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Typing of *Helicobacter pylori* with Monoclonal Antibodies against Lewis Antigens in Lipopolysaccharide

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Recently, it has been shown that the lipopolysaccharide (LPS) O antigen of *Helicobacter pylori* contains Lewis x (Le^x), Lewis y (Le^y), or both Le^x and Le^y antigens. We applied a serotyping method for *H. pylori* by an enzyme-linked immunosorbent assay with monoclonal antibodies (MAbs) specific for these antigens and the related fucosylated H type 1 (H1) antigen. The selected MAbs recognized the Le^x and/or Le^y structures in the LPS of *H. pylori*. The agreement between the results of biochemical compositional analysis and the serological data validated our serotyping system. A total of 152 strains from different geographic origins (The Netherlands, Canada, Poland, Italy, and People's Republic of China) were examined for typeability based on the presence of Lewis antigens. One hundred twenty-nine (84.9%) strains were typeable, and 12 different serotyping patterns were observed; 80.9% of the strains contained Le^x and/or Le^y antigens, and 18.4% reacted with the MAb against the related H1 antigen either alone or in combination with the Le^x and/or Le^y antigen. Our results show that the Le^x and Le^y antigens are frequently encountered in the LPS of *H. pylori* strains from various geographic origins. This typing method is an easy-to-perform technique, which can be used for strain differentiation in epidemiological studies of *H. pylori* infections.

Helicobacter pylori has been implicated in the etiology of human gastritis and in the development and recurrence of gastric and duodenal ulcers (5, 7). More recently, infection by *H. pylori* is thought to be causatively related to gastric adenocarcinoma and to B-cell lymphoma of the stomach (MALT lymphoma) (11, 24, 29). Despite the high incidence and wide distribution of the microorganism in human populations (50% of the world's population is infected), little is known about the epidemiology of *H. pylori*. The source of infection, natural reservoirs, and modes of transmission have not been established so far, although there is mounting evidence for human-to-human transmission. For research purposes, a reliable and simple typing system for *H. pylori* strains is required.

The existing typing systems, based on agglutination, biotyping, or enzyme profiles, show considerable homogeneity among *H. pylori* strains (14, 17, 18). By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of bacterial proteins (9, 21) and immunoblotting (8), strain differences can be detected, although variations in SDS-PAGE gels and differentiation of the majority of strains into a limited number of serogroups by immunoblotting are major problems. Molecular techniques such as DNA fingerprinting by restriction enzyme endonuclease analysis and ribotyping show a large genomic diversity and allow the discrimination of *H. pylori* strains by a unique pattern for each strain (12, 16, 26).

Serotyping systems are very limited for *H. pylori*, probably because little is known about the antigenic structure of *H. pylori*. Mills et al. (19) have shown by antigenic analysis of lipopolysaccharide (LPS) extracts from *H. pylori* both a common antigen and strain-specific antigens that are sufficiently diverse to be used in an O antigen serotyping system. With passive hemagglutination, six serotypes (O groups 1 through 6) can be differentiated. However, the use of polyclonal antisera, the need for absorption of these antisera, and the laborious technique for purification of LPS as antigenic material are drawbacks of this serotyping system.

Recently, it has been shown by biochemical and spectroscopic analyses that the LPS O antigen of *H. pylori* ATCC 43504 contains fucosylated *N*-acetyllactosamino-glycans with Lewis x (Le^x) determinants (2). The LPSs of two other *H. pylori* strains, MO19 and P466 (6), contain Lewis y (Le^y) and Le^x plus Le^y, respectively (1). These antigens are known as blood group antigens and are also present in human mucosa. We tested clinical isolates of *H. pylori* with monoclonal antibodies (MAbs) with specificity for Lewis and the related H1 antigens to demonstrate the presence of these fucosylated blood group antigens, and we evaluated them as the basis for a serotyping system.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. In total, 155 isolates of *H. pylori* were used. With the exception of the type strain, ATCC 43504 (=NTCC 11637), the strains were clinical isolates from patients with different gastroduodenal diseases (gastritis, peptic ulcer disease, gastric carcinoma, or B-cell lymphoma). The strains were from different geographic origins: The Netherlands (n = 81), Canada (n = 6), Poland (n = 5), Italy (n = 20), and People's Republic of China (n = 40). The six Canadian strains were serotype O1 to O6, as described by Mills et al. (19). Strains P466 and MO19 were obtained from T. Boren, Umeå, Sweden (6).

The clinical isolates from the University Hospital, Vrije Universiteit, Amsterdam, The Netherlands, were identified as *H. pylori* by the following criteria:

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TABLE 1. MAbs used in this study

MAb	Specificity[a]	Isotype	Source[b]	Reference
4D2 (HpN16)	H1	IgM	R. Negrini	20, 21
6H3 (CB-10)	Le^x (mono-/trimeric)	IgM	R. Negrini	20, 21
54.1F6A	Le^x (tri-/polymeric)	IgM	G. J. van Dam, A. N. Deelder	28
540 (HpN35)	Le^y	IgG1	R. Negrini	20, 21
7Le	Le^a	IgG1	Bioprobe BV	3
2.25Le	Le^b	IgG1	Bioprobe BV	4

[a] Validation of the MAbs was done by testing them by ELISA for the protein-linked neoglycoconjugates Le^x, Le^y, H1, Le^a, and Le^b and for the purified LPS of strain ATCC 43504 (polymeric Le^x).

[b] R. Negrini, Biotechnology Laboratory, General Hospital, Brescia, Italy; G. J. van Dam and A. N. Deelder, Department of Parasitology, University of Leiden, Leiden, The Netherlands; Bioprobe BV, Amstelveen, The Netherlands.

atmosphere required for growth (10% CO_2 and 5% O_2), incubation time (48 to 72 h), morphology in Gram stain (spiral shape), and positive catalase, oxidase, and urease tests. The strains were kept frozen at −80°C in 20% glycerol. Strains were grown on sheep blood agar (Oxoid no. 2; Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) at 37°C for 48 h in 10% CO_2, checked for purity, and subcultured in *Brucella* broth supplemented with 5% fetal calf serum. After incubation at 37°C in 10% CO_2 for 48 h, cells were checked for spiral shape, harvested in sterile phosphate-buffered saline (PBS), centrifuged at 2,800 × *g* for 10 min, and washed three times. The pellet was diluted with PBS to a cell concentration of 3.75×10^8/ml. The other strains were identified as *H. pylori* in the laboratory of origin. They were sent to us as whole-cell suspensions of approximately 3.75×10^8 bacteria per ml. From this point, all strains were handled similarly for enzyme-linked immunosorbent assays (ELISA).

MAbs. The MAbs used in this study are listed in Table 1. MAbs 4D2 (HpN16), 6H3 (CB-10), and 540 (HpN35) are *H. pylori*-induced MAbs, described by us previously (22, 23). MAb 54.1F6A is directed against the schistosomal circulating cathodic antigen, a glycoprotein containing polymeric Le^x (9). MAbs 7Le and 2.25Le (Bioprobe B.V., Amstelveen, The Netherlands) were obtained after vaccination of mice with mammalian antigens (3, 4). The above-described MAbs were tested for specificity by ELISA against synthetic Lewis antigens (Isosep AB, Tullinge, Sweden). These synthetic Lewis antigens are neoglycoconjugates (6). With the exception of Le^y, the oligosaccharides were prepared from human milk, coupled to a spacer (*p*-aminophenylethyl or acetylphenylenediamine), and covalently linked with protein (human serum albumin or bovine serum albumin [BSA]). Le^y was prepared synthetically and coupled to human serum albumin. About 20 to 25 oligosaccharide chains are linked to one molecule of human serum albumin or BSA. The synthetic Lewis antigens used were Le^x (monomeric and trimeric), Le^y, Lewis-related H type 1, Lewis a (Le^a), and Lewis b (Le^b) antigens. Furthermore, the MAbs were tested against LPS of strain ATCC 43504, which contains polymeric Le^x antigens. The specificities of the MAbs are listed in Table 1. MAb 6H3 binds to monomeric and trimeric Le^x but not to polymeric Le^x; MAb 54.1F6A binds to trimeric and polymeric Le^x but not to monomeric Le^x.

ELISA. The ELISA used to detect Lewis and related antigens was performed according to conventional procedures. Bacterial whole-cell concentrations of 7.5×10^6 CFU/ml of PBS were used to coat 96-well flat-bottom polystyrene microtiter plates (Immulon ll, medium binding capacity; Greiner Labor Technik, Frickenhausen, Germany) with 100 μl per well, after which the plates were incubated overnight at room temperature. The plates were washed three times with PBS containing 0.07% Tween 80 and 0.001% merthiolate (PBST). MAbs were added to the wells in a concentration of 100 ng/ml (100 μl per well). The plates were incubated overnight at room temperature and then washed three times with PBST. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin M (IgM) or IgG (American Qualex, San Clemente, Calif.), diluted 1:1,000 in PBST with 0.5% normal goat serum, was added to the plates. The plates were incubated at 37°C for 2 h and washed. Color was developed with phosphate citrate buffer containing 1 mg of *ortho*-phenylenediamine dihydrochloride (Sigma Chemical Co, St. Louis, Mo.) per ml and 0.5 μl of H_2O_2 (30% [vol/vol]) per ml. This staining solution (100 μl) was added to each well, and the plates were incubated at room temperature in the dark for 30 min. The reaction was stopped with 50 μl of 10% (vol/vol) H_2SO_4. The color change was measured at 492 nm with a Dynatech model MR 7000 plate reader. The optical density at 492 nm (OD_{492}) values were classified as negative (OD of <0.3) or positive (OD of ≥0.3) reactions. The breakpoint from a negative to a positive reaction (OD of 0.3) was chosen on the basis of the sum of nonspecific background binding values for MAbs and for the conjugate, which never exceeded an OD of 0.2. Controls in the ELISA were the binding of MAbs to PBS-coated wells and the binding of the conjugate to antigen-coated wells. The synthetic protein-linked Lewis antigens and the LPS of strain ATCC 43505 (polymeric Le^x) were the positive controls for the MAbs. To optimize antigen-antibody concentrations, a variety of antigen-antibody concentrations were tested. A concentration of 100 ng/ml was chosen for the MAbs, because concentrations of <100 ng/ml showed reduced reactivities and concentrations of >100 ng/ml did not really optimize the reactions.

Proteinase K digestion and SDS-PAGE. Proteinase K digestion of bacterial whole-cell lysates was done by a modification of a procedure from Hitchcock and Brown (13) as described by Mills et al. (19). The enzyme-treated cells were fractionated by SDS-PAGE with the discontinuous buffer system described by Laemmli (15). Electrophoresis was conducted with a constant current of 20 mA, a stacking gel of 5% acrylamide, and a separation gel of 12% acrylamide containing 0.1% SDS. After SDS-PAGE, the gels were fixed and stained for LPS by silver staining (27).

RESULTS

Recognition of Le^x and Le^y antigens. By ELISA, we were able to demonstrate the recognition by MAbs of Le^x and Le^y antigens in the strains known, by biochemical compositional analysis, to carry these epitopes. Strains ATCC 43504 and MO 19 express polymeric Le^x and Le^y, respectively, whereas strain P466 expresses both polymeric Le^x and Le^y. Strains ATCC 43504 and P466 also showed reactivity with the anti-Lewis H1 MAb and with the anti-Le^y MAb, respectively (Table 2).

Typing clinical strains. Initially, we typed the 152 clinical strains with the six MAbs listed in Table 2. Of the 152 strains, 3 strains gave a positive reaction with MAb 7Le, with specificity for Le^a, and 11 strains showed reactivity with MAb 2.25Le, specific for Le^b. On the basis of the facts that Le^a and Le^b antigens have never been detected by biochemical compositional analysis of LPSs of *H. pylori* strains and that Le^a and/or Le^b immune responses have not been found by us after immunization of rabbits with *H. pylori* (unpublished results), we decided to exclude these two MAbs from the screening battery of MAbs. Although the H1 antigen, which is closely related to the Lewis antigens, also has never been found biochemically in *H. pylori* LPS, we included the anti-H1 MAb in the typing scheme, because we found an immunological response in rabbits immunized with an H1-positive strain of *H. pylori* and because several strains reacted only with the anti-H1 MAb. The results of typing of the clinical strains by the four MAbs are shown in Table 3. All strains were typed at least in dupli-

TABLE 2. Detection of Lewis epitopes by MAbs in LPS of *H. pylori* strains with biochemically defined Lewis structures

MAb	Specificity	Reactivity in strain[a]:		
		ATCC 43504	P466	MO19
4D2	H1	+	−	−
6H3	Le^x	−	−	−
54.1F6A	Le^x	+	+	−
540	Le^y	−	+	+
7Le	Le^a	−	−	−
2.25 Le	Le^b	−	+	−

[a] −, OD_{492} value of <0.3; +, OD_{492} value of ≥0.3. By biochemical compositional analysis, strain ATCC 43504 contains polymeric Le^x, strain MO19 contains Le^y, and strain P466 contains polymeric Le^x and Le^y.

TABLE 3. Serotyping patterns of *H. pylori* strains

Serotype	No. of strains	Origin (no. of strains)[a]	Specificity of MAb			
			4D2 (H1)	6H3 (Le^x monomer)	54.1F6A (Le^x polymer)	540 (Le^y)
1 (=O1)	3	T (1), A (2)	+	−	+	−
3/4 (=O3/O4)[b]	56	T (2), A (34) L (2), B (10) F (8)	−	+	+	+
5 (=O5)	4	T (1), A (3)	+	−	+	+
6 (=O6)	5	T (1), A (4)	−	−	−	+
7	6	A (6)	+	−	−	−
8	14	A (2), B (2)	+	+	+	+
9	1	A (1)	+	−	−	+
10	8	A (3), B (4), F (1)	−	+	+	−
11	2	A (2)	−	+	−	−
12	27	A (8), L (2) B (1), F (16)	−	−	+	+
13	2	A (1), F (1)	−	−	+	−
14	1	L (1)	−	+	−	+
NT (=O2)[c]	23	T (1), A (5) B (3), F (14)	−	−	−	−

[a] T, Toronto, Canada; A, Amsterdam, The Netherlands; L, Łódź, Poland; B, Brescia, Italy; F, Fujian, People's Republic of China.
[b] Strains O3 and O4 (Mills et al. [19]) could not be differentiated with the MAbs that we used, but strain O3 reacted with MAb Hp138, an *H. pylori*-induced MAb with specificity for an unknown epitope in the LPS of *H. pylori*, whereas strain O4 did not.
[c] NT, nontypeable.

cate. One hundred twenty-nine of the 152 strains (84.9%) were typeable, and 12 different serotype patterns were observed; 15 strains (9.9%) contained only the Le^x antigen, 6 strains (3.9%) contained only the Le^y antigen, and 102 strains (67.1%) contained both Le^x and Le^y antigens. Twenty-eight strains (18.4%) reacted with the anti-H1 MAb. The strains designated O1 to O6 have been described by Mills et al. (19), and these serotypes could be differentiated (with the exception of O3 and O4) by our anti-Le^x and -Le^y MAbs. Strain ATCC 43504, which contained Le^x, is serotype O1; serotype O2 was nontypeable; serotypes O3 and O4 showed the same reaction pattern with both Le^x and Le^y; serotype O5 contained both Le^x and Le^y but differed from serotype O3 by a reactivity with the anti-H1 MAb; and serotype O6 showed only Le^y reactivity (Table 3). The numbering of serotypes by our method has been started from the reaction patterns of strain O1 to O6, with the omission of serotype number 2, as strain O2 was nontypeable by our system, and with the combination of serotypes O3 and O4 as serotype 3/4, as these strains were not differentiated in our system (Table 3).

Nontypeable strains. Overall, 15.1% of the strains were nontypeable. From one of the nontypeable strains, we fractionated a proteinase K-treated cell lysate by SDS-PAGE. A characteristic ladderlike pattern was seen, indicating the presence of an O side chain (data not shown). A relatively large number of *H. pylori* strains from the People's Republic of China were nontypeable (14 of 40 strains).

Assignment of serotypes. The reactivities of the four MAbs selected with all (n = 152) strains (Table 3) were determined in two independent tests. For 138 strains, the outcomes of the two experiments were identical and the serotypes were assigned on the basis of these data. On four additional independent occasions we tested the reactivity of the MAbs with 21 strains randomly selected from the group of 138 strains. On all four occasions, the 21 strains yielded outcomes identical to those obtained in the two initial experiments, indicating that our decision to assign a serotype when the outcomes of the initial two typing experiments were concordant was correct. For the remaining 14 of the 152 strains, the results of the two initial tests were discordant. Eight strains were randomly selected from this group of 14 strains and tested again on four independent occasions: six of the eight strains yielded outcomes, on all four occasions, identical to the result for one of the two tests done initially (see above), and serotypes were assigned. Two of the eight strains tested yielded results that two and three times, respectively, were identical to the result of one of the two initial tests. Likewise, the remaining six of the 14 strains were serotyped by repeating the assays. For these six strains a third typing run was always decisive. A higher number of typing runs did not change the serotype determination.

Of the discordant reactions observed, 67% had OD_{492} values that deviated 0.006 to 0.100 OD unit from the cutoff point (OD of 0.3); the remaining 23% of the disccordant reactions deviated 0.110 to 0.200 OD unit from the cutoff point.

Eventually, 144 (95%) of the 152 strains proved to be the same serotype, as determined by the very first test.

DISCUSSION

We have demonstrated that Le^x and Le^y antigens are frequently encountered in *H. pylori* and that a serotyping system based on these antigens is feasible. An ELISA with MAbs with specificity for Lewis epitopes in the LPS of *H. pylori* was used to develop such a typing system for clinical strains of *H. pylori*. Although the antigenic heterogeneity in *H. pylori* strains, which has been shown in a few studies (8, 10, 25), has been suggested as a basis for a serotyping system, the only formal scheme for differentiation of *H. pylori* strains on the basis of O antigens is the serotyping system described by Mills et al. (19). Six serotypes (O1 to O6) were differentiated by these investigators, but the passive hemagglutination technique used for this serotyping requires a labor-intensive LPS preparation for production of antigenic material and is therefore not practical for routine typing of numerous samples in the clinical laboratory. The development of an easy-to-perform and reliable serotyping system could facilitate strain classification for epidemiological analysis. With the recent biochemical compositional analysis of *H. pylori* LPS and the detection of Lewis blood group struc-

tures in this LPS (1a, 2), the way was paved for the development of a serotyping system with MAbs with specificity for the Lewis antigens.

The selected MAbs were tested for specificity with the protein-linked neoglycoconjugates Le^x (monomeric and trimeric), Le^y, H1, Le^a, and Le^b and polymeric Le^x antigens (LPS of strain ATCC 43504) by ELISA. In our typing system these MAbs recognized Le^x antigens in the LPS of strain ATCC 43504, Le^y antigens in the LPS of strain MO19, and both Le^x and Le^y antigens in the LPS of strain P466; the presence of these antigens in these strains has been confirmed by biochemical compositional analysis (Table 2). These data demonstrate that the serological and structural data are in agreement with each other and validate our serotyping system. The presence of H1, Le^a, and Le^b antigens in *H. pylori* strains has not yet been confirmed by biochemical analysis. This is possibly due to the fact that, in contrast to biochemical analysis, serological analysis is able to detect structures that represent, on a molar basis, only a small fraction of the total. However, the possibility of cross-reactivity of the anti-H1, anti-Le^a, and anti-Le^b MAbs with an undetermined epitope present in the LPS core region cannot be excluded.

The serotyping system described in this study is simple and reliable and has good discriminatory power. The technique can start with one colony of the primary isolation of *H. pylori*, does not require laborious sample preparation, and is not time-consuming. This serotyping system extends the results of Mills et al. (19) and confirms the differentiation of the six serotypes described by these investigators. In the initial biochemical compositional analysis, strain O3 contained only Le^x antigens, but, more recently, α-1,2-linked fucose, which indicates the presence of the Le^y antigen, has also been detected in the LPS of this strain (1). The serological typing of strain O3 by our system agreed with these biochemical data.

With our method 85% of the strains could be typed. The majority of the nontypeable strains (14 of 23) were of Chinese origin. Of the total number of Chinese strains, 35% (14 of 40) were untypeable versus only 8% (9 of 112) of the strains from other areas ($\chi^2 = 14.65$; $P < 0.001$). Nontypeability might be due to the loss of the O side chain, which is known to occur when isolates are subjected to a number of in vitro passages (19, 20). Loss of the O side chain can be confirmed by electrophoretic analysis by the absence of a ladderlike pattern of LPS. The presence of a ladderlike pattern in one of our nontypeable strains, however, showed that at least one other serotype, which was not reactive with our MAbs, exists and that other serotypes might be an explanation for the nontypeability. Le^x and/or Le^y antigens were detected in over 80% of the strains; therefore, we conclude that these blood group antigens are frequently occurring antigens in *H. pylori*. These Le^x and Le^y epitopes occur in all *H. pylori* strains independently of their geographic origins.

The assignment of serotypes by ELISA was reproducible. By repeating the assays with those strains for which there were discrepant results between the initial typing patterns, the serotype could be determined reliably. When strains are typed for epidemiological purposes, we do not recommend duplicate typing, since only 5% of the strains showed discrepancies in serotype determination after the first test. However, when the serotype of a single isolate needs to be determined with a higher degree of accuracy, repeated typing is recommended.

We conclude that Lewis blood group antigens are also antigenic epitopes of *H. pylori* LPS and that they are frequently encountered in *H. pylori* strains. These antigens are suited for serological typing and may therefore be useful for epidemiologic studies.

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