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Helicobacter pylori Expresses a Complex Surface Carbohydrate, Lewis X

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Monoclonal antibodies (MAbs) specific for Lewis X (Le^x) reacted with whole cells of *Helicobacter pylori* NCTC11637, UA799, UA802, UA825, UA861, UA1182, and UA1206 in immunoelectron microscopy and enzyme-linked immunosorbent assay (ELISA) experiments. These MAbs have documented specificity to Le^x, whereas MAbs for Le^a and Le^b were negative in both immunoelectron microscopy and ELISA. *H. pylori* coccoid forms also reacted with the MAbs, whereas the flagellum lacking the sheath showed no reactivity. The Le^x structures were associated with membrane fractions in the ELISA experiments, and silver-stained sodium dodecyl sulfate-polyacrylamide gels confirmed the presence of lipopolysaccharides which reacted with the MAbs in immunoblots. Serum from an *H. pylori*-infected individual contained immunoglobulins which blocked the binding of the Le^x MAbs, indicating that part of the host immune response to *H. pylori* is to the Le^x structure. The ability of this gastric pathogen to mimic an oncofetal antigen (self) could explain the down regulation of anti-*H. pylori* T-cell response seen in *H. pylori*-infected individuals.

Chronic infection of the gastric mucosa with *Helicobacter pylori* is the most frequent cause of recurrent gastroduodenal inflammatory disease, including gastric and duodenal ulcers (17, 28, 44). A causative role for *H. pylori* in the development of both gastric adenocarcinoma (14, 27) and lymphoma of mucosa-associated lymphoid tissue (46) has also recently come to light. The bacterium colonizes human gastric mucosa, where it can persist, with or without symptoms, for many years (22). Interest in this bacterium has increased since the National Institutes of Health (13) recently recommended that *H. pylori* infections of the stomach be treated with antibiotics.

The requirements for attachment of *H. pylori* to gastric epithelial surfaces and for subsequent colonization of the gastric mucosa are unique and, because of the hostile environment involved, require exceptional strategies. Borén and coworkers (5) recently demonstrated that the Lewis B (Le^b) blood group antigen on human gastric epithelium can mediate attachment by *H. pylori*. A reciprocal adhesin on the bacterial cell may be the sialic acid-specific hemagglutinin on the surface of *H. pylori* (12).

Once established in the stomach, *H. pylori* elicits local as well as systemic antibody responses (30). Subsequent tissue damage occurs by one of several mechanisms, including the production of a vacuolating cytotoxin (23) by some strains. Cloning of the *vacA* gene (8, 29, 33, 41) and expression of the toxin has demonstrated that the toxin resembles an immunoglobulin A (IgA) protease (33). In mice, oral administration of a sonic extract of *H. pylori* cells, or the purified cytotoxin, resulted in gastric lesions such as mucosal erosion, necrosis, and ulceration (41) which resemble those seen in humans colonized by *H. pylori*. Other virulence factors include a mucinase (35, 36).

IgG has been shown not to be protective and does not prevent colonization of the stomach (4, 16). *H. pylori* has been reported to induce autoantibodies which cross-react with human gastric mucosa (26). Autoantibodies were detected in 89.6% of *H. pylori*-infected patients but were absent in *H. pylori*-negative individuals.

It has been known for several years that A, B, and H blood group structures are present on specific gram-negative bacteria (37). Until recently, complex carbohydrate structures such as Le^x have been thought to occur only on higher eukaryotic cells. Aspinall and coworkers (1) reported structural analysis of the carbohydrates which mimic human cell surface glycoconjugates present in lipopolysaccharide (LPS) from *H. pylori* NCTC 11637, using one- and two-dimensional nuclear magnetic resonance spectroscopy. They identified a structural similarity to glycolipid antigens from group O human erythrocyte membranes (19) which bear the Le^x determinant.

The goal of this study was to examine *H. pylori* cells by immunoelectron microscopy and enzyme-linked immunosorbent assay (ELISA) for reaction to antibodies directed against Lewis antigens, using mouse isotype IgM monoclonal antibodies (MAbs) specific for Le^a (Galβ1→3[Fucα1→4]GlcNAc), Le^b (Fucα1→2Galβ1→3[Fucα1→4]GlcNAc), Le^x (Galβ1→4[Fucα1→3]GlcNAc), antidimeric X (Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4[Fucα1→3]GlcNAc), and human CD15 (Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4Glc), also called hapten X, Le^x, and lacto-*N*-fucopentaose (LNFP) III.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. pylori* NCTC11637, UA799, UA802, UA825, UA861, UA1182, and UA1206 were isolated from endoscopic biopsy specimens obtained from patients attending the University of Alberta Hospital, using methods of isolation and characterization described by Taylor et al. (39, 40). *H. pylori* strains were grown on brain heart infusion agar (Oxoid, Basingstoke, England) containing 5% yeast extract, 5% bovine serum, vancomycin (15 μg/ml), and amphotericin (15 μg/ml). Cultures were incubated in 5% carbon dioxide–5% hydrogen–90% nitrogen at 37°C. Control cultures of *Campylobacter coli* and *Campylobacter fetus* were grown on Mueller-Hinton agar (Oxoid) and incubated in 5% carbon dioxide at 37°C.

Primary antibodies. The antibodies used were mouse isotype IgM MAbs from three different sources. Anti-Le^x (85-5000; Synaff-Chembiomed) is specific for (Galβ1→4[Fucα1→3]GlcNAc) and is referred to as the Le^x structure. Anti-human CD15 (CNM 1336; Cedarlane Laboratories Ltd.) has documented specificity for Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4Glc (LNFP III [21]; also called hapten X and Le^x). The antidimeric X MAb (BCR 90/45; Accurate Chemical, Westbury, N.Y.) is specific for Galβ1→4[Fucα1→3]GlcNAcβ1→

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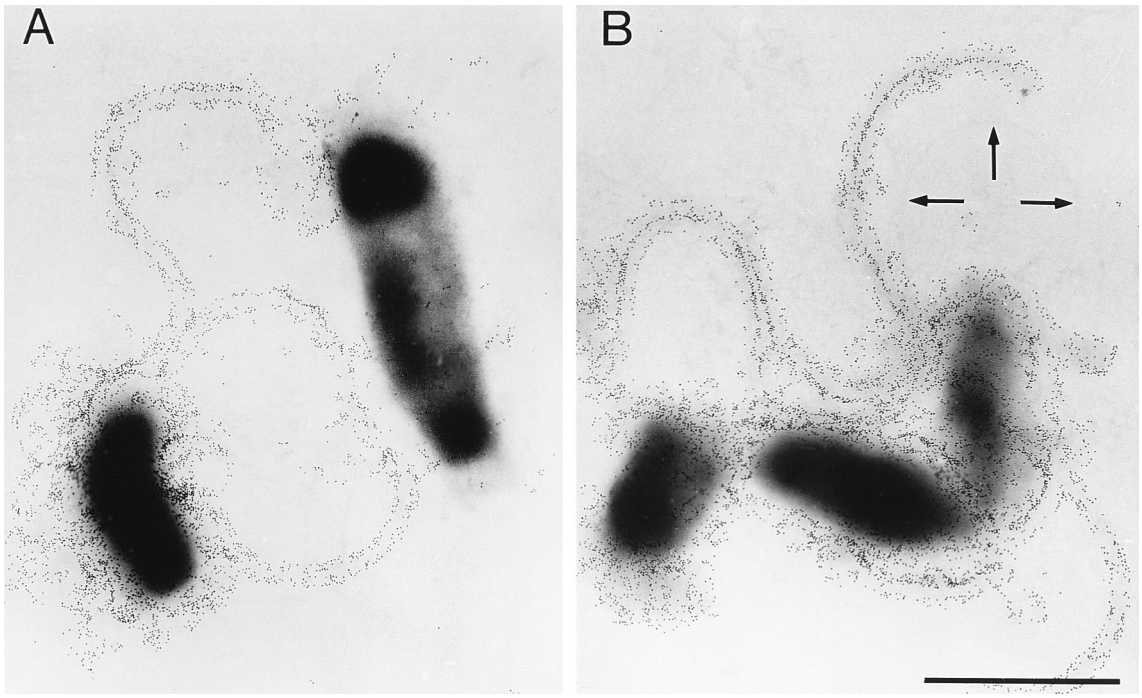

FIG. 1. Transmission electron micrographs of unstained unfixed *H. pylori* UA1206 cells. (A) Cells incubated with an anti-human CD15 IgM MAb and goat anti-mouse IgM–10-nm colloidal gold particles; (B) *H. pylori* incubated with the anti-human CD15 MAb and 10-nm colloidal gold particles, showing unsheathed flagellum (solid arrow) not labeled with the Le^x MAbs. Bar = 1.0 μm.

3Galβ1→4[Fucα1→3]GlcNAc. This MAb also reacts with LNFP III. Anti-Le^a MAbs (Synaff-Chembiomed) are specific for Galβ1→3[Fucα1→4]GlcNAc, and anti-Le^b MAbs (Synaff-Chembiomed) are specific for Fucα1→2Galβ1→3[Fucα1→4]GlcNAc.

ELISA. Microtiter plates (Titertek) were coated with *H. pylori* whole cell antigen diluted to 50 μg of protein per ml as estimated by a Bradford protein assay (Bio-Rad), using standard coating procedures. *H. pylori* UA1182 cells were separated into cytoplasmic and membrane fractions by the procedure of Yamato et al. (47). The primary antibodies used were as mentioned above, and the secondary MAbs were goat isotype IgM anti-mouse IgG-IgM (heavy and light chains)-horseradish peroxidase (HRP) conjugate (1215-035-068; Jackson Immunoresearch Laboratories), MAb sheep isotype IgM anti-human IgG (NA933; Amersham Life Science)-HRP conjugate, and 2,2′-azino-di-[3-ethyl-benzthiazoline-sulfonate] (756407; Boehringer Mannheim) (HRP substrate).

For the ELISA anti-Le^x, anti-human CD15, and anti-dimeric X unprocessed whole cell blocking experiment, we used polyclonal antibodies from *H. pylori*-infected patients which had been verified by culture of *H. pylori* from an antral biopsy specimen. Polyclonal antibodies from *H. pylori*-negative individuals were used as negative controls.

Immunoelectron microscopy. *H. pylori* whole cells removed from agar plates were suspended in phosphate-buffered saline (pH 7.3) and washed twice. The bacterial suspension was then adsorbed onto Formvar-coated electron microscope grids by floating the grid on a drop of suspension, transferred to a primary mouse IgM MAb, and incubated. Samples were then blocked with 1% bovine serum albumin (BSA) and further incubated with a goat anti-mouse IgM–10-nm colloidal gold conjugate (GAF-445; EY Laboratories Inc.). The sample was again blocked with 1% BSA and then incubated with a 20-nm colloidal gold conjugate (GAF-444; EY Laboratories), washed in distilled water, and examined unstained. Positive labeling was determined by the presence of gold particles on unfixed and unstained *H. pylori* cells. Images were recorded on Kodak 4489 electron microscope film, using a Philips model 410 transmission electron microscope.

SDS-PAGE and Western blot (immunoblot) analysis of LPS. Proteinase K-treated *H. pylori* whole cell lysate LPS was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (20) and silver stained as described by Tsai and Frasch (42). A gel run in parallel was electrophoretically transferred onto a nitrocellulose membrane (2) and immunostained.

RESULTS AND DISCUSSION

Seven *H. pylori* strains were tested: NCTC11637, UA799, UA802, UA825, UA861, UA1182, and UA1206. All reacted with the three different MAbs specific for Le^x structures as determined by the presence of gold particles (Fig. 1) but not antibodies against Le^a or Le^b in both immunoelectron microscopy and ELISA. *H. pylori* coccoid forms also reacted with the MAbs for Le^x structures. Transmission electron microscopy and immunogold labeling experiments of *H. pylori* UA1182 cells incubated with anti-human CD15 demonstrated strong reactivity (Fig. 1). Prior incubation of *H. pylori* cells with anti-Le^x antibodies in a double-labeling experiment inhibited labeling with anti-human CD15 (data not shown), implying that the two MAbs react with the same structure. The *H. pylori* flagellum which lacked the outer sheath as a result of mechanical disruption (Fig. 1B) showed no such reactivity. NCTC11637 cultures contained some cells which labeled with anti-Le^x MAbs, whereas many cells within the same field were negative as determined by electron microscopy gold labeling. Cells of both *C. coli* and *C. fetus* were negative for immunogold labeling with Le^a, Le^b, and Le^x antibodies.

To confirm our findings, we used the ELISA technique with unprocessed whole cells of *H. pylori* UA1182 and anti-Le^x monoclonal sera from two different sources (Fig. 2A). The presence of the Le^x antigen on the surface of *H. pylori* UA1182 cells was confirmed with these two Le^x-specific monoclonal antisera (columns 5 and 6). Polyclonal antibodies from *H. pylori*-infected patients (columns 3 and 4) and *H. pylori*-negative individuals (columns 1 and 2) were used as positive and negative controls.

To determine the location of the Le^x antigens in the *H. pylori* cells, we separated *H. pylori* UA1182 cells into cytoplasmic and membrane fractions by using the procedure of Yamato et al. (47) and repeated the ELISAs.

The reactivities of anti-Le^x and anti-dimeric X antibodies to the cytoplasmic fractions of *H. pylori* UA1182 were markedly reduced (Fig. 2B, columns 5 and 6), whereas the binding of

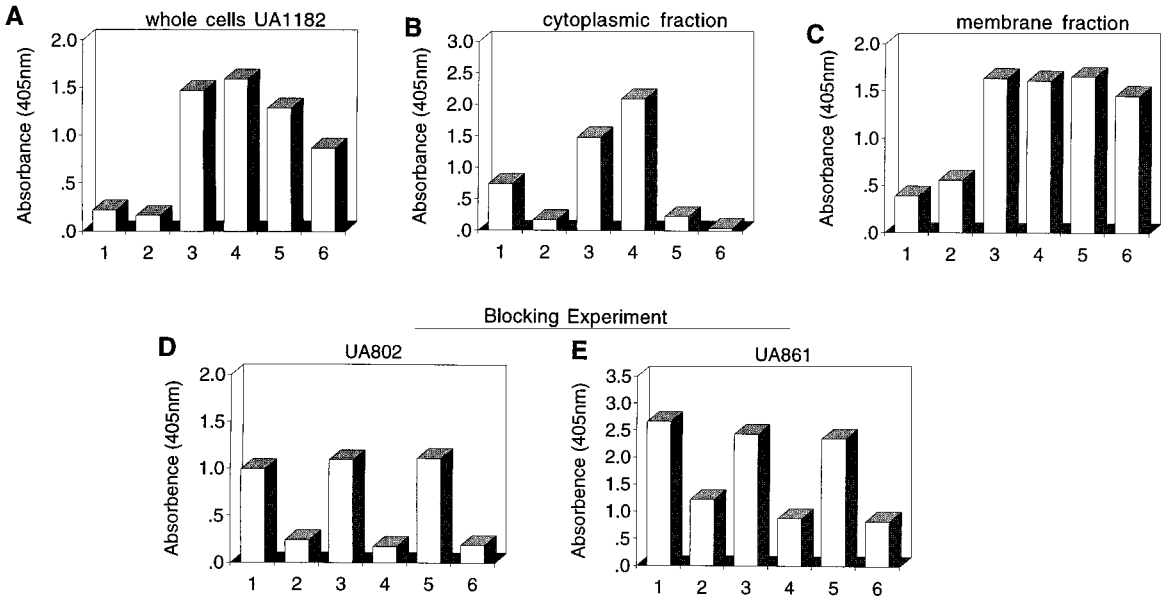

FIG. 2. ELISA antibody response to *H. pylori*. (A) Response to unprocessed whole cells of strain UA1182. Columns: 1 and 2, sera from *H. pylori*-negative patients; 3 and 4, sera from *H. pylori*-positive patients; 5, MAb specific for Le^x; 6, MAb specific for dimeric X. (B) Response to the cytoplasmic fraction of UA1182. Columns are as in panel A. (C) Antibody response to the membrane fraction of UA1182. Columns are as in panel A. (D and E) ELISA anti-Le^x unprocessed whole cell blocking experiment using *H. pylori*-positive human serum. Columns: 1, anti-Le^x; 3, anti-dimeric X; 5, anti-human CD15; 2, 4, and 6, prior incubation with *H. pylori*-positive human serum. ELISA readings are calculated by averaging duplicate test wells, using a Titertek Multiscan Plus spectrophotometer at 405 nm. The final numbers have been adjusted by deducting the average of triplicate control wells incubated with the antibodies but no HRP conjugate or the enzyme substrate.

polyclonal patient sera remained the same or even increased (columns 3 and 4). A small increase in binding was also observed with the serum from the *H. pylori*-negative control (column 1). We have previously observed by immunoelectron microscopy minor nonspecific binding with sera from non-*H. pylori*-infected individuals. This presumed cross-reactivity could be reduced by prior adsorption with *C. jejuni* and/or *Escherichia coli* cells (34).

The binding of anti-Le^x and anti-dimeric X to membrane fractions of *H. pylori* UA1182 (Fig. 2C, columns 5 and 6) was greater than the binding to whole cells (Fig. 2A, columns 5 and 6). The binding of anti-Le^x and anti-dimeric X to the cytoplasmic fraction (Fig. 2B, columns 5 and 6) was negligible, and in contrast, there was little change in response of the positive control sera. The Le^x activity, therefore, appears to be associated with the membrane components of *H. pylori* which are enriched for LPS attached to the cell membrane and show maximum reactivity with Le^x MAbs.

We wondered if antibodies produced by *H. pylori*-positive individuals (4, 16) would be directed toward the same structures as those which were reacting with the anti-Le^x MAb. Therefore, we performed an ELISA blocking experiment in which *H. pylori* whole cells from strains UA802 and UA861 (Fig. 2D and E) were incubated with anti-Le^x, anti-dimeric X, and anti-human CD15 MAbs (columns 1, 3, and 5). Columns 2, 4, and 6 represent samples incubated with *H. pylori*-positive patient serum prior to incubation with anti-Le^x MAbs. Prior incubation with *H. pylori*-positive patient serum reduced the binding activity of the anti-Le^x MAbs tested. Other strains examined showed similar results (data not shown). Therefore, our evidence is consistent with the notion that a portion of IgG present in the patient sera is directed to Le^x determinants in *H. pylori* (26).

Silver-stained SDS-polyacrylamide gels of proteinase K-treated whole cell lysates of *H. pylori* demonstrated the presence of LPS in strains UA861, UA1182, UA1206, and NCTC 11637 (Fig. 3A), although UA1182 and NCTC11637 (lane 2 and 4) lacked the LPS ladderlike pattern at >22-kDa. Anti-human CD15 reacted with the LPS ladder at >22 kDa in strains UA861 and UA1206 in immunoblot analysis (Fig. 3B).

Moran et al. (24) noted strain-to-strain differences in mobilities and banding patterns of *H. pylori*, reflecting possible structural differences in the LPS. They found that multiple passages resulted in the loss of O side chains and the production of rough forms as determined by SDS-PAGE. Strains UA1182 and NCTC11637 have been repeatedly subcultured in our laboratory, which may explain the lack of the LPS ladderlike pattern at >22 kDa in SDS-PAGE. In addition, strain UA1182 ELISA readings were reduced compared with those of more recent isolates, but cross-reaction was found in the ELISA (Fig. 2A) and the immunogold labeling experiments (Fig. 1). This observation may also reflect differences in sensitivity be-

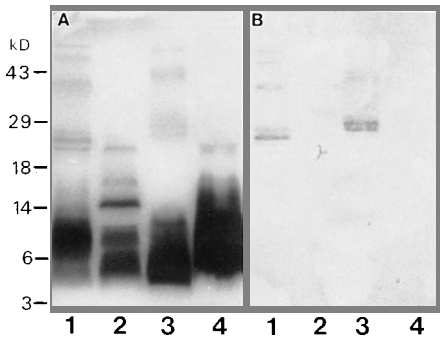

FIG. 3. (A) SDS-PAGE silver stain of proteinase K-treated whole cell lysates of *H. pylori*. Lanes: 1, UA861; 2, UA1182; 3, UA1206; 4, NCTC11637. (B) Immunoblot analysis using antibodies directed against anti-human CD15.

tween the two techniques. It has also been noted that anti-Le^x MAbs, which react poorly with whole *H. pylori* cells in immunoelectron microscopy, show strong reactivity to the supernatant from *H. pylori* on nitrocellulose immuno-dot blots (data not shown). The presence of structures reactive with anti-Le^x antibodies is also therefore associated with the *H. pylori* cell supernatant. It is likely that the Le^x antigen is secreted from the cells and can subsequently enter gastric tissue.

The Le^x antigen or its di- or trimeric form is tumor associated in that many human adenocarcinomas accumulate a large quantity of Le^x bound to a novel ceramide (15). Maximum expression of Le^x and its di- or trimeric structure occurs between 38 and 85 days of gestation at the epithelia of various organs, particularly gastrointestinal epithelium. The expression of Le^x apparently declines during later development.

Recently Velupillai and Harn (43) described the first example of a Le^x structure (LNFP III) in a lower eukaryote, in the surface antigen from the eggs of the parasitic worm *Schistosoma mansoni*. This antigen was shown to play a signaling role in the interaction of the parasite with its human host. By regulating its immune response via elaboration of a mammalian cytokine, the eggs trigger an alteration in the lymphoid T-cell response, shifting the immunity from cell mediated to antibody mediated, which is ineffective in combating infection (25). The Le^x trisaccharide present in LNFP III was found to stimulate a B-cell-enriched population of spleen cells to produce large amounts of interleukin 10 (IL-10) and prostaglandin E_2, which down regulate T helper 1 responses, thereby suppressing cell-mediated immunity (43). It has been suggested that oligosaccharide ligands may operate in other diseases, such as human immunodeficiency virus infection and malignant tumors (31).

This observation may also apply to *H. pylori* infection, as there is good evidence (11) that down regulation of the T-cell response occurs in *H. pylori* gastritis. *H. pylori* can directly augment natural killer activity in lymphocytes through stimulation of gamma interferon production (38). In *H. pylori* gastritis, the gastric epithelial cells express major histocompatibility class II antigens (45), and local expression of cytokines such as tumor necrosis factor alpha, IL-6 (10), and IL-8 (9) is increased. However, purified *H. pylori* LPS has been shown to induce production of the cytokines tumor necrosis factor, IL-1, and IL-6 in human peripheral mononuclear blood cells (3), although to a much lesser extent than LPS preparations from other bacteria.

Le^x structures on *H. pylori* cells could potentially mask *H. pylori* for a time, which might explain the autoantibodies that *H. pylori* has been reported to induce which cross-react with human gastric mucosa (26). It might also explain a phenomenon recognized long ago by physicians, that pregnancy has a beneficial effect on ulcers, although reflux symptoms continue and the ulcer often returns after birth (7, 32). During pregnancy, the expression of Lewis antigens on erythrocytes is masked as a result of increased binding by plasma lipoproteins (18). Modification of immunity due to pregnancy and possible coating of the plasma lipoproteins to *H. pylori* cells may also mask Le^x on these cells at least for a time.

Our studies suggest explanations for a number of phenomena associated with *H. pylori* infection and suggest that an ability to mimic a human oncofetal antigen not only may assist colonization and long-term infection but also may augment the development of ulcers and gastric carcinoma.

Chan et al. (6) have recently described purification of two enzyme activities (α1,3-fucosyltransferase and β1,4-galactosyltransferase) and calculated their K_m and V_{max} values. These two enzymes are the same as those used in mammals to produce the Le^x antigen. These activities were found in the *H. pylori* cells and were also shown to be secreted into the medium. These data confirm and extend the finding that we report in this paper.

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