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Characterization of an Immunoreactive Species-Specific 19-Kilodalton Outer Membrane Protein from *Helicobacter pylori* by Using a Monoclonal Antibody

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Immunoblotting experiments on hyperimmune rabbit serum and sera from patients with *Helicobacter pylori* gastritis showed a consistent antibody response to a 19-kDa outer membrane protein antigen. A monoclonal antibody, designated HP 40, which reacted by Western immunoblotting with this protein was produced. It was shared by all *H. pylori* strains tested (D 273, NCTC 11637, and 24 wild strains) but not by the thermophilic *Campylobacter* species, *Campylobacter fetus*, *Helicobacter mustellae*, or *Helicobacter fennelliae*. Immunogold staining suggested that the 19-kDa antigen was exposed on the outer surface of the bacteria. Its functional role and effectiveness as a serological diagnostic tool are under study.

Helicobacter pylori is closely associated with type B antral gastritis and peptic ulceration in humans (17, 24, 29). Its etiological role in these disorders remains questionable but is supported by significant therapeutic trials with antimicrobial agents (9, 10) and experiments with animal models (14). Moreover, possible virulence factors of *H. pylori* have been described previously (2).

Initial serological investigations indicate that patients with *H. pylori* infections mount a significant circulating antibody response against the organism (11, 13, 16), and several immunogenic conserved major proteins have been identified by Western immunoblotting and radioimmunoprecipitation techniques (18, 22). In this report, we describe the production and characterization of a monoclonal antibody (MAb) reacting against a 19-kDa antigen which appeared to be *H. pylori* species specific.

MATERIALS AND METHODS

Bacterial strains. *H. pylori* D 273 (from E. Drouet) (6) was used for immunization procedures. The following strains were also included in this study: *Campylobacter* (*Helicobacter*) *pylori* NCTC 11637, *Campylobacter coli* NCTC 11349, *Campylobacter jejuni* NCTC 11351, *Campylobacter fetus* NCTC 5850, and *Campylobacter laridis* ATCC 35221. *Helicobacter fennelliae* CLO-2 and *Helicobacter mustellae* F-CLO F3 (27) (kindly provided by F. Megraud, Bordeaux, France) and 24 *H. pylori* wild strains isolated from patients with gastritis or duodenal ulcer were also tested. All strains were cultured on tryptocasein soy agar supplemented with 1% starch and 10% horse blood and were incubated in microaerobic conditions (Anaerocult C; Merck, Darmstadt, Federal Republic of Germany) for 72 h at 37°C.

Preparation of antigenic extracts. *H. pylori* acid extracts were prepared by treatment of whole bacteria with 0.2 M glycine-hydrochloride buffer (pH 2.2), as described by Goodwin et al. (11). Outer membranes were obtained by the method of Blaser et al. (3). Briefly, washed bacterial suspensions (10^9 CFU/ml) in 0.01 M Tris buffer (pH 7.4) were sonicated and then pelleted by centrifugation at 5,000 × *g* for 20 min. The supernatant was centrifuged at 100,000 × *g* for 60 min at 4°C. The resulting pellet was treated by a sodium *N*-lauroylsarcosinate solution (Sarkosyl) (1% in 7 mM EDTA) (23) and incubated for 20 min at 37°C. Outer membranes representing the Sarkosyl-insoluble fraction were collected by centrifugation at 50,000 × *g* for 60 min at 4°C, resuspended in sterile distilled water, and stored at −20°C.

Preparation of bacterial LPS. The extraction of lipolysaccharide (LPS) from *H. pylori* D 273 was performed by the phenol-water (50% final concentration) extraction method of Westphal and Jann (30) at 65°C for 30 min. After dialysis of the aqueous phase, LPS was recovered by repeated centrifugation at 100,000 × *g* for 4 h.

Polyclonal antisera. Adult New Zealand White rabbits were inoculated intravenously with a washed suspension of *H. pylori*. Inoculations were repeated twice weekly with increasing concentrations (10^3 to 10^6 cells per ml) and volumes (0.3 to 1.5 ml) of the antigens during 2 months. Sera were collected 6 days after the last injection and stored at 4°C. Sera were also taken from 20 adult patients undergoing gastroduodenal endoscopy. Among them, 10 patients had clinical and histological signs of gastritis and an *H. pylori* strain was isolated from biopsy. The remaining 10 patients had neither clinical symptoms nor histological signs of gastritis and were bacteriologically negative.

MAb production. Female BALB/c mice were immunized intraperitoneally with a whole-cell washed suspension of *H. pylori* D 273 (10^8 CFU/ml). The first inoculation was with complete Freund's adjuvant. Immunization was repeated twice with incomplete Freund's adjuvant at 3-week interval. The mice were boosted 2 weeks after the last inoculation. Four days later, their spleen lymphocytes were fused with SP2/O-Ag14 myeloma cells (25) by the polyethylene glycol 4000 method (8). The fused cells were suspended in RPMI 1640 medium with 10% fetal calf serum and hypoxanthine-aminopterine-thymidine and plated into microtiter wells. After 10 days, supernatants of hybrid cultures were screened by immunofluorescent antibody assay and enzyme-linked immunosorbent assay (ELISA) (see below). Positive hybrids were cloned twice by limiting dilution, and then monoclones

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were grown in tissue culture bottles for further analysis of secreted antibodies. The MAbs were isotyped by gel immunodiffusion with rabbit anti-mouse immunoglobulins (Nordic Immunologicals).

Immunofluorescence assay. Fifteen microliters of a bacterial suspension (10^7 cells per ml) from each strain mentioned above was allowed to dry at room temperature on acetone-resistant 10-well immunofluorescence glass slides. Slides were fixed in cold acetone for 10 min and air dried. Then 25 μl of MAb was added to each well and incubated at 37°C for 30 min. The slides were gently rinsed with phosphate-buffered saline (PBS), pH 7.2, and air dried. Fluorescein-labeled anti-mouse immunoglobulins (Diagnostics Pasteur, Marnes la Coquette, France) at a suitable dilution were added, and the slides were incubated for 30 min at 37°C, washed twice with PBS, and dried on filter paper. Reading was with a Zeiss Axioskop equipped with an HBO 50 mercury incident light source (Carl Zeiss, Oberkochen, Federal Republic of Germany).

ELISA. The ELISA procedure with acid-glycine extract was performed as previously described (11).

Immunoblotting experiments. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a linear gradient of 8 to 18% acrylamide by the method of O'Farrell (20). Equal volumes of sample buffer (5% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue in 0.01 M Tris-HCl, pH 8) and outer membrane protein (OMP) preparation were mixed and boiled for 5 min. Then a 10-μg amount of protein (as determined by the method of Markwell et al. [15]) was loaded and electrophoresed (10 mA per gel) until the tracing dye migrated near the bottom of the gel. The molecular weights of the components resolved were calculated on the basis of a calibration curve of known protein markers (electrophoresis calibration kit; Pharmacia, Uppsala, Sweden). After electrophoresis, the proteins were either silver stained by the method of Oakley et al. (19) or transferred from the gel onto a nitrocellulose sheet (26). The blots were soaked in blocking solution (5% nonfat milk, 0.01% thimerosal, and 0.01% antifoam in PBS) and then incubated for 2 h at room temperature in antibody (rabbit and human sera diluted 1 in 100, hybridoma supernatant fluids undiluted). After washings in PBS with 0.05% Tween 20 (PBS-T), incubation was done for 1 h at 37°C with peroxidase-conjugated anti-species immunoglobulins suitably diluted in blocking solution. After washings in PBS-T and then rinsing with 0.05 M Tris-buffered saline, pH 7.4, staining was performed with 60 ml of 0.3% 4-chloro-1-naphthol in methanol in 100 ml of PBS with 0.02% H_2O_2. The separation and the transfer to nitrocellulose of components from the bacterial LPS and the determination of their reactivity with antibody (rabbit serum and hybridoma supernatant fluid) were performed as described above.

Immunoelectron microscopy. A suspension of *H. pylori* containing approximately 10^7 cells per ml was used for immunoelectron microscopy. Formvar-coated grids were floated on 50-μl drops of the suspension for 5 min and washed on 3 successive drops of PBS with 1% bovine serum albumin (BSA). The grids were then incubated for 30 min with MAb (undiluted supernatant of cell culture). After washings in PBS-BSA and then in 20 mM Tris-HCl (pH 8.2) with 1% BSA, the grids were incubated for 20 min with anti-mouse immunoglobulin G coupled to 5-nm gold particles (Amersham International, Amersham, United Kingdom). The grids were washed in Tris buffer and in deionized water, air dried, and then stained by phosphotungstic acid. For ultrathin sections, 200 μl of bacterial suspension was centrifuged at 800 × *g* for 10 min in siliconized tubes and then resuspended with MAb. After 1 h, samples were centrifuged and resuspended in Tris buffer. After pelleting, the cells were incubated with gold conjugate for 30 min. After washings, samples were fixed and embedded in epoxy resin. Ultrathin sections were made and then poststained with uranyl acetate and lead citrate before examination in a Philips EM 300 transmission electron microscope.

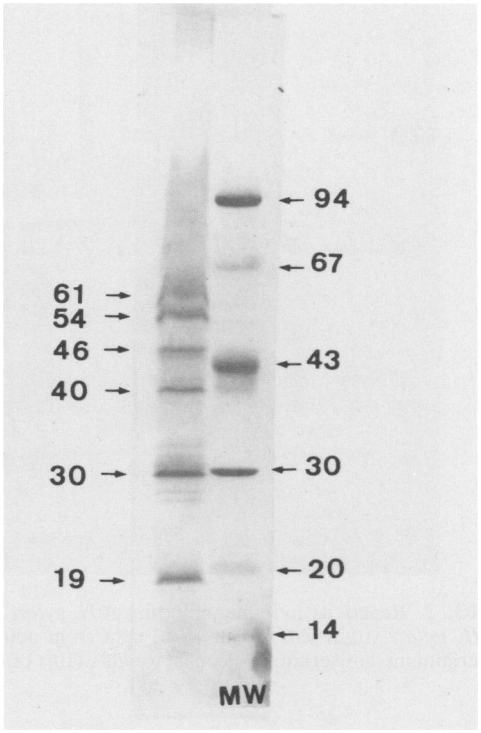

FIG. 1. PAGE profile of *H. pylori* D 273 OMPs after silver staining. MW, molecular weight markers (sizes are indicated in kilodaltons).

RESULTS

Characterization of *H. pylori* OMP by SDS-PAGE. The OMP profiles of *H. pylori* D 273, *H. pylori* NCTC 11637, and 24 *H. pylori* strains isolated from patients with gastritis or duodenal ulceration were similar. Silver staining showed six major bands at 61, 54, 46, 40, 30, and 19 kDa. A typical SDS-PAGE pattern of OMP from strain D 273 is shown in Fig. 1.

Reactivity of polyclonal sera. Figure 2 shows the results of immunoblot analysis of an OMP preparation of *H. pylori* D 273 with a rabbit hyperimmune serum sample and 10 serum samples from patients with clinical symptoms of gastritis and from whom an *H. pylori* strain was isolated. The rabbit antiserum strongly reacted with polypeptides at 120, 88, 61, 54, 30, 25, and 19 kDa. A similar response was obtained with human sera, with special mention of the 61-, 25-, and 19-kDa polypeptides. The serum samples from 10 noninfected individuals showing no sign of gastritis did not react.

MAbs. A total of 257 hybrid cultures were tested by immunofluorescence assay and ELISA. Forty-four were positive by both methods. After cloning, 13 stable hybridomas were established. We selected a highly reactive clone,

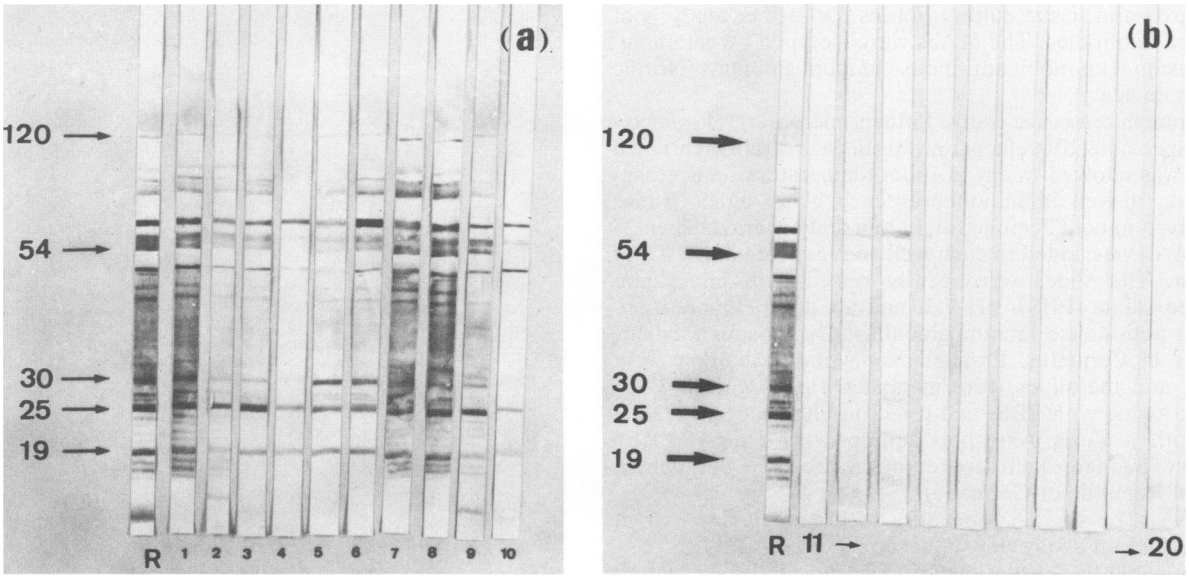

FIG. 2. Reactivity in immunoblotting of *H. pylori* D 273 OMPs. (a) Sera from adult patients with clinical signs of gastritis and from whom an *H. pylori* strain was isolated; (b) sera from adult patients with no clinical or bacteriological signs of *H. pylori* infection. R, rabbit hyperimmune antiserum. Molecular weights (10^3) of major immunoreactive proteins are indicated on the left.

designated HP 40, which produced an immunoglobulin G2a antibody. This reacted in the immunofluorescence assay and ELISA with the immunizing *H. pylori* D 273 but also with all strains of *H. pylori* tested, i.e., NCTC 11637 and 24 wild strains from patients with gastritis. No reaction was detected when *C. fetus*, *C. jejuni*, *C. coli*, *C. laridis*, *H. mustellae*, and *H. fennelliae* were used as antigens. Immunoblotting experiments on OMP preparations showed that the antibody reactivity was monoclonal and directed against a 19-kDa protein shared by all strains of *H. pylori* tested but not by the *Campylobacter* strains, *H. mustellae*, or *H. fennelliae* (Fig. 3). When immunoblotting was performed with separated LPS components from *H. pylori* D 273, rabbit anti-*H. pylori* antiserum detected multiple diffuse bands, whereas MAb HP40 did not react (Fig. 4). Immunoelectron microscopy performed on either whole bacteria or ultrathin sections demonstrated that the concerned epitope was located at the bacterial surface (Fig. 5).

DISCUSSION

It has been demonstrated that whole-cell and outer membrane profiles observed in various strains of *H. pylori* were nearly identical and that none was similar to those of *C. jejuni* and *C. fetus*. Perez-Perez and Blaser (21) found that major whole-cell bands migrated at 62, 56, 29, and 26 kDa. Newell (18) and Czinn et al. (5) detected constant OMPs at 61, 54, and 31 kDa, and Hawtin et al. (12) showed that the 61- and 28-kDa (probably equivalent to the 31-kDa) polypeptides were closely linked to the native urease protein. Our results are in agreement with previous findings, since major proteins from the outer membrane preparation were located at 61, 54,

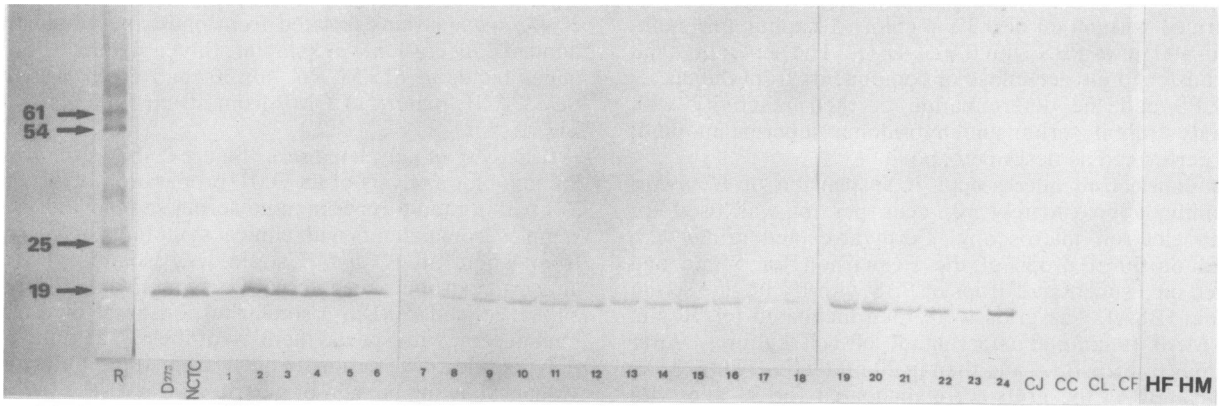

FIG. 3. Reactivity in immunoblotting of MAb HP 40 on OMPs from various *H. pylori*, *Campylobacter*, and *Helicobacter* strains. Lanes: D273, D 273 strain; NCTC, *H. pylori* NCTC 11637; 1 to 24, wild *H. pylori* strains; CJ, *C. jejuni* NCTC 11531; CC, *C. coli* NCTC 11349; CL, *C. laridis* ATCC 35221; CF, *C. fetus* NCTC 5850; HF, *H. fennelliae* CLO-2; HM, *H. mustellae* F-CLO F3. R, rabbit hyperimmune antiserum reacting with D 273. Molecular weights (10^3) are indicated on the left.

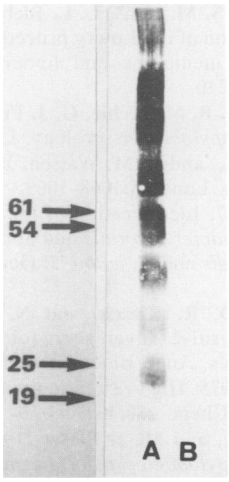

FIG. 4. Reactivity in immunoblotting on LPS from *H. pylori* D 273. Lanes: A, rabbit hyperimmune antiserum; B, MAb HP 40. Molecular weights (10^3) are indicated on the left.

and 30 kDa, additional bands being seen at 46, 40, and 19 kDa. Previous workers studied the immune responses of *H. pylori*-infected patients and demonstrated that major immunogens were at 61 to 63 kDa (18, 28) and 110 to 120 kDa (1, 28). Our immunoblotting experiments with a hyperimmune rabbit serum sample demonstrated a strong antibody response with the major OMPs (61, 54, and 30 kDa) but also an additional reactivity with bands at 120, 88, 25, and 19 kDa. Similar results were obtained with sera from *H. pylori*-infected patients, whereas sera from noninfected individuals did not react. Our data indicated that the 19-kDa protein was consistently reactive. This fact did not clearly appear in other works except that of Clayton et al. (4), who noted such a reactivity with their hyperimmune rabbit serum. However, they did not consider the 19-kDa polypeptide as a major immunogen. Dunn et al. (7) also characterized a 19-kDa protein from *H. pylori* by two-dimensional gel electrophoresis, but no further information was provided.

In this study, it was found that a 19-kDa protein was obtained with OMP preparations from all *H. pylori* strains tested. We raised the MAb HP 40 directed against this protein which reacted in immunoblotting with all of the *H. pylori* strains but not with the *Campylobacter* ones and two other *Helicobacter* species, indicating that it was specific for *H. pylori*. When MAb HP 40 was used in an indirect immunogold technique, the 19-kDa protein was identified on the surface-exposed structures. In addition, ultrathin sections of bacteria were also immunogold labeled. Although a sharp definition of bacterial morphology was not obtained, it was clear that the 19-kDa protein was closely linked to the outer membranes. The OMPs of gram-negative bacteria are immunologically important structures because of their accessibility to host defense mechanisms. We showed that the 19-kDa OMP of *H. pylori* contained a common antigen among isolates of this bacterium. Since antibody to this protein was consistently present in symptomatic patients, the antigen could be considered a candidate for developing a serological diagnostic test for *H. pylori* infection. Further studies to purify the 19-kDa OMP and to determine its functional role, if one exists, are in progress.

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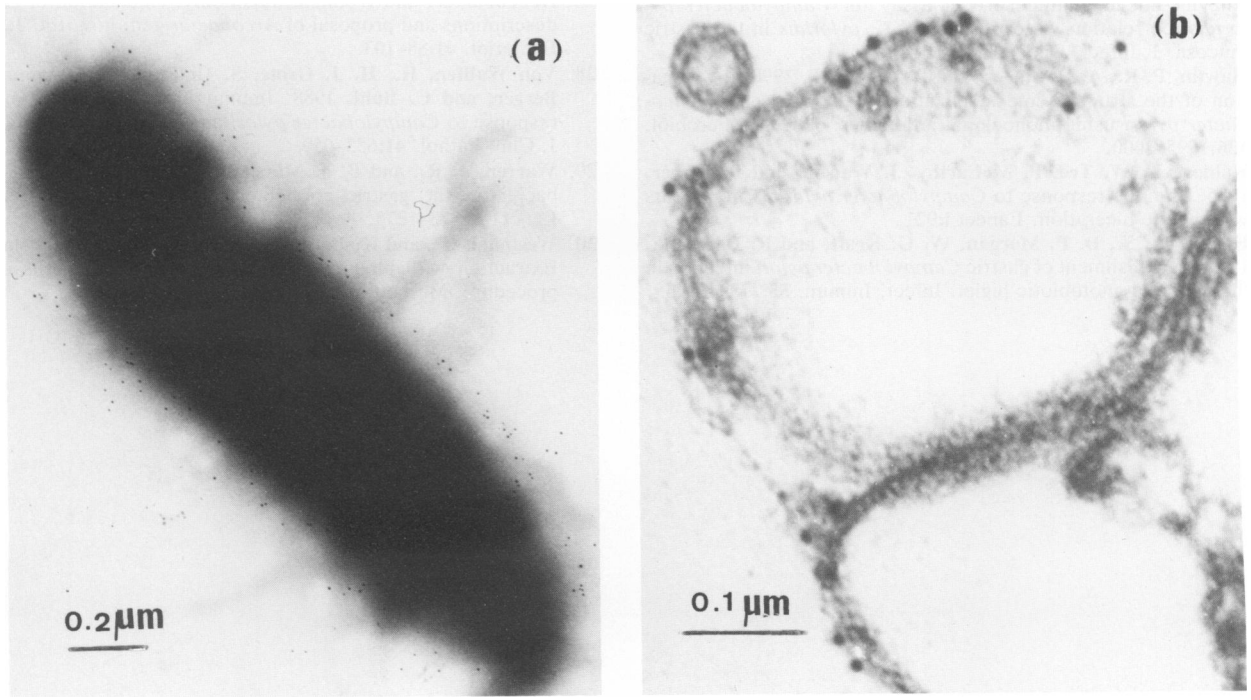

FIG. 5. Transmission electron microscopy of *H. pylori* D 273 after reacting with MAb HP 40 and indirect immunogold labeling. (a) Whole bacteria; (b) ultrathin sections.

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