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Identification of *Helicobacter pylori* by Immunological Dot Blot Method Based on Reaction of a Species-Specific Monoclonal Antibody with a Surface-Exposed Protein

INGRID BÖLIN,[1]* HANS LÖNROTH,[2] AND ANN-MARI SVENNERHOLM[1]

Department of Medical Microbiology and Immunology, Göteborg University, S-413 46 Göteborg,[1] and Department of Surgery II, Sahlgren's Hospital, S-413 45 Göteborg,[2] Sweden

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Monoclonal antibodies (MAbs) against membrane preparations of *Helicobacter pylori* were produced. One MAb was found to be specific for *H. pylori*, because it did not react with a number of other bacterial species, including *Helicobacter felis* and *Campylobacter jejuni*. This MAb reacted with a 30-kDa protein found in outer membrane preparations of *H. pylori*. The protein was also detected on the cell surface on intact bacteria when analyzed by immunoelectron microscopy. To facilitate the identification of *H. pylori* isolates after culturing of biopsies, an immunodot blot assay based on the reaction of this MAb was developed. This assay was found to be highly specific for *H. pylori*. Sixty-six clinical isolates typed as *H. pylori* by conventional biochemical tests were found to be positive, whereas no other bacterial species tested gave a positive result. By this method, reliable and rapid identification of *H. pylori* could be accomplished.

Helicobacter pylori is the causative agent of type B gastritis and is also closely associated with duodenal ulceration in humans. Several assays have been developed to demonstrate the presence of *H. pylori* in clinical specimens (3), including microbiological cultures, histological examination, rapid gastric biopsy urease tests, serologic tests, and PCR. Culturing of biopsies is still commonly used for diagnosis and may be of increasing importance as a tool to monitor antibiotic resistance of *H. pylori* when antibiotic therapy regimens become widely used. It has also been reported to be as sensitive as PCR for detecting *H. pylori* (19). The identification of *H. pylori* after cultivation of biopsies usually involves the use of different biochemical tests, as well as morphological examination of the clinical isolates. The bacterial properties detected by these methods may be shared by other bacteria and are therefore not totally specific for *H. pylori*. In this report, we describe the production and characterization of a monoclonal antibody (MAb) specific for *H. pylori* and the development of an immunological dot blot assay that allows for rapid and highly specific identification of *H. pylori.*

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. The strains were kept at −70°C until used. *H. pylori* and *Helicobacter felis* strains were grown on Columbia agar plates with 8% horse blood in an atmosphere of 10% CO_2 and 5% O_2 and incubated for 48 to 72 h. *H. pylori* strains from biopsies were isolated on Skirrow blood agar plates and identified by colony morphology appearance and urease and oxidase production. Biopsies were obtained from patients with duodenal ulcers and from one asymptomatic volunteer. *Campylobacter jejuni* strains and *Capnocytophaga sputigena* were grown as *Helicobacter* strains but were incubated for shorter time. *Campylobacter rectus* was grown anaerobically. *Escherichia coli* strains were grown on Casamino Acids-yeast extract agar, while *Salmonella*, *Shigella*, *Yersinia*, and *Vibrio cholerae* strains were grown on Columbia agar plates containing human blood. All strains were incubated at 37°C overnight.

Antigen preparations. Total membranes and Triton X-100 insoluble outer membranes were produced as described previously (1). Briefly, bacteria were scraped from plates of confluently growing *H. pylori*, sonicated, and subjected to low-speed centrifugation to remove intact bacteria. Total membranes were pelleted by centrifugation for 30 min at 10,000 × *g*. Outer membranes were prepared by suspending the total membranes in Triton X-100, followed by ultracentrifugation to pellet the insoluble outer membranes. Lipopolysaccharide (LPS) was prepared by the hot phenol-water extraction method according to the procedure of Westphal and Jann (20), followed by repeated ultracentrifugation. Flagellin protein was purified as described previously (10).

Production of MAbs. MAbs against *H. pylori* E50 were produced as described previously (4) by immunizing BALB/c mice with total membrane preparations. In the initial screening, the 23 hybrids reacting in the highest titers with the *H. pylori* membranes were selected. Of these, nine stable antibody-producing hybridomas were cloned and expanded by cultivation in 100 ml of Iscove's medium–10% fetal calf serum in tissue culture bottles (Nunc). Culture fluids from established, antibody-secreting hybridomas were harvested and frozen in aliquots at −30°C until further tested, and the specific *H. pylori*-antibody-producing cells were frozen in liquid nitrogen for long-term storage.

The isotypes of the different MAbs were determined by means of single radial immunodiffusion with mouse immunoglobulin (Ig) isotype-specific antisera (IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3) by the method of Mancini et al. (14). The Ig concentration was determined by the same method by assaying an appropriate dilution of the MAb in isotype-specific anti-mouse Ig; preparations with known contents of the respective mouse Ig isotype were used as standards.

ELISA. The enzyme-linked immunosorbent assay (ELISA) used for screening of the MAbs was performed as described previously (13), with 25 μg of total membrane preparations, 10 μg of LPS, and 5 μg of flagellin protein per ml for coating.

SDS-PAGE and immunoblotting. Whole bacteria, total membranes, and outer membranes of strain E50 and whole bacteria of *C. jejuni* CB37 and *H. felis* strains were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (11). Ten microliters of the respective samples was applied to the gel, and Rainbow molecular weight markers (Amersham Sweden AB, Solna, Sweden) were used. After immobilization of the protein profiles on nitrocellulose sheets, reaction with the MAb diluted 1:10 was carried out and bound antibodies were detected by using anti-mouse IgG labelled with peroxidase. Filters were developed with hydrogen peroxide substrate and 4-chloronaphthol chromogen (Bio-Rad Laboratories AB, Solna, Sweden).

Dot blot test. Bacterial strains to be tested were suspended in phosphate-buffered saline (PBS) to an optical density of 1.5 at 600 nm. Two microliters of each suspension was applied to a nitrocellulose filter presoaked in PBS and air dried for 15 min. The filter was blocked in 1% bovine serum albumin (BSA) in PBS for 30 min and then was incubated with MAb HP30-1:1 diluted 1:10 in PBS containing 0.1% BSA and 0.05% Tween for 1.5 h. After being washed, the filter was developed with the appropriate enzyme conjugate and substrate, as described for immunoblotting. Stained dots on a white background indicated positive results.

Immunoelectron microscopy. Fresh *H. pylori* cells grown for 2 days were suspended in 1% BSA in PBS to ca. 10^{11} cells per ml and applied to Formvar-carbon-coated grids for 2 min. After being washed, the grids were incubated on

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, Göteborg University, Guldhedsgatan 10A, S-413 46 Göteborg, Sweden. Phone: 46 31 604499. Fax: 46 31 820160.

TABLE 1. Bacterial strains used in this study

Bacterium	Strain	Source
Helicobacter pylori	E32, E50	Kindly provided by J.-P. Butzler, World Health Organization Collaborating Center for Enteric Campylobacter, Brussels, Belgium
	Hepy2–Hepy19	Own isolate
	Hel29, -43, -48, -54	Own isolate
	U⁻#4 (urease negative)	Kindly provided by G. Perez-Perez (15)
	Hel3, -15, -23, -24, -30	Astra Hässle AB
	CCUG17874 (=NCTC 11637)	Culture Collection, University of Göteborg
Helicobacter felis	CCUG28539	Culture Collection, University of Göteborg
	CCUG28540	Culture Collection, University of Göteborg
	Czinn	Kindly provided by S. Czinn
Campylobacter jejuni	CB37	J.-P. Butzler
	Campy2, -3, -4	Departmental Strain Collection
Campylobacter rectus	CCUG 20446A	Kindly provided by M. Wikström, Department of Oral Microbiology, Göteborg University
Capnocytophaga sputigena	Caps 1	Own isolate
Escherichia coli	E11881/9	Departmental Strain Collection
	E17018/A	Departmental Strain Collection
	E11881/5	Departmental Strain Collection
	H10407+	Departmental Strain Collection
	E1392-75+	Departmental Strain Collection
Shigella flexneri	21244	Departmental Strain Collection (originally obtained from D. Niesel)
	2120	Departmental Strain Collection
	3995	Departmental Strain Collection
	TmLR66	Departmental Strain Collection
Salmonella enteritidis	IHS 53068	Departmental Strain Collection
	IHS 53069	Departmental Strain Collection
	IHS 53070	Departmental Strain Collection
	IHS 53071	Departmental Strain Collection
Vibrio cholerae	569B	Departmental Strain Collection
	X25049	Departmental Strain Collection
	TI9479	Departmental Strain Collection
Yersinia enterocolitica	12253	Departmental Strain Collection
	12709	Departmental Strain Collection
	12776	Departmental Strain Collection

drops of MAb HP30-1:1 solution (diluted 1:2 in PBS-BSA-Tween) for 15 min. Incubation with gold anti-mouse IgG (Amersham Sweden AB) and negative staining were performed as described previously (9).

RESULTS

MAbs. Hybrids, obtained after fusing lymphocytes from mice immunized with a total membrane preparation from *H. pylori* E50 with myeloma cells, were tested for production of antibodies reacting with the homologous membrane preparation in the ELISA. After the initial screening, hybridomas giving the highest titers in the ELISA, with the membrane preparation used as an antigen, were selected for further analysis. Twenty-three of these hybridomas were additionally tested by ELISA for reactivity with *H. pylori* LPS and flagellin protein. Five hybridomas were found to react with LPS, and six were found to react with flagellin. After recloning, nine stable hybridomas were established and retested against the three different antigen preparations. The MAbs produced by three of them were found to react only with membrane proteins and not with LPS or flagellin. One of these MAbs, designated HP30-1:1 of the IgG1 subclass, was also found to react in high titers with membranes from strains CCUG 17874 and E32. The other two MAbs were specific for strain E50. Because MAb HP30-1:1 recognized an antigen common to different strains of *H. pylori* and therefore could be of potential value for diagnostic purposes, it was further tested for reactivity with *H. pylori* proteins.

Characterization of the antigen reacting with the MAb. The specificity of MAb HP30-1:1 was further investigated in immunoblotting experiments with whole-cell, total membrane, and outer membrane preparations from homologous *H. pylori* E50. The MAb reacted with a 30-kDa protein found in whole cells, as well as in outer membranes, as shown in Fig. 1. No reactivity was found against proteins of *H. felis* or *C. jejuni* (Fig. 1). It has been reported that the small subunit of the urease produced by *H. pylori* has a molecular mass of 30 kDa (18). A urease-negative mutant that does not produce any of the subunits of this enzyme (16) was therefore included in the experiment. The MAb reacted with a protein of the same size in the urease-negative mutant and in the wild-type strain (Fig. 1, lane 6).

Dot blot assay. We also wanted to determine if the 30-kDa protein was exposed on the surface of intact bacteria and if MAb HP30-1:1 reacted with the native 30-kDa protein. This was tested by a dot blot assay with bacterial suspensions spot-

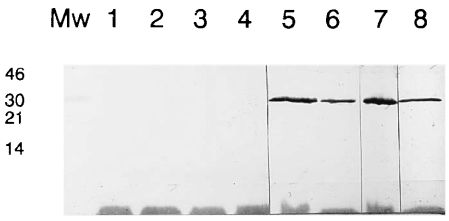

FIG. 1. Immunoblotting of protein profiles with MAb HP30-1:1. Lanes: 1, *C. jejuni* CB37; 2, *H. felis* 28540; 3, *H. felis* 28539; 4, *H. felis* Czinn; 5, whole cells of *H. pylori* E50; 6, whole cells of *H. pylori* U⁻#4; 7, total membranes of strain E50; 8, outer membranes of strain E50. Mw, molecular mass markers (sizes in kilodaltons are indicated to the left).

ted on nitrocellulose filters and then incubated with the MAb. Included in the assay were strains of *H. felis*, *C. jejuni*, and a number of other intestinal pathogens. The MAb reacted strongly with all *H. pylori* strains tested but not with any of the other bacterial species (Fig. 2A). Three strains of *H. felis* did not react with the MAb (Fig. 2B).

The assay was also compared with conventional biochemical tests, i.e., the urease test and the oxidase test, for the purpose of identifying clinical isolates from biopsies as *H. pylori*. Sixty-six strains isolated from biopsies and identified as *H. pylori* by

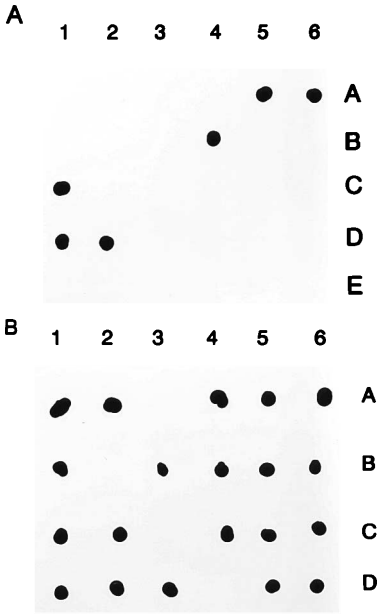

FIG. 2. Dot blot assay with MAb HP30-1:1. Suspensions of different bacteria were applied in the following order: filter A; position 1A, *S. flexneri* 21270; 1B, *C. jejuni* CB37; 1C, *H. pylori* CCUG 17874; 1D, *H. pylori* Hel3; 1E, *V. cholerae* 569B; 2A, *E. coli* E1392-75+; 2B, *V. cholerae* TI9479; 2C, *V. cholerae* X25049; 2D, *H. pylori* Hepy5; 2E, *C. jejuni* Campy2; 3A, *E. coli* H110407+; 3B, *E. coli* E11881/9; 3C, *E. coli* E11881/5; 3D, *Y. enterocolitica* 12253; 3E, *S. enteritidis* IHS53068; 4A, *V. cholerae* TI9766; 4B, *H. pylori* E50; 4C, *E. coli* E17018/A; 4D, *S. flexneri* 3995; 4E, *C. jejuni* Campy4; 5A, *H. pylori* Hepy4; 5B, *S. flexneri* TmlR66; 5C, *S. flexneri* 21244; 5D, *S. enteritidis* IHS53068; 5E, *S. enteritidis* IHS53069; 6A, *H. pylori* E32; 6B, *Y. enterocolitica* 12776; 6C, *Y. enterocolitica* 12709; 6D, *C. jejuni* Campy3; 6E, *S. enteritidis* IHS 53070; filter B; position A1, *H. pylori* U⁻#4; A2, *H. pylori* Hepy9; A3, *H. felis* 28539; A4, *H. pylori* Hepy10; A5, *H. pylori* Hepy23; A6, *H. pylori* Hepy24; B1, *H. pylori* Hepy27; B2, *C. jejuni* CB37; B3, *H. pylori* Hel28; B4, *H. pylori* Hel29; B5, *H. pylori* Hel30; B6, *H. pylori* Hel31; C1, *H. pylori* Hepy13; C2, *H. pylori* Hepy16; C3, *H. felis* 28539; C4, *H. pylori* Hepy17; C5, *H. pylori* Hepy18; C6, *H. pylori* Hepy19; D1, *H. pylori* Hepy29; D2, *H. pylori* Hel43; D3, *H. pylori* Hel45; D4, *H. felis* Czinn; D5, *H. pylori* Hel48; D6, *H. pylori* E50.

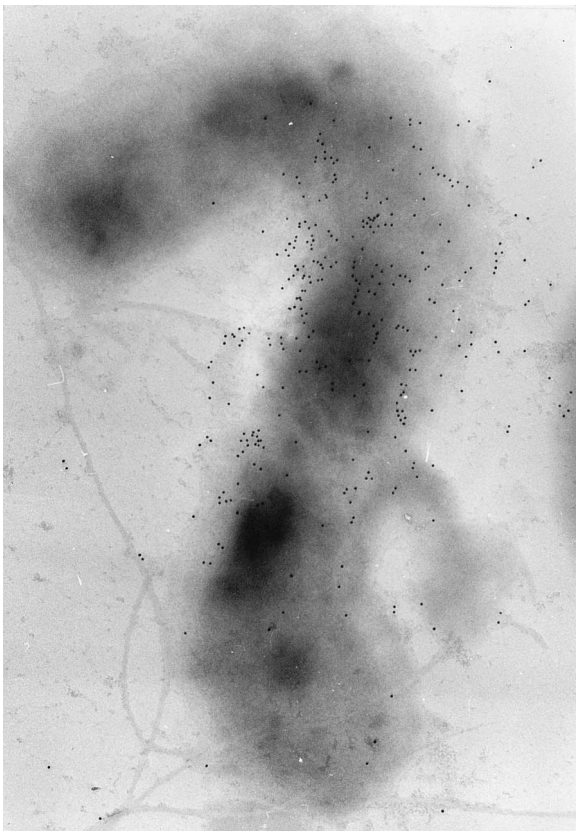

FIG. 3. Immunogold electron microscopy with MAb HP30-1:1. Gold-labelled MAbs bound to the surface of *H. pylori* E50. Note the absence of gold particles around the flagella. Original magnification, ×25,000.

morphological criteria and urease and oxidase tests were all strongly positive in the dot blot assay. Material from one single colony was sufficient for a weak but clearly positive result (data not shown). The results for 18 of these *H. pylori* isolates are shown in Fig. 2B. When 27 strains of other bacterial species were tested, including the strains in Fig. 2A and the oral bacteria *C. rectus* and *C. sputigena* (data not shown), they were all found to be negative. The sensitivity, specificity, and positive predictive values for the dot blot test were calculated to be 100%.

Immunoelectron microscopy. The surface-exposed localization of the 30-kDa protein was confirmed by immunogold electron microscopy. MAb HP30-1:1 bound to the surface of the bacterial cell in an irregular pattern (Fig. 3). The sheet surrounding the flagella did not bind the gold-labelled antibodies.

DISCUSSION

The MAb described here is highly specific for *H. pylori* and recognizes a 30-kDa protein exposed on the surface of the bacterium. The specificity of the MAb in detecting *H. pylori* antigen in the immunodot blot assay makes it suitable for diagnostic purposes. We have employed the dot blot assay as a reliable method for the identification of *H. pylori* after culturing of biopsies, without any false-positive or false-negative results. Other bacterial species, particularly those from oral flora, may be isolated when biopsies are used for diagnosis of *H. pylori* infection. Production of urease and oxidase is a common trait among bacteria. The dot blot test eliminates the need for biochemical tests for typing of bacterial isolates with prop-

erties similar to those of *H. pylori* and can therefore be regarded as a more specific method for identification. Other applications with MAb HP30-1:1 may also be developed, i.e., detection of *H. pylori* antigen in gastric fluid or biopsies by inhibition ELISA or immunofluorescence microscopy.

The antigen recognized by the MAb is only found in *H. pylori*, and preliminary data show that *H. pylori*-infected individuals have serum antibodies toward this antigen, indicating that it is produced in vivo. This suggests that it may also be suitable as an antigen in ELISAs for detection of serum antibodies. The 30-kDa protein seems to be produced by all strains of *H. pylori*, because all of the strains we have tested so far are positive in the dot blot assay, including one strain from an asymptomatic carrier. Although there is extensive genetic variation among strains of *H. pylori* (2), the protein profiles have been found to be quite similar (15), in agreement with the observation that the epitope recognized by MAb HP30-1:1 is conserved among *H. pylori* strains.

Several other MAbs which react with *H. pylori*-specific antigens have been described (3, 5–8, 17). Some of these MAbs have been used for diagnostic purposes and have been employed in immunofluorescence tests (7, 8, 17). Protein antigens with molecular weights of 19,000 and 25,000 recognized by two of these MAbs have been identified (6, 7). The function of the 30-kDa protein recognized by MAb HP30-1:1 has yet to be determined. For this purpose, we are in the process of cloning and characterizing the structural gene of this protein. When analyzed by immunoelectron microscopy, the binding pattern of the MAb to the surface of the *H. pylori* resembled the results obtained for nonfimbrial adhesins like CS3 of *E. coli* (12). The possible adhesive function of the 30-kDa protein will be further investigated.

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