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Expression of the Human Cell Surface Glycoconjugates Lewis X and Lewis Y by *Helicobacter pylori* Isolates Is Related to *cagA* Status

HANS-PETER WIRTH,[1] MANQIAO YANG,[1] MIKIO KARITA,[1] AND MARTIN J. BLASER[2]*

Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2605,[1] and Infectious Disease Section, Department of Veterans Affairs Medical Center, Nashville, Tennessee 37212[2]

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Monoclonal antibodies were used in an enzyme-linked immunosorbent assay for the detection of human Lewis immunodeterminants in the lipopolysaccharide of *Helicobacter pylori*. In 94 *H. pylori* isolates, expression of Lewis^x (Le^x) and Le^y was a stable phenotypic marker independent of the growth medium and cell age; 46 (49%) of the isolates expressed both and 34 (36%) of the isolates expressed either Le^x or Le^y; 14 (15%) were negative for both determinants. Twelve (13%) isolates expressed Le^b, 3 (3%) expressed Le^a, and 2 (2%) expressed sialyl-Le^x. *H. pylori* isolates positive for both Le^x and Le^y were predominantly *cagA*$^+$ ($P < 0.001$) and possessed the s1 signal sequence ($P < 0.05$) and the m1 midregion type ($P = 0.033$) of *vacA*. Isogenic mutants of *H. pylori* CPY3401 were created by interruption of the *cagA*, *picB*, or *ureA* gene. The *cagA*-ablated strain (but not the *picB*- and *ureA*-ablated mutant strains) had significantly ($P < 0.01$) diminished expression of Le^y compared with that of the wild-type strain; for all four strains, expression of Le^x was similar. In conclusion, 89% of *H. pylori* isolates express Le determinants in their lipopolysaccharide, mimicking human cell surface glycoconjugates. Strong expression of Le^x and Le^y by *cagA*$^+$ isolates could counterbalance their enhanced proinflammatory activities and thereby facilitate persistence.

Helicobacter pylori is an important human pathogen causing chronic gastritis (30) which may lead to peptic ulcer disease (31), gastric adenocarcinoma (37, 39), or gastric lymphoma (57). Although *H. pylori* infection induces strong local and systemic immune responses (43, 58), these are insufficient for eliminating the pathogen, since it persists for life in most hosts. Its localization in the gastric lumen permits *H. pylori* to reduce the influence of host phagocytes, antibodies, and complement, whereas other bacterial features, including motility, superoxide dismutase, and catalase activity further protect against the immune response.

Microbial expression of host antigens is another mechanism to escape elimination by the immune response. For example, *Neisseria gonorrhoeae* lipopolysaccharides (LPS) resemble host glycolipids of the paragloboside group (28), and LPS epitopes can be modified by sialylation with cytidine monophospho-*N*-acetylneuraminic acid present in human blood (29). *Campylobacter jejuni* LPS has structural similarity with glycosphingolipids of the ganglioside group (4). Several gram-negative bacteria can express human AB0-blood group antigens (49), *Streptococcus bovis* expresses sialyl-Lewis^x (sialyl-Le^x) (21), and ova of *Schistosoma mansoni* and adult worms express Le^x as antigen (24, 51).

Aspinall and colleagues showed that the O-antigen regions of the LPS purified from four *H. pylori* strains contained partially fucosylated *N*-acetyllactosaminoglycan chains terminated by a Lewis type 2 immunodeterminant, either Lewis^x (Le^x) or Lewis^y (Le^y) (3, 5–7). Using immunoelectron microscopy and enzyme-linked immunosorbent assaying (ELISA), Sherburne and colleagues (11, 48) have confirmed the presence of Le^x in seven *H. pylori* strains. Recently, Appelmelk and colleagues pointed to a possible pathogenic role of anti-Le antibodies in *H. pylori*-induced gastritis (2). The aims of the present study were to characterize the expression of Le antigens in *H. pylori* and to assess whether this expression is related to features of the infection or to particular *H. pylori* genotypes.

* Corresponding author. Mailing address: Vanderbilt University School of Medicine, A-3310 Medical Center North, Nashville, TN 37232-2605. Phone: (615) 322-2035. Fax: (615) 343-6160. Electronic mail address: Martin.Blaser@mcmail.vanderbilt.edu.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* reference strains P466, MO19, C1, and NCTC11637 (ATCC43504) (3, 5–7) were obtained from J. L. Penner or from the American Type Culture Collection. The other isolates were obtained from gastric antral biopsies of patients undergoing upper gastrointestinal endoscopy at the Nashville Department of Veterans Affairs Medical Center, Yamaguchi University Hospital (Yamaguchi, Japan), or the University Hospital of Zurich, Switzerland. We studied 94 *H. pylori* isolates (numbers given in parentheses) from patients originating from 19 different countries as follows: Asian and Australian isolates were from China (3), Japan (19), Thailand (4), Turkey (3), and Australia (1); North American isolates were from the United States (35) and Canada (1); South American isolates were from Peru (3); African isolates were from Algeria (2), Ghana (1), Morocco (3), Sudan (1), and Zaire (1); European isolates were from Italy (2), the former Yugoslavia (3), Norway (1), Portugal (2), Spain (1), and Switzerland (8). Stock cultures were maintained at −70°C in brucella broth (BBL Becton Dickinson Microbiology Systems, Cockeysville, Md.) supplemented with 15% glycerol. Routinely, the isolates were cultured on Trypticase soy agar plates with 5% sheep blood (TSB [BBL]) in a humid microaerobic atmosphere with 5% CO_2 at 37°C for 48 h. A series of isolates was cultured either in brucella broth supplemented with 5% fetal bovine serum (FBS) under continuous shaking or on brucella agar (BBL) plates supplemented with 5% FBS and incubated under the same conditions. The identity of *H. pylori* was confirmed by characteristic colony morphology, urease activity, and presence of *ureA* determined by PCR with extracted chromosomal DNA (see below). Forty-five isolates used in this study had previously been characterized with respect to *vacA* subtype (8). To examine the effect of the age of the cells on Le expression, isolates also were grown on TSB plates and in brucella broth supplemented with 5% FBS for 24, 48, 72, or 96 h.

ELISA with bacterial whole cells. Cells of *H. pylori* isolates grown for 48 h on TSB plates were harvested and washed twice with 3 ml of 0.15 M NaCl per plate. After centrifugation for 10 min at 3,000 × *g* at 4°C, pellets were suspended in 0.15 M NaCl–15% glycerol. Unless Le antigen determinations were done immediately, the suspensions were frozen at −70°C until testing. Protein concentrations of cell suspensions were determined by bicinchoninic acid protein assaying (Pierce, Rockford, Ill.). Immulon 2 flat-bottom microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated with 100 μl of twofold dilutions of *H. pylori* cell suspensions (starting with 1 μg of protein per well) in 0.05 M sodium bicarbonate coating buffer (pH 9.6) overnight at room temperature. The wells were blocked for 1 h at room temperature with 200 μl of 2% bovine serum

TABLE 1. Characteristics of mouse MAbs used to examine *H. pylori* cells

Specificity	Clone	Isotype	Reciprocal-use dilution	Immunogen	Reference
Le^a (type 1 chain)	T174	IgG1	500	SK-CO-10 colon cancer cell line	47
Le^b (type 1 chain)	T218	IgM	500	SK-CO-10 colon cancer cell line	47
Le^x (type 2 chain)	P12	IgM	500	Human placenta cells	44
Le^y (type 2 chain)	F3	IgM	1,000	SK-LC-3 lung cancer cell line	27
Sialyl-Le^x	CSLEX1	IgM	500	Gastric adenocarcinoma cells	19

albumin (BSA) in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Subsequently, the wells were incubated for 1 h at room temperature with 100 μl of PBS with 1% BSA and mouse monoclonal antibodies (MAbs) (all from Signet Laboratories, Inc., Dedham, Mass.) to Le^a, Le^b, Le^x, Le^y, and sialyl-Le^x (Table 1) that have been extensively characterized for detection of Le antigens in immunohistochemistry (16, 19, 27, 44, 47). Bound immunoglobulin M (IgM) or IgG antibodies were detected with 100 μl of goat anti-mouse IgM coupled with alkaline phosphatase (Sigma Chemical Company, St. Louis, Mo.) or goat anti-mouse IgG antibodies coupled with alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, Ind.) diluted 1:2,500 or 1:1,000 in PBS–0.05% Tween 20 (PBS-T). As a substrate, 0.5% *p*-nitrophenylphosphate in 0.1 M diethanolamine (pH 9.8)–1 mM $MgCl_2$ was used. Between incubation steps, the wells were washed three times for 5 min with PBS-T. In addition to controls without primary or secondary antibody, whole-cell preparations of *Escherichia coli* HB101 were included in each assay. To determine the level of nonspecific antibody binding, the optical density (OD) values of the wells coated with *E. coli* cells were subtracted from the values of the wells coated with equal protein concentrations of *H. pylori* cells. The results were expressed as OD values (at 410 nm) × 1,000 Units (ODU) for a whole-cell coating concentration corresponding to 1 μg of protein per well. In experiments comparing *H. pylori* growth on different media, a mean Le^y-to-Le^x ratio was calculated by averaging the three individual Le^y-to-Le^x ratios for the assays with cells corresponding to 1 μg and 500 and 250 ng of protein per well.

In other experiments, microtiter plates were coated with twofold dilutions of 1 μg of protein per well of Le^b coupled to human serum albumin (Le^b-HSA), Le^x-trisaccharide coupled to BSA (Galβ1→4[Fucα1→3]GlcNAc-3CSpacer-NH-lysine-BSA; Le^x-BSA), or Le^x antigen (CD15) coupled to BSA (Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4Glc-3CSpacer-NH-BSA; lacto-*N*-fucopentaose III-BSA) (V-LABS, Inc., Covington, La.) and exposed to the primary MAbs.

ELISAs for the *cagA* product (CagA) and the vacuolating cytotoxin (VacA) of *H. pylori* were performed as described previously (13, 23).

ELISA with sodium metaperiodate-treated whole cells. In a modification of the ELISA, wells were coated with whole-cell suspensions of *E. coli* HB101, *H. pylori* reference strains P466 and C1, or isolates CPY3401, J182, J174, HPK1, 88-22, and M-8037 and were blocked with 2% BSA in PBS. Before incubation with the primary antibody (anti-Le^x or anti-Le^y), the cells were exposed for 1 h at room temperature in the dark to 50 mM sodium acetate (pH 4.5) alone (control) or containing 10 mM sodium metaperiodate. As additional controls, the sodium metaperiodate oxidizing procedure was carried out on cells of *H. pylori* P466, C1, and CPY3401, with subsequent detection of CagA and VacA by ELISA. The ODU values for wells coated with *E. coli* (HB101) whole-cell suspensions of equal protein concentrations were subtracted to correct for nonspecific antibody binding.

Determination of Le^x and Le^y of *H. pylori* whole cells after saline washing. Cells of *H. pylori* isolate CPY3401 were harvested as described above and centrifuged for 10 min at 3,000 × *g* at 4°C, the pellet was resuspended in 0.15 M NaCl by vigorous vortex mixing, and the cells were centrifuged again. The washing procedure was repeated six times, and aliquots of each pellet and each supernatant were saved for Le^x and Le^y determinations by ELISA, as described above. The coating concentration of the cell pellets was standardized according to protein concentrations as described above, and supernatants were diluted 100- to 2,000-fold prior to assay.

Determination of Le^x and Le^y in crude membrane preparations of *H. pylori*. Cells of *H. pylori* reference strains P466 and C1 were harvested and centrifuged twice as described above, and pellets were resuspended in distilled water. For further disruption, the cells were sonicated (Braun SonicU, Braun, Germany) three times for 30 s with 15-s intervals on ice. After centrifugation for 10 min at 5,000 × *g* at 4°C to remove intact cells and large cell fragments, the supernatant was subjected to centrifugation at 100,000 × *g* for 90 min at 4°C. The resulting supernatant, representing the cytoplasmic fraction, was removed, and the pellet representing the crude membrane fraction (50) was resuspended in distilled water. The amounts of protein in all fractions were determined by the bicinchoninic acid assay, and Le^x and Le^y amounts were determined by ELISA, as described above.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with *H. pylori* (or *E. coli* HB101) whole-cell preparations was done as described elsewhere (9). After treatment with proteinase K (50 μg/200 μg of protein of the cell suspension) for 1 h at 60°C, aliquots originally corresponding to 0.5 to 30 μg of protein per lane were electrophoresed at 100 V, with a stacking gel of 3% acrylamide and a separating gel of 10% acrylamide (22). After SDS-PAGE, the gels were fixed and LPS was detected by silver staining (52). Electroblotting to nylon transfer membranes (0.22-μm pore size; MSI, Westboro, Mass.) was performed in a Trans-Blot Cell (Bio-Rad, New York) at 1 A for 30 min with 20 mM Tris base–0.15 M glycine–20% methanol as electroblotting buffer. Le determinants were visualized by subsequent incubation with MAbs to Le^x and Le^y (Signet Laboratories) and goat anti-mouse IgM antibodies coupled with alkaline phosphatase (Sigma), and the substrate was 0.01% nitroblue tetrazolium and 0.005% 5-bromo-4-chloro-indolyl phosphate in 50 mM Tris base–3 mM $MgCl_2$.

Molecular techniques. Cells of *H. pylori* isolates grown for 24 h on TSB plates were lysed with 60% guanidinum thiocyanate–0.1 M EDTA–0.5% sarcosyl to isolate chromosomal DNA (53). DNA was purified by chloroform extraction and was concentrated by isopropanol precipitation. Oligonucleotide primers (23, 25, 53, 54) were synthesized with a 392 DNA synthesizer (ABI, Foster City, Calif.). PCR for *ureA* (positions 2659 to 2678, 5′-ATGAAACTCACCCCAAAAGA-3′; 3281 to 3261, 5′-TTGTCTGCTTGTCTATCAACC-3′), *cagA* (1170 to 1191, 5′-GATAACGCTGTCGCTTCATACG-3′; 1578 to 1557, 5′-CTGCAAAAGATT GTTTGGCAGA-3′), and *picB* (1327 to 1341, 5′-TGTTTGGTTTCCCTG-3′; 2663 to 2649, 5′-ACGCATTCCTTAACG-3′) utilized reaction conditions of 94°C for 1 min, 50°C for 2 min, 72°C for 2 min with 35 amplification cycles, and the products were analyzed by ethidium bromide agarose gel electrophoresis. By PCR, all isolates studied had *ureA* present, and *picB* and *cagA* statuses were completely concordant, as expected (54). Isogenic mutants of *cagA*$^+$ *H. pylori* isolate CPY3401 were created as described elsewhere (23). Briefly, a 3.2-kb *Sca*I fragment from pMKQ containing promotorless *xylE* and a kanamycin resistance cassette (Kan^r) was blunt-end ligated into Klenow-filled *Hin*dIII sites in the open reading frames of *cagA* or *picB* in pMT1 as well as into the unique *Eco*RV site in the *ureA* open reading frame in pHPT178. The recombinant plasmids were introduced into wild-type CPY3401 cells by natural transformation and by selection for kanamycin resistance to create the following isogenic mutants: 3401A$^-$ (CPY3401; *cagA*::[*xylE*-Kan^r]), 3401B$^-$ (CPY3401; *picB*::[*xylE*-Kan^r]), and 3401U$^-$ (CPY3401; *ureA*::[*xylE*-Kan^r]). Colony hybridization for *cagA* was performed as previously described (53). A 408-bp PCR product from *cagA* was labelled with ^{32}P by using the random-primed DNA labelling kit (Boehringer Mannheim). Both bacterial whole cells and extracted chromosomal DNA UV cross-linked to Protran BA85 nitrocellulose membranes were used; *E. coli* HB101 served as a negative control.

Statistics. Results were expressed as means ± standard errors of the means (SEM). Proportions were compared by using the χ^2 test with Yates' correction or Fisher's exact test, when appropriate. Distributions of ODs were compared by using Student's *t* test for comparison of means of independent samples. All *P* values were calculated for two-tailed significance levels, and values of <0.05 were considered significant.

RESULTS

Characterization of the antibodies to Le^x and Le^y. Anti-Le^b but not anti-Le^x and anti-Le^y antibodies recognized purified Le^b-HSA by a dot immunoblot technique. Although both molecules are difucosylated, the structure of Le^b differs from Le^y in the linkage between β-D-Gal and D-GlcNAc (1→3 in Le^b versus 1→4 in Le^y). Le^x differs from Le^b both with respect to the binding between β-D-Gal and D-GlcNAc (1→4 in Le^x) and because Le^x is monofucosylated. That anti-Le^x recognized Le^x antigen (CD15) coupled with BSA (lacto-*N*-fucopentaose III-BSA, Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4 Glc-3CSpacer-NH-BSA) but not Le^x-trisaccharide-BSA (Galβ1→4[Fucα1→3]GlcNAc-3CSpacer-NH-lysine-BSA) indicates that β-D-Gal or D-Galβ1→4D-Glc in addition to the Le^x-trisaccharide was essential for recognition. As expected, the anti-Le^y antibodies bound to neither of the two compounds. By

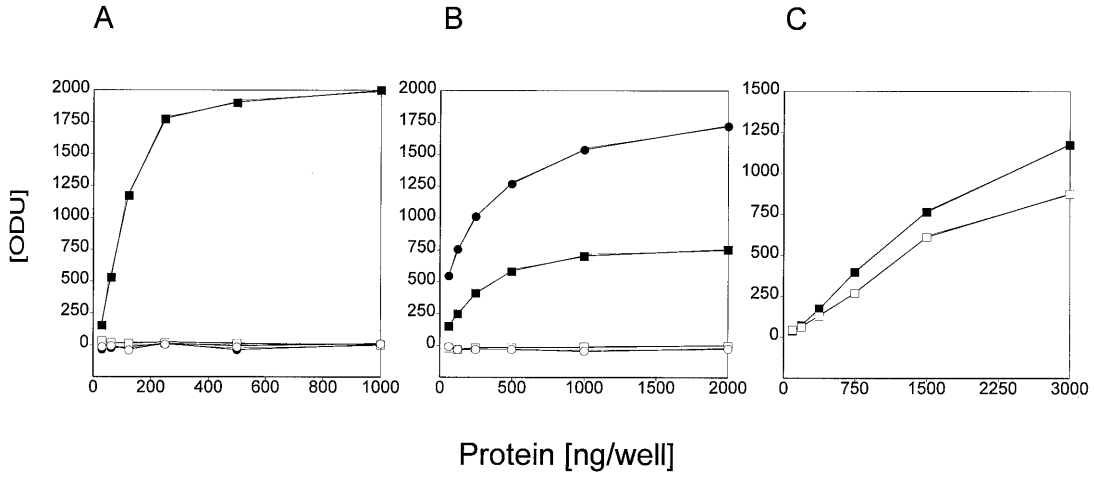

FIG. 1. Determination of *H. pylori* cellular antigens by ELISA. (A) Native or oxidized purified Le^x. The wells of microtiter plates were coated with Le^x antigen (CD15) coupled to BSA, standardized according to protein concentration. To the immobilized Le^x antigen, 10 mM sodium metaperiodate in 50 mM sodium acetate (pH 4.5) (open symbols) or 50 mM sodium acetate buffer alone (black symbols) as a control was added for 1 h. Subsequently, the wells were incubated with MAbs to Le^x (squares) or Le^y (circles). Bound antibodies were detected by incubation with goat anti-mouse IgM conjugated with alkaline phosphatase, and *p*-nitrophenylphosphate was the substrate. ODU values for wells coated with *E. coli* (HB101) whole-cell suspensions of equal protein concentrations were subtracted to correct for nonspecific antibody binding. The results are the means of two determinations. (B) Le^x or Le^y detection in native or oxidized *H. pylori* whole cells. The wells were coated with *H. pylori* (isolate J182) whole-cell suspensions expressed as amounts of protein per well. Immobilized *H. pylori* cells were treated for 1 h with 10 mM sodium metaperiodate in 50 mM sodium acetate (pH 4.5) (open symbols) or sodium acetate buffer alone (black symbols) as a control. Le^x or Le^y was detected by subsequent incubation with antibodies to Le^x (squares) or Le^y (circles). ODU values for wells coated with *E. coli* whole-cell suspensions of equal protein concentrations were subtracted to correct for nonspecific antibody binding. (C) CagA detection in native or oxidized *H. pylori* whole cells. Wells were coated with whole-cell suspensions of *cagA*$^+$ *H. pylori* isolate CPY3401 expressed as amounts of protein per well. Immobilized *H. pylori* cells were treated for 1 h with 10 mM sodium metaperiodate in 50 mM sodium acetate (pH 4.5) (open squares) or with sodium acetate buffer alone (black squares) as a control. The wells were subsequently incubated with rabbit anti-CagA IgG, goat anti-rabbit IgG conjugated with alkaline phosphatase, and substrate as described. ODU values for wells coated with *E. coli* (HB101) whole-cell suspensions of equal protein concentrations were subtracted to correct for nonspecific antibody binding.

ELISA, the anti-Le^x but not anti-Le^y antibodies recognized purified Le^x antigen (CD15) coupled to BSA (Fig. 1A), but no binding occurred with Le^x-trisaccharide-BSA. In total, these results indicate specificity of the anti-Le^x antibodies to Le^x antigen (CD15) and no cross-reactivities of the anti-Le^y antibodies to Le^x-trisaccharide, Le^x antigen, and Le^b.

In agreement with the structural findings (3, 5), *H. pylori* reference strains MO19 and P466 were typed Le^y+ 1,629 ODU and 1,319 ODU, respectively), and strain C1 was typed Le^x+/Le^y(+) (1,534/116 ODU); however, strain P466 was negative for Le^x but positive for sialyl-Le^x and strain NCTC11637 (ATCC43504) obtained from two different sources consistently did not show reactivity with the MAb to Le^x but showed low-level reactivity for Le^y (Le^x/Le^y, 0/263 ODU). Neither repetitive saline washing of the cells nor growth on different media revealed immunodetectable Le^x. According to the structural results (6, 7), the terminal Le^x-trisaccharide in the LPS of strain NCTC11637 is immediately followed by another Le^x-trisaccharide (instead of Galβ1→4D-Glc), which could interfere with binding of the anti-Le^x MAb. However, *H. pylori* C1 (3) also carries dimeric Le^x, and the ELISA typing of this strain was concordant with the structural findings.

Characterization of the *H. pylori* antigen recognized by anti-Le^x and anti-Le^y antibodies. As expected, sodium metaperiodate treatment of immobilized purified Le^x antigen coupled to BSA completely abolished anti-Le^x antibody binding (Fig. 1A). Similarly, sodium metaperiodate treatment of *H. pylori* whole cells completely eliminated binding of anti-Le^x and anti-Le^y antibodies (Fig. 1B). This finding was consistent for six *H. pylori* strains, representing all patterns of Le expression. In contrast and as expected, sodium metaperiodate treatment of *H. pylori* whole cells only slightly reduced antibody binding to protein antigens such as CagA (Fig. 1C) or VacA (data not shown) in strains C1, P466, and CPY3401.

Further characterization of the antigens recognized by antibodies specific to Le^x and Le^y showed the following. First, the level of Le^x and Le^y detection was not increased by using *H. pylori* sonicate instead of equivalent amounts of whole cells (data not shown), indicating that the Le determinants must be located on the cell surface and are easily accessible to the antibodies. Second, the antigen was stable even after six repetitive saline washings of *H. pylori* whole cells. Extensive washing did not result in a loss of detectable Le^x or Le^y per protein content of the whole-cell preparation (962, 1,005, 1,222, 1,275, 1,244, and 1,263 ODU for Le^x and 683, 688, 798, 811, 860, and 849 ODU for Le^y, respectively, after one to six washes) and the Le^y-to-Le^x ratio for the cells remained unchanged (0.63 to 0.71), indicating similar resistance of both determinants to the washing procedure. Interestingly, detection of the Le antigens was enhanced after three washes, in agreement with the removal of cellular components that blocked antibody recognition in the native cells. That supernatants showed progressively less antigen in successive washes indicates the stability of the antigen in the cellular fraction. Third, in a crude membrane preparation of *H. pylori* cells after osmotic and ultrasound disruption of the cells and ultracentrifugation, the Le-to-protein ratios were significantly higher than those of the initial sonicate and the cytosolic fraction. For *H. pylori* Le^x reference strain C1 after ultracentrifugation, Le^x was 8.46-fold more concentrated in the pellet than in the supernatant. The pellet-to-supernatant ratio for *H. pylori* Le^y reference strain P466 after ultracentrifugation was 6.81. Fourth, neither boiling in SDS nor proteinase K digestion of *H. pylori* whole cells destroyed the immunodetection of the Le^x (in strain C1) and the Le^y (in strains P466 and MO19) antigens. Both Le^x- and Le^y-carrying *H. pylori* determinants migrated chiefly at approximately 30 kDa (Fig. 2A and B), although the molecular mass of LPS cannot be estimated very accurately with protein molec-

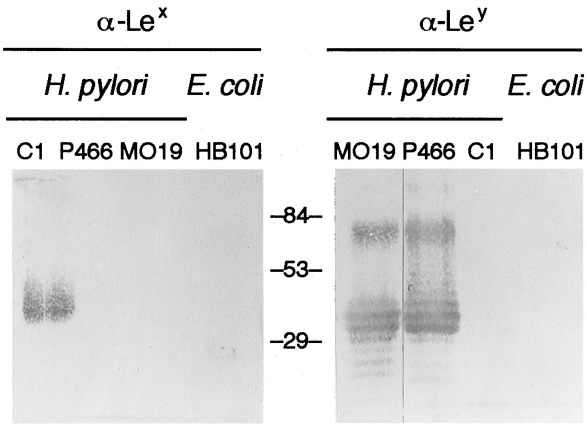

FIG. 2. SDS-PAGE and immunoblotting of proteinase K-treated whole cells of *H. pylori*. (A) For Le^x detection, whole cells of *H. pylori* reference strains C1 (lane 1), P466 (lane 2), MO19 (lane 3), and *E. coli* HB101 (lane 4) corresponding to 10 μg (lane 1) and 20 μg (lanes 2 to 4) of protein were electrophoresed and electroblotted. Le^x was detected by incubation with mouse IgM MAb to Le^x (α-Le^x), goat anti-mouse IgM conjugated with alkaline phosphatase, and substrate as described. (B) For Le^y detection, whole cells of *H. pylori* reference strains MO19 (lane 1), P466 (lane 2), C1 (lane 3), and *E. coli* HB101 (lane 4) corresponding to 2 μg (lanes 1 and 2) and 20 μg (lanes 3 and 4) of protein were electrophoresed and electroblotted. Le^y was detected by incubation with mouse IgM MAb to Le^y (α-Le^y), goat anti-mouse IgM conjugated with alkaline phosphatase, and substrate.

ular mass markers (52). *H. pylori* high-molecular-mass LPS has been described, and its occurrence seems to be associated with strains that have not been extensively passaged (32, 34, 48). The ladder-like pattern typical for LPS was not discernible for the higher-molecular-mass Le^y-carrying bands as well as for Le^x-carrying LPS, which is compatible with the formation of homopolymers, as has been proposed elsewhere (34). In total, these findings confirm that the Le determinants detected are part of the *H. pylori* LPS.

Le^x and Le^y expression of *H. pylori* under various growth conditions. *H. pylori* reference strains MO19, P466, and C1 and isolates 84-183, 88-23, T-8832, and HPK5 were grown in parallel for 48 h on TSB plates and on brucella agar plates supplemented with 5% FBS. Mean (± SEM) Le^x expression after growth on brucella agar plates (95 ± 43 ODU) was minimally lower than that after growth on TSB plates (116 ± 47 ODU; *P*, not significant). Le^y expression was slightly higher for growth on brucella agar plates (1,009 ± 145 ODU) than for that on TSB plates (965 ± 165 ODU; *P*, not significant). Four Le^x-negative strains (88-22, ATCC43504, CH5, and P466) and one Le^y-negative strain (88-22) when grown on TSB plates were also negative when grown in brucella broth. The effects of differences in growth media (brucella broth plus 5% FBS; TSB plates) and cell age on Le expression in seven *H. pylori* isolates studied were minimal. For three isolates, Le^x of 24- to 96-h cells varied between 94% ± 19% and 117% ± 10% (mean percent 48-h values) for broth and between 96% ± 12% and 103% ± 11% for plates. For seven isolates, Le^y varied between 105% ± 15% and 120% ± 13% for broth and between 107% ± 5% and 121% ± 7% for plates. The mean broth-to-plate ratios for 24-, 48-, 72-, and 96-h cultures were 1.00 ± 0.20 for Le^x and 1.25 ± 0.10 for Le^y. The mean Le^y-to-Le^x ratios of cells grown in the two media were 5.18 versus 5.45 for *H. pylori* 88-23, 0.46 versus 0.44 for J182, and 0.16 versus 0.10 for CH2. In summary, under the growth and media conditions examined, expression of Le antigens by *H. pylori* strains was a highly stable phenotype.

Single-colony Le typing. To confirm that individual *H. pylori* strains can express both Le^x and Le^y, Le typing was performed on eight single colonies of each of five isolates and reference strains P466 and C1. The ranges of Le^x/Le^y expression (in ODU) for the single colonies were as follows: CPY3401, 1,369 to 1,689/788 to 992; P-9040, 724 to 891/1,393 to 1,745; T-8832, 457 to 694/1,673 to 1,896; T-8828, 645 to 926/1,212 to 1,620; J174 811 to 991/563 to 897; P466, 9 to 30/1,183 to 1,450; and C1, 1,032 to 1,347/61 to 85. Thus, the Le^x and Le^y values of the single colonies of individual parental strains varied within a small ODU range, indicating both the reproducibility of the method and the phenotypic homogeneity of a given strain.

Detection of Le^x and Le^y in *H. pylori* isolates. Having confirmed the stability of Le antigen expression, we then surveyed 94 *H. pylori* isolates from different parts of the world to determine the distribution of phenotypes. Among the surveyed isolates, both Le^x and Le^y were expressed over a broad ODU range (Fig. 3A and B). On the basis of the distribution of the values in ELISA, a cutoff ODU of ≥100 was selected to reflect positivity. With this cutoff, 46 (49%) isolates were positive for both Le^x and Le^y, 10 isolates were positive for Le^x only, 24 isolates were positive for Le^y only, and 14 (15%) isolates were negative for both determinants. Therefore, 85% of the *H.*

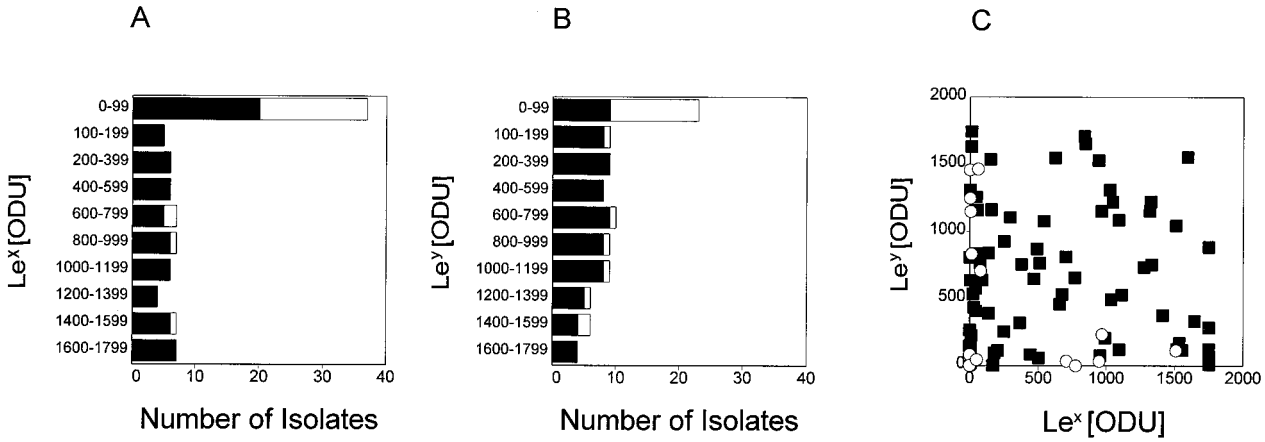

FIG. 3. (A and B) Determination by ELISA of Le^x and Le^y expression, respectively, of 94 clinical *H. pylori* isolates. The abscissa shows the numbers of isolates yielding ODU values within the intervals indicated on the ordinate. Isolates were characterized as *cagA*$^+$ (black boxes) or *cagA* (open boxes) as described. (C) ODU values for the 94 *H. pylori* isolates for both Le^x and Le^y. Open circles, *cagA* isolates; black squares, *cagA*$^+$ isolates.

TABLE 2. Relationship between Le expression and ulcer disease for 90 *H. pylori* clinical isolates[a]

Associated disease[b]	No. of isolates showing specified Le expression[c]				Total no.
	Le^x-/Le^y-	Le^x+/Le^y-	Le^x-/Le^y+	Le^x+/Le^y+	
Ulcer	5	8	17	33	63
Duodenal	5	5	12	27	49
Gastric	0	3	5	6	14
Nonulcer	9	2	7	9	27
Total	14	10	24	42	90

[a] For 4 of the 94 *H. pylori* isolates studied, no clinical data were available.
[b] According to the findings at upper gastrointestinal endoscopy and the clinical records of each patient.
[c] Determined by ELISA, as described; negativity for Le^x or Le^y was defined as ODU of <100.

TABLE 4. Expression of Le antigens in 94 *H. pylori* clinical isolates

Le antigen	% of isolates expressing antigen			% of isolates expressing as sole antigen		
	All (n = 94)	*cagA*$^+$ (n = 71)	*cagA* (n = 23)	All (n = 94)	*cagA*$^+$ (n = 71)	*cagA* (n= 23)
Le^x	60	72	22	10	8	13
Le^y	74	87	35	19	18	22
Sialyl-Le^x	2	3	0	0	0	0
Le^a	3	1	9	0	0	0
Le^b	13	11	17	3	0	13
≥1 antigen	89	97	65			
≥2 antigens				57	70[a]	17[b]

[a] 1 sialyl-Le^x/Le^x, 1 sialyl-Le^x/Le^y, 4 Le^b/Le^y, 39 Le^x/Le^y, 1 Le^a/Le^x/Le^y, and 4 Le^b/Le^x/Le^y antigens.
[b] 1 Le^a/Le^b, 1 Le^a/Le^y, and 2 Le^x/Le^y antigens.

pylori isolates expressed at least one Le type 2 immunodeterminant in their LPS. No obvious geographic trends due to the origin of the isolates were observed.

Then, we examined Le^x and Le^y expression in 90 *H. pylori* isolates from patients known to have or to be free of ulcer disease (Table 2). Of 63 isolates from ulcer patients, 58 (92%) were positive for at least one Le determinant (44 [90%] of 49 duodenal ulcer isolates and all 14 gastric ulcer isolates), compared with 18 (67%) of 27 isolates from patients without ulcer disease ($\chi^2 = 7.45$; $P < 0.01$). Since ulcer disease has previously been found to be associated with *H. pylori* strains of certain genotypes (12, 15, 53), we then explored these relationships with Le expression.

Correlation of Le^x and Le^y expression with *cagA* status of *H. pylori* isolates. Expression of Le^x and Le^y differed markedly between *cagA*$^+$ and *cagA* *H. pylori* isolates (Fig. 3A and B; Table 3). The mean level of Le^x expression for the *cagA*$^+$ isolates (651 ± 71 ODU) was significantly ($P < 0.01$ [Student's t test]) higher than that of the *cagA* isolates (223 ± 90 ODU). The mean Le^y expression level for the *cagA*$^+$ isolates (684 ± 60 ODU) also was significantly ($P < 0.01$) higher than that of the *cagA* isolates (324 ± 109 ODU). Of the 71 *cagA*$^+$ isolates, 69 (97%) were positive for at least one Le determinant, compared with 11 (48%) of 23 *cagA* isolates ($\chi^2 = 29.6$; $P < 0.001$). Only 2 (9%) of the 23 *cagA* isolates were positive for both Le^x and Le^y, compared with 44 (62%) of the 71 *cagA*$^+$ isolates ($\chi^2 = 17.5$; $P < 0.001$). Isolates with strong (>250 ODU) expression levels of both determinants (Fig. 3C) were exclusively *cagA*$^+$ ($\chi^2 = 14.5$; $P < 0.001$).

Because of the correlation of *vacA* type and *cagA* status, we examined Le^x and Le^y expression in 45 *H. pylori* isolates that had been characterized according to *vacA* signal sequence and midregion type (8). At least one Le determinant was detected in 29 (94%) of 31 s1 isolates versus 7 (50%) of 14 s2 isolates ($\chi^2 = 8.9$; $P < 0.01$), 19 (95%) of 20 ml isolates versus 14 (64%) of 22 m2 isolates ($P = 0.013$; Fisher's exact test), and 18 (95%) of 19 s1m1 isolates versus 6 (46%) of 13 s2m2 isolates ($P = 0.003$; Fisher's exact test). Seventeen (55%) of 31 s1 isolates versus 2 (14%) of 14 s2 isolates ($\chi^2 = 4.9$; $P < 0.05$) and 12 (60%) of 20 m1 versus 6 (27%) of 22 m2 isolates ($P = 0.033$; Fisher's exact test) were positive for both Le^x and Le^y. Only one (8%) of 13 s2m2 isolates versus 11 (58%) of 19 s1m1 isolates was positive for both Le^x and Le^y ($P = 0.009$; Fisher's exact test). These results indicate that expression of Le determinants also correlates with *vacA* genotype.

TABLE 3. Relationship between Le expression and *cagA* status for 94 *H. pylori* clinical isolates

cagA status[a]	No. of isolates showing specified Le expression[b]				Total no.
	Le^x-/Le^y-	Le^x+/Le^y-	Le^x-/Le^y+	Le^x+/Le^y+	
cagA	12[c]	3	6	2	23
cagA$^+$	2	7	18	44[d]	71
Total	14	10	24	46	94

[a] Determined by PCR and colony hybridization, as described.
[b] Determined by ELISA, as described; negativity for Le^x or Le^y was defined as ODU of <100.
[c] Proportion of isolates that are *cagA* is significantly ($\chi^2 = 29.6$; $P < 0.001$) different from those of all other groups of isolates.
[d] Proportion of isolates that are *cagA*$^+$ is significantly ($\chi^2 = 17.7$; $P < 0.001$) different from those of all other groups of isolates.

Detection of Le^a, Le^b, and sialyl-Le^x in *H. pylori* isolates. Because of the importance of Le^a, Le^b, and sialyl-Le^x in human biology (38, 42, 59), we also determined expression of these antigens in the 94 *H. pylori* isolates (Table 4). Expression of Le^b, Le^a, and sialyl-Le^x was found in 13, 3, and 2%, respectively, of the isolates surveyed. Le^a was coexpressed with Le^b, Le^y, or Le^x and Le^y; sialyl-Le^x was coexpressed with Le^x or Le^y. Le^b was expressed together with Le^a, Le^y, or Le^x and Le^y or as a sole determinant. Sialyl-Le^x was found in only two *cagA*$^+$ isolates; Le^a and Le^b occurred in both *cagA*$^+$ and *cagA* isolates. Expression of Le^b as a sole determinant was exclusively found in *cagA* isolates, whereas in *cagA*$^+$ isolates Le^b was regularly coexpressed with Le^y (Le^b/Le^y in four isolates and Le^b/Le^x/Le^y in four isolates).

Le expression in wild-type CPY3401 and isogenic mutants. To determine whether *cagA* status was a marker of a cell lineage that correlated with Le expression or whether the *cagA* product had a role in this expression, we created a series of isogenic mutants of wild-type isolate CPY3401, which is a high-level expressor of Le^x and Le^y. Isogenic mutants 3401A$^-$, 3401B$^-$, and 3401U$^-$ were created by insertion of a promotorless *xylE*-kanamycin cassette into *cagA*, *picB*, or *ureA*, respectively, of isolate CPY3401, as described. Mutant 3401A$^-$ had a diminished (47%) level of expression of Le^y (533 ± 121 ODU) compared with that of the wild-type isolate (1,136 ± 113 ODU; $P < 0.01$) and those of mutants 3401B$^-$ and 3401U$^-$ (Fig. 4). In contrast, expression levels of Le^x for wild-type 3401 and for isogenic mutants 3401A$^-$, 3401B$^-$, and 3401U$^-$ were similar. The similar expression levels of Le^x and Le^y in wild-type 3401 and in 3401B$^-$ and 3401U$^-$ indicate that the genetic transformation with the promotorless *xylE*-kana-

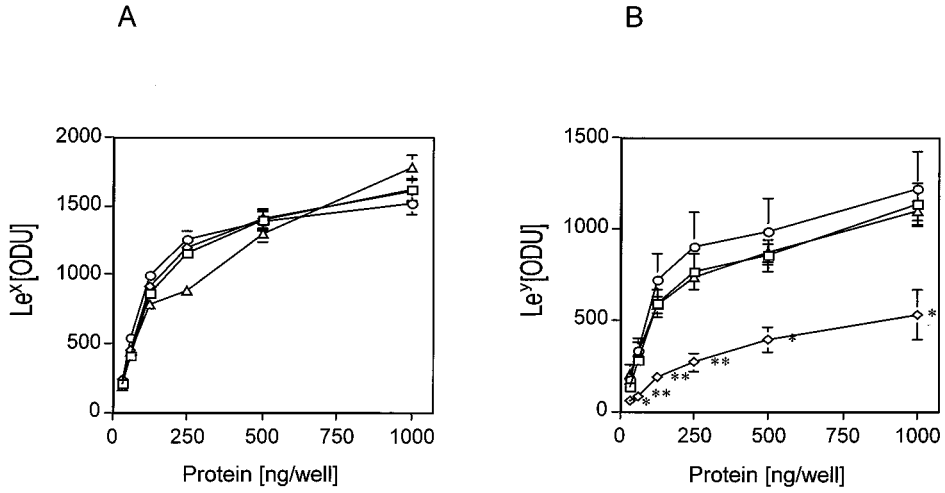

FIG. 4. (A and B) Determination of Le^x and Le^y expression, respectively, by ELISA in wild-type *H. pylori* isolate CPY3401 (squares) and isogenic mutants. The wells were coated with *H. pylori* whole-cell suspensions represented as amount of protein per well. Isogenic mutants $3401A^-$ (diamonds), $3401B^-$ (circles), and $3401U^-$ (triangles) were created by insertion of a promotorless *xylE*-kanamycin cassette into *cagA*, *picB*, and *ureA*, respectively. ODU values are expressed as means ± SEM of five determinations. *, $P < 0.01$; **, $P < 0.001$ (Student's *t* test).

mycin cassette itself did not influence Le expression and point to a specific role for the *cagA* product in Le^y expression.

DISCUSSION

Our findings confirm the presence of Le antigens in the LPS of *H. pylori* cells (2, 6, 48) and extend our understanding of their characteristics. First, we showed that most (85%) but not all of a large series of *H. pylori* isolates obtained from different parts of the world express more that minimal amounts of either Le^x or Le^y and that nearly half (49%) express both. Second, we established a quantitative assay and showed that the expression characteristics were stable under a variety of growth conditions. For three of the four reference strains for which structure had been previously analyzed (3, 5), our results were completely consistent. The discrepancy for the fourth strain (NCTC11637) may reflect the presence of a mixed population in the initial culture, as suggested by previous immunoelectron microscopic analysis (48), or the transition of the LPS to a rough form, as suggested by electrophoretic analysis (34, 48). Alternatively, since the anti-Le^x MAb used is specific for the Le^x antigen (CD15) and does not recognize the Le^x-trisaccharide itself, it may not be sufficiently sensitive to detect Le^x under all confirmations induced by neighboring structures. Third, we showed for the first time that *H. pylori* isolates may express other host cell surface antigens, such as Le^a, Le^b, or sialyl-Le^x. Fourth, we demonstrated that Le expression is related to other *H. pylori* strain characteristics. One reason that no Le determinants are detected in some isolates could be that the antibodies we used may not be fully sensitive, as was previously described for *H. pylori* (2). This is a limitation of all studies with MAbs to define cellular antigens that may be diverse. Use of a wider panel of antibodies may permit greater detection of Le determinants.

That most *H. pylori* isolates express Le antigens is unlikely to be accidental. *Schistosoma mansoni*, another microbe that causes persistent infections in which most infected persons remain without clinical consequences, expresses Le^x antigen on both the surface of adult worms and on ova (24, 51). Le^x antigen expression by *S. mansoni* appears to both downregulate the T helper 1 cell response (56) and to induce IgM autoantibodies binding to Le^x on granulocytes (55); such data are currently not available for *H. pylori*. Although it causes a luminal infection likely with immunoregulatory mechanisms different from those of *S. mansoni*, we nevertheless speculate that expression of Le antigens by *H. pylori* could have similar effects on the host response. These could parallel the observed low level of proinflammatory activity of the lipid A moiety of *H. pylori* LPS (35, 41) and could help explain why *H. pylori* is unique among gram-negative pathogens in not increasing CD15 or CD11c expression on granulocytes (17). Cross-linking of CD15 on granulocytes induces CD11b upregulation, and both CD11b and CD11c act as complement receptors and adhesion molecules. Lack of activation is consistent with the low level of proinflammatory activity of *H. pylori* LPS and is possibly related to the expression of CD15 (Le^x antigen) and other host Le determinants by *H. pylori*. Conversely, Le expression by *H. pylori* could lead to autoimmunity against host cells expressing similar antigens (2). Since Le expression is altered in epithelial cells that have undergone malignant transformation (1, 20, 46), specific Le expression by *H. pylori* should be explored in gastric neoplasia, which may follow prolonged *H. pylori* infection (33).

Le expression appears more common among isolates from patients who have ulcer disease than from those without the disease. Since ulcer risk has been previously associated with *H. pylori* strain characteristics (12, 15, 53), it was not surprising to find an association of Le expression with both *cagA* and *vacA* genotypes. This linkage may merely reflect common ancestry or could suggest natural selection of strains with particular phenotypic characteristics. Wild-type *cagA* strains lack a 30- to 40-kb region (26) that has been called the *cag* pathogenicity island. That ablation of *cagA*, but not the adjacent *picB*, which also is part of this island, or the highly conserved *ureA*, reduced Le^y expression suggests that the *cagA* product, a hydrophilic, highly basic, immunogenic, and surface-exposed high-molecular-mass molecule (12, 53), specifically affects either Le^y expression or localization on the cell surface. The basis for the specificity of its role in Le^y but not Le^x expression is not currently understood. Since $cagA^+$ strains induce more inflammation than do *cagA* strains (14, 36, 40), and assuming that persistence increases opportunities for transmission to new

hosts, we speculate that high-level expression of host Le antigens may be particularly adaptive in *cagA*$^+$ strains as a means of counterbalancing their proinflammatory effects. That lack of Le expression by *cagA*$^+$ isolates is uncommon (3%) is consistent with this idea.

Although attachment of *H. pylori* to gastric epithelial cells has been suggested to be mediated by host Le^b determinants (10, 18), finding that Le^b can be expressed by *H. pylori* may argue against this hypothesis. Future studies of *H. pylori* adhesion should include the Le phenotypes of both the microbe and the host cells.

For most gram-negative bacteria, the terminal components of the LPS polysaccharide side chains usually show the greatest diversity (45). That *H. pylori* strains express a small number of terminal antigens on their LPS has several implications. First, that host cell (Le) antigens are present suggests selection of *H. pylori* strains possessing these attributes and implies a long coevolutionary history. Second, the limited number of antigens detected suggests that as a species, *H. pylori* may not need to generate substantial antigenic diversity in order to survive the host immune response, perhaps because of its residence in the gastric lumen, in which immune mechanisms are not optimally effective. Third, the selection in *H. pylori* for cellular antigens that exist as allelic variants in their hosts suggests that strains expressing particular phenotypes may be best adapted to hosts of particular genotypes. Such selection could reflect the ability of *H. pylori* to adhere to the gastric epithelium or to minimize neutrophil activation, among other possibilities.

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ADDENDUM IN PROOF

Since submission of this work, we have become aware of the article by Simoons-Smit et al. (I. M. Simoons-Smit, B. J. Appelmelk, T. Verboom, R. Negrini, J. L. Penner, G. O. Aspinall, A. P. Moran, S. F. Fei, S. Bi-Shan, W. Rudnica, A. Sanio, and J. de Graff, J. Clin. Microbiol. **34:**2196–2200, 1996).

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