 3, 3', 5, 5'-tetramethylbenzidine and H₂O₂ (TMB 1 component Microwell peroxidase substrate; BioFX Laboratories) was added. The reaction was stopped after 10 min by adding 50 μl of 1 N H₂SO₄, and the absorbance was measured on a microplate reader (model 550; Bio-Rad Laboratories) at dual wavelengths (450 and 630 nm). Direct sandwich ELISAs with the single MAb (41A5 or 82B9) were developed and performed as described above.

Immunoblotting (Western blotting) was performed as described previously (10). For dot blotting, 2 μl of samples was dotted on a nitrocellulose membrane. The samples denatured by heat treatment (100°C, 5 min) in the presence of 1% SDS were also used. The membrane was blocked with 4% (wt/vol) milk and Tween 20 (blocking buffer) for 1 h at room temperature, washed in washing buffer, and incubated in the MAb solution (50 μg/ml in Block Ace). The membrane was then washed in washing buffer, incubated with peroxidase-conjugated antiserum immunoglobulin G (G; Cappel; dilution, 1:800 in Block Ace) for 1 h at room temperature, and the peroxidase activity was developed in substrate solution (0.6 mM 3, 3'-diaminobenzidine and 4 mM H₂O₂ in PBS).

Catalase activity was determined at 25°C by the spectrophotometric method of Beers and Sizer (2) with a molar absorption coefficient of 43.48 liters/mole/cm at 240 nm (7). Protein concentration was determined with a bicinchoninic acid protein assay reagent (Pierce Chemical Co.) with bovine serum albumin as a standard.

Cross-reactivity with other catabases. Human erythrocyte catalase and bovine liver catalase were obtained from Sigma and Amersham Pharmacia Biotech, respectively. Cell extract containing catalase of other bacteria was prepared from the following catalase-positive type cultures: _H. pylori_ ATCC 43504, _Helicobacter felis_ ATCC 49179, _Helicobacter hepaticus_ ATCC 51448, _Helicobacter mustelae_ ATCC 43772, _Helicobacter cinaedi_ ATCC 35683, _Campylobacter jejuni_ ATCC 29428, and _Escherichia coli_ ATCC 25922. _Helicobacter_ species and _C. jejuni_ were cultured on BHI agar plates containing 5% horse blood in a microaerobic environment for 4 days. _E. coli_ was cultured aerobically on BHI agar plates for 3 days. All cultures were done at 37°C. Bacterial cells were harvested, washed in 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 as described above. The cell extract containing catalase was obtained by ultracentrifugation at 90,000 × g for 30 min. Reactivity of the MAbs 21G2,
41A5, and 82B9 with other catalases was tested in the direct sandwich EIAs with the single MAb.

**RESULTS**

**Purification and identification of cellular antigen from *H. pylori*.** We purified the *H. pylori* cellular antigen from the soluble fraction in the cytoplasm by immunoaffinity chromatography. An elution profile of the antigen is shown in Fig. 1A. The cellular antigen was eluted in fractions 14 to 19. SDS-PAGE of each fraction showed that the cellular antigen was purified to a single band, and the molecular mass of the antigen was estimated to be 59 kDa (Fig. 1B). The native molecular mass of the purified antigen was estimated to be 260 kDa by gel filtration chromatography (data not shown), suggesting that the antigen consists of a homotetramer structure. The absorption maxima of the purified antigen were 278 and 406 nm (data not shown).

We concluded that the purified antigen is native *H. pylori* catalase from the following facts. (i) The native molecular mass of the antigen was close to that of *H. pylori* catalase (200 kDa), which is a homotetramer (7). (ii) The molecular mass of the subunit of the antigen (59 kDa) was in agreement with the value predicted from the amino acid sequence of the *H. pylori* catalase gene product (58,599 Da) (14) and was also close to the experimentally determined value (50 to 57 kDa) (7, 15, 21). (iii) Eight amino acids from the amino terminus of the purified antigen were determined to be Met-Val-Asn-Lys-Asp, which are identical to those in the reported sequence of *H. pylori* catalase (13, 14, 21). (iv) Catalase activity was eluted parallel with the antigenicity from the immunoaffinity column (Fig. 1A). (v) The absorption maxima of the purified antigen were very similar to that of *H. pylori* catalase (280 and 405 nm) (7). (vi) The specific catalase activity of the purified antigen (68 mmol of H₂O₂/min/mg of protein) was almost the same as that of the purified catalase (60 ± 3 mmol of H₂O₂/min/mg of protein) (7).

**Purification of human fecal antigen from two *H. pylori*-positive subjects.** By comparing the reactivity of the ultracentrifugal fecal supernatant with that of the *H. pylori* cell extract in the EIA, as described previously (17), the relative amount of the antigen in feces was estimated to be extremely low (ca. 5 × 10⁻⁶). Therefore, the partial purification by ammonium sulfate precipitation and cation-exchange chromatography on the CM-Sephadex column was performed before the immunoaffinity chromatography. The cation-exchange chromatography was chosen because the isoelectric point of the *H. pylori* catalase is relatively high (pI = 9.0 to 9.3) (7). The antigenicity was adsorbed on a CM-Sephadex column and eluted with PBS, indicating that the fecal antigen was cationic (data not shown). Elution profiles on the immunoaffinity column are shown in Fig. 2. The antigenicity was detected in the eluate fractions, and both peak fractions of the antigenicity showed catalase activity. SDS-PAGE of the purified human fecal antigen from subject A showed a single band (59 kDa), and five amino acids from the amino terminus of the purified antigen were determined to be Met-Val-Asn-Lys-Asp, which are identical to the reported sequence of *H. pylori* catalase. The facts described above show the existence of intact catalase originating from *H. pylori* in fecal samples from human subjects infected with *H. pylori*.

**Specificity of the MAbs with the antigen.** To guarantee the high specificity of the MAbs for *H. pylori* catalase, we examined the reactivity of the MAbs with other catalases. The *H. pylori* catalase reacted dose dependently in the EIAs that use one kind of MAb—21G2, 41A5, or 82B9 (data not shown)—and the lower limit of the detection for *H. pylori* catalase was 6 ng of protein/ml. Whereas, no reaction of human erythrocyte catalase and bovine liver catalase was observed up to 100,000 ng/ml in the EIAs. Furthermore, no cross-reactivity with other bacterial catalases was observed in the EIAs (Table 1). Immunoblotting of the *H. pylori* catalase with the MAbs 21G2, 41A5, and 82B9 showed no detectable band. The MAbs 21G2, 41A5, and 82B9 reacted with native *H. pylori* catalase, but did not react with denatured *H. pylori* catalase by dot blotting. The results indicate that the MAbs recognized a conformational epitope consisting of a homotetramer structure, because *H. pylori* catalase should be converted to a subunit structure in the process of immunoblotting.

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FIG. 2. Purification of *H. pylori* fecal antigen from subject A (A) and subject B (B) by immunoaffinity chromatography with MAb 21G2. Six milliliters of partially purified fecal antigen (2.4 mg of protein and 1 mg of protein for panels A and B, respectively) was loaded onto an immunoaffinity column. The column was washed with PBS, and the antigen was eluted with 0.2 M glycine-HCl buffer (pH 3.0). Fractions of 10 ml were collected. The arrows indicate the start of elution. ○, antigenicity (1:10 dilution for panel A and no dilution for panel B); △, catalase activity.
DISCUSSION

We purified *H. pylori* cellular antigen recognized by MAb 21G2 from the soluble fraction by immunoaffinity chromatography and identified the antigen as *H. pylori* catalase. Similarly, the human fecal antigens were purified from the two *H. pylori*-positive human subjects. The amount of the purified fecal antigens was extremely low. Thus, we could not determine the specific catalase activity of the purified fecal antigens precisely. However, from the results obtained and the fact that native molecular masses of the fecal antigen from three positive subjects were the same, 260 kDa (17), we concluded that the existence of intact catalase originated from *H. pylori* in fecal samples from human subjects infected with *H. pylori*. The interesting fact that native catalase is excreted in intact form without being denatured in the gut is reported first in the present paper. The catalase activity of *H. pylori* was significantly greater than that of the related bacterium *C. jejuni* (7). We also estimated that the *H. pylori* catalase content was 10% of the total soluble protein from the results of the antigen purification (data not shown). *H. pylori* catalase activity was positive for aged cultures (the coccoid cells) even up to 160 days (22). *H. pylori* catalase in whole cells, the supernatant, and the two cell extracts containing catalase (Table 1). Newell et al. reported the production of an anti-*H. pylori* catalase MAb, CP30, which could react with the subunit of the catalase (D. G. Newell, P. Nuijten, A. R. Stacey, and S. L. Hazell, abstract from Microb. Ecol. Health Dis. 4(Suppl.):S120, 1991), and the MAB was used for immunoblotting analysis of the *H. pylori* catalase in other reports (13, 15). Thus, the target epitope of the MAB CP30 is considered to be on the primary sequence; however, the specificity of the MAB has not been shown. The high specificity of the MABs (21G2, 41A5, and 82B9) for *H. pylori* catalase, which may refer to the properties of recognizing a conformational epitope with a homotetramer structure of *H. pylori* catalase, could be emphasized.

For several pathogenic bacteria, catalase is involved in the defense mechanisms against in vivo killing by polymorphonuclear granulocytes. *H. pylori* catalase activity was also shown to apparently be essential for the survival of *H. pylori* at the phagocytes’ cell surface (16). *H. pylori* catalase-deficient mutants occurred spontaneously in vitro (13, 21); however, there has been no report on the isolation of catalase-deficient strains clinically (21). Moreover, a vaccine study indicates that *H. pylori* catalase is a highly effective antigen, suggesting that it may be essential in vivo (15). The findings suggest that *H. pylori* catalase is necessary for *H. pylori* infection. Therefore, the EIA that used the well-characterized MABs with high specificity for native *H. pylori* catalase would be a useful diagnostic test for *H. pylori* infection. A clinical study for the evaluation of the EIA that used the MAB 21G2 is in progress and will be reported elsewhere.

### REFERENCES

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<th>Origin</th>
<th>Catalase activity (µmol of H₂O₂/min/ml)</th>
<th>A₄₅₀/₆₃₀²</th>
<th>21G2</th>
<th>41A5</th>
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* Data represent mean values in duplicate.

² Cell extracts were used as bacterial catalases. Specific activities in micromoles of H₂O₂ per minute per milligram of cellular protein are shown in parentheses.


