Analysis of *Helicobacter pylori* isolates from Chile: occurrence of selective type 1 Lewis b antigen expression in lipopolysaccharide

Eleonora Altman,¹ Heriberto Fernández,² Vandana Chandan,¹ Blair A. Harrison,¹ Myra Wilson Schuster,² Laura Otth Rademacher² and Claudio Toledo³

Correspondence

Eleonora Altman

Eleonora.altman@nrc-cnrc.gc.ca

¹Institute for Biological Sciences, National Research Council of Canada, Ottawa, ON K1A 0R6, Canada

²Instituto de Microbiología Clínica, Edificio de Ciencias Biomédicas, Facultad de Medicina, Universidad Austral de Chile, PO Box 567, Valdivia, Chile

³Instituto de Medicina, Facultad de Medicina, Universidad Austral de Chile, PO Box 567, Valdivia, Chile

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Previous studies have shown that the LPS of *Helicobacter pylori* isolated from North American and European hosts predominantly expresses type 2 Lewis x (Le^x) and Le^y epitopes, whilst the LPS from Asian strains has the capacity to express type 1 Le^a and Le^b structures. The aim of this study was to evaluate the expression of Le antigens and the cytotoxin-associated antigen (CagA) by *H. pylori* isolates from Chile. A total of 38 isolates were screened. The expression of Le antigens and CagA was determined by whole-cell indirect ELISA, using commercially available monoclonal anti-Le and polyclonal anti-CagA antibodies. LPS profiles of *H. pylori* isolates were assessed by gel electrophoresis and Western blotting. Expression of Le^x and/or Le^y epitopes was confirmed in 32/38 isolates (84 %), whilst 9/38 isolates (24 %) expressed type 1 Le^b blood group determinants, in addition to type 2 Le^x and Le^y structures. Six strains (16 %) were non-typeable. The majority of *H. pylori* strains examined were CagA-positive (83.3 %).

INTRODUCTION

Helicobacter pylori is recognized as the most common bacterial pathogen associated with chronic gastritis and peptic ulcers in humans and with increased risk of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. It has been estimated to infect the gastric mucosa of more than 60 % of adults over the age of 60 in industrialized countries (Dunn *et al.*, 1997). Recent studies suggest that, in Latin America, *H. pylori* infection is most commonly acquired in childhood. The prevalence rates of *H. pylori* vary from 30 to 90 %, depending on the socioeconomic status of a given population, with most children being infected by 10 years of age (Coelho *et al.*, 2000).

H. pylori produces several putative colonization factors, including urease, adhesin, flagella, cytotoxin-associated antigen (CagA), VacA and LPS (Covacci *et al.*, 1999). *H. pylori* LPS from a number of strains from North American and European populations expresses type 2 Lewis x (Le^x) and Le^y blood group epitopes that mimic cell-surface

fucose polygalactosamine-containing structures present on human gastric and tumour cells (Monteiro, 2001) (Fig. 1). Previous studies have shown a strong correlation between the expression of Le^x and Le^y antigens and CagA by *H. pylori* isolates contributing to inflammation and persistence (Wirth *et al.*, 1996). More recently, the detection of type 1 Le^a and Le^b structures in the LPS of *H. pylori* strains obtained from Asian symptomatic hosts compared with LPS from Western strains was reported (Monteiro *et al.*, 2000) (Fig. 1). Studies on host gastric Le expression of Taiwanese *H. pylori*-infected patients have provided evidence for a role of gastric Le^b and Le^x determinants in the bacterial density of *H. pylori* in the stomach that could be correlated with clinicohistological outcome (Sheu *et al.*, 2003). In this study, we report on the distribution of Le antigens and CagA in Chilean clinical isolates of *H. pylori* and the occurrence of Le^b blood group antigen in the LPS of these strains.

METHODS

Patients. *H. pylori* was cultured from two biopsies each taken for culture from the antrum and corpus during endoscopy performed at the County Hospital and at a private clinic in Valdivia, Chile (by

Abbreviations: CagA, cytotoxin-associated antigen; HRP, horseradish peroxidase; Le, Lewis; WCE, whole-cell indirect ELISA.

Fig. 1. Structure of the O-chain and core oligosaccharide LPS regions of Western *H. pylori* strains. Reproduced from Monteiro *et al.* (2000) by permission of Oxford University Press. The O-chain is covalently linked to the core oligosaccharide through a side chain DD-Hep. Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; AEP, 2-aminoethylphosphate; P, phosphate.

C. T.). Written informed consent to participate in this study was obtained from all patients. The Ethics Committee of the Facultad de Medicina, Universidad Austral de Chile, approved this study. Of the 196 biopsy samples collected, 59 were identified as *H. pylori* (isolation frequency 30.1 %). The median age of patients was 51.4 years (range 17–87 years) with a male-to-female ratio of 0.51 (20 males and 39 females). Thirteen adult patients had gastritis, ten had gastric ulcers, six had oesophagitis, two had reflux and five had duodenal ulcers. Fourteen isolates were from patients without lesions and nine isolates were from patients with no registered symptoms. A total of 38 *H. pylori* isolates were chosen at random and examined in the present study.

Culture. Biopsy samples were homogenized and inoculated onto antibiotic-supplemented (Oxoid Dent Supplement) blood agar plates and incubated at 37 °C under microaerophilic conditions for 4–5 days. Characteristic colonies were tested for culture purity by a urease assay and Gram staining. Single colonies were propagated further, and frozen stocks were prepared using brain heart infusion broth containing 20 % glycerol. They were stored at –80 °C. *H. pylori* isolates were cultured at 37 °C on Columbia blood agar (Difco Laboratories) containing 7 % defibrinated horse blood in a microaerophilic environment for 48 h.

Whole-cell indirect ELISA (WCE). *H. pylori* strains were cultivated on plates as described previously (Logan *et al.*, 2000). Cells were harvested and washed with 5 ml 10 mM PBS (pH 7.4) per plate. Following centrifugation (5000 g, 5 min, 4 °C), pellets were suspended in 25 ml PBS to give a final concentration of 10^8 cells ml⁻¹. Microtitre plates (ICN) were coated with 100 µl bacterial suspension and incubated overnight at 4 °C. The bacteria were removed and the wells were fixed for 5 min with methanol and then dried for 15 min. The wells were blocked with 200 µl milk diluent/blocking solution (MDB; KPL) for 2 h at 37 °C. Subsequently, the wells were incubated for 2 h at 37 °C with 100 µl anti-Le^a, -Le^b, -Le^x or -Le^y monoclonal antibody (mAb) solution (Signet Laboratories) diluted 1:200 in MDB or rabbit anti-CagA antibody (Austral Biologicals) diluted 1:100 in MDB. Corresponding secondary antibody, either horseradish peroxidase (HRP)-conjugated anti-mouse IgG + IgM or HRP-conjugated anti-rabbit IgG (Cedarlane Laboratories) diluted 1:1000 in MDB, was added and the plates were incubated for 1 h at room temperature. Between incubation steps, the wells were washed four times with PBS. After a final washing step, the substrate 3,3',5,5'-tetramethylbenzidine (KPL) was added and the reaction was stopped with 1 M phosphoric acid. The A₄₅₀ was determined using a microtitre plate reader (Dynatech). Non-specific background values were determined as A₄₅₀ of the negative-control wells containing bacterial cells, secondary antibody conjugate and substrate. These A₄₅₀ values were ≤0.2. Values of A₄₅₀ <0.2 were classified as negative and

values of $A_{450} \geq 0.2$ were classified as positive reactions. To ensure plate-to-plate consistency, *H. pylori* strain 26695 cells were used as a positive control for Le^x, Le^y and CagA. Assays did not vary by more than 10 %.

Electrophoresis and Western blotting. SDS-PAGE was performed with a mini-slab apparatus (Bio-Rad) using the Laemmli method. LPS samples were prepared from whole cells according to a previously described method (Logan & Trust, 1984) and equivalent amounts were loaded in each lane and stained according to Tsai & Frasch (1982) or transferred to nitrocellulose for immunological detection with anti-Le^x and anti-Le^y mAbs as described previously (Logan *et al.*, 2000). To ensure consistency, *H. pylori* strain 26695 was used as a positive control for anti-Le^x and anti-Le^y mAbs. For detection of Le^b in bacterial whole-cell samples, nitrocellulose membranes were blocked with 1% skimmed milk in 10 mM PBS (pH 7.4) overnight at 4 °C. Subsequently, membranes were incubated with anti-Le^b mAb solution diluted 1:100 in 10 mM PBS (pH 7.4) overnight at 4 °C. Corresponding secondary antibody, HRP-conjugated anti-mouse IgG + IgM diluted 1:3000 in 10 mM PBS (pH 7.4), was added and the nitrocellulose membranes were incubated for 1 h at room temperature. The membranes were washed three times with 10 mM PBS (pH 7.4) between the incubation steps and developed using SuperSignal West Pico Chemiluminescent substrate (Pierce) following the manufacturer's instructions. The synthetic Le^b antigen Le^b-hexasaccharide-BSA (lacto-*N*-difucohexaose I-BSA or LNDFHI-BSA; V-Labs) was used as a positive control. Whole cells of the Le^y antigen-expressing *H. pylori* Sydney (SS1) strain were used as a negative control in Western blots to ensure specificity of anti-Le^b mAbs. In no case did we find any cross-reactivity with Le^y for the Le^b-specific mAbs under Western blot conditions.

RESULTS AND DISCUSSION

In the present study, we examined *H. pylori* isolates from Chile by WCE, gel electrophoresis and Western blotting. Similar approaches have been applied previously to the analysis of *H. pylori* isolates from gastric biopsies (Rasko *et al.*, 2000) and typing of *H. pylori* isolates from different geographical regions (Simoons-Smit *et al.*, 1996). However, the distribution of Le antigens in *H. pylori* strains from Latin America has not been examined.

Screening of 38 Chilean isolates of *H. pylori* by WCE resulted in the detection of Le^x and/or Le^y epitopes in 32 strains. Co-expression of Le^x and Le^y antigens was detected in 14 strains. Three isolates expressed only Le^x antigen

(7.9 %), whilst exclusive Le^y expression was identified in six isolates (15.8 %). Le^b antigen was identified in nine isolates (23.7 %), either co-expressed with Le^x and Le^y (four isolates) or just with Le^y (five isolates) (Table 1). No isolate expressing Le^a antigen was identified. Expression of the CagA protein was detected in 83.3 % of isolates studied. Six isolates (15.8 %) contained no identifiable Le antigen and were non-typeable. A previous survey of *H. pylori* isolates from different geographical regions by Simoons-Smit *et al.* (1996) reported a similar distribution of

typeable (85 %) and non-typeable strains (15 %), with most of the non-typeable isolates originating from China.

We were unable to demonstrate a significant correlation between the expressed Le antigen type and the associated disease (Table 1). Previous studies by Wirth *et al.* (1996) suggested that Le expression appeared to be more common among *H. pylori* isolates from patients with ulcer disease. In the present study, 9/10 analysed isolates from patients with ulcer disease (five gastric ulcer isolates and five

Table 1. Expression of Le and CagA antigens in *H. pylori* isolates from Chile

Strain	Disease	WCE (A ₄₅₀)					O-chain*
		Le ^x	Le ^y	Le ^a	Le ^b	CagA	
002CL	Oesophagitis	—	0.915	—	—	—	+ (Le ^y)
004CL	Gastric ulcer	1.065	—	—	—	0.957	+ (Le ^x)
006CL	Duodenal ulcer	—	—	—	—	0.989	—
011CL	Dyspepsia	—	1.282	—	—	0.426	+ (Le ^y)
016CL	Gastric ulcer	—	0.280	—	0.83	1.248	+ (Le ^b)
017CL	Dyspepsia	0.366	0.836	—	—	1.674	+ (Le ^y)
018CL	Dyspepsia	—	—	—	—	—	+
023CL	Duodenal ulcer	—	0.787	—	—	—	+ (Le ^y)
026CL	Dyspepsia	0.378	1.303	—	0.556	0.491	+ (Le ^y , Le ^b)
027CL	Dyspepsia	—	—	—	—	—	—
029CL	Duodenal ulcer	—	1.140	—	—	—	+ (Le ^y)
047CL	Dyspepsia	0.376	0.759	—	—	1.603	+ (Le ^y)
048CL	Dyspepsia	—	1.135	—	0.592	—	+ (Le ^y , Le ^b)
049CL	Dyspepsia	—	1.305	—	0.453	0.465	+ (Le ^y)
058CL	Gastro-oesophageic reflux	1.308	0.798	—	—	1.320	+ (Le ^x , Le ^y)
060CL	Duodenal ulcer	0.290	0.993	—	—	1.590	+ (Le ^y)
066CL	Gastric ulcer and reflux	—	0.308	—	—	1.828	+ (Le ^y)
068CL	Oesophagitis	0.958	0.701	—	—	1.442	+ (Le ^x , Le ^y)
069CL	Gastritis	—	—	ND	—	ND	ND
076CL	Gastritis	1.724	0.343	—	—	1.770	+ (Le ^x)
077CL	Gastric ulcer	1.769	0.314	—	—	1.227	+ (Le ^x)
082CL	Gastritis	1.882	1.024	—	—	1.949	+ (Le ^x , Le ^y)
084CL	Oesophagitis	0.768	—	ND	—	ND	ND
085CL	Oesophagitis	1.299	—	—	—	0.345	+ (Le ^x)
094CL	Gastritis	0.249	0.454	—	—	0.636	+ (Le ^y)
098CL	Dyspepsia	1.363	0.377	—	—	1.514	+ (Le ^x)
099CL	Gastritis	1.768	0.747	—	0.688	1.790	+ (Le ^x , Le ^y , Le ^b)
101CL	Gastric ulcer	0.381	0.989	—	—	1.629	+ (Le ^y)
104CL	Gastritis	—	0.711	—	0.262	0.442	+
109CL	Antro-pyloric deformation	0.276	0.759	—	1.143	1.512	+ (Le ^y , Le ^b)
110CL	Dyspepsia	1.280	1.003	—	—	1.659	+ (Le ^x , Le ^y)
113CL	Dyspepsia	—	1.046	—	—	0.357	+ (Le ^y)
114CL	Dyspepsia	—	—	—	—	0.363	—
151CL	Dyspepsia, weight loss	0.587	1.131	—	—	1.504	+ (Le ^y)
153CL	Dyspepsia	—	0.896	—	1.243	1.575	+ (Le ^b)
160CL	Dyspepsia	—	—	—	—	0.361	+
217CL	Dyspepsia	1.360	0.530	—	—	1.586	+
240CL	Duodenal ulcer	0.256	0.374	—	0.527	0.436	+

ND, Not determined.

*SDS-PAGE analysis was performed on proteinase K-treated bacterial cells. The Le antigen content of the O-chain is shown in parentheses and is based on Western blot analysis (see Figs 2 and 3).

duodenal ulcer isolates) tested positive for at least one Le determinant. However, the expression of at least one Le determinant was also confirmed in 24/28 (85.7 %) surveyed isolates of non-ulcer origin (16 dyspepsia isolates, six gastritis isolates, four oesophagitis isolates, one antropyloric deformation isolate and one gastro-oesophagic reflux isolate). Interestingly, four of the nine Le^b-expressing isolates identified in this study originated from dyspepsia patients (Table 1). Four of six non-typeable strains also originated from dyspepsia patients. The prognostic significance of these findings remains to be established. Le^b expression was also found in two isolates from patients with ulcer disease (one duodenal ulcer and one gastric ulcer isolate) in combination with either type 2 Le^x and/or Le^y expression. Exclusive Le^x expression was confirmed in 2/4 isolates from patients with oesophagitis and one gastric ulcer isolate, whilst exclusive Le^y expression was established in six isolates (6/38, 15.8 %) from patients with diverse clinical symptoms and associated diseases (Table 1).

WCE findings were corroborated by SDS-PAGE and Western blot analyses with anti-Le^x, anti-Le^y and anti-Le^b mAbs. As expected, most typeable isolates exhibited typical high-molecular-mass ladder-like patterns indicative of smooth-form LPS (Table 1, Figs 2 and 3). Both WCE (Table 1) and silver staining revealed that strains 104CL (Fig. 2a, panel iii), 153CL (Fig. 2a, panel iv) and 217CL (Fig. 2a, panel iv) possessed an O-chain polysaccharide expressing Le^y and/or Le^x antigens, although their presence could not be detected by Western blotting with their respective anti-Le mAbs. The detection of a high-molecular-mass ladder-like pattern by silver staining in strains 018CL (Fig. 2a, panel i) and 160CL (Fig. 2a, panel iv), which tested negative for the presence of Le antigens by WCE and Western blotting, was indicative of the expression of an additional antigen that did not react with anti-Le mAbs (Table 1, Fig. 2).

Previous attempts to carry out Western blotting experiments with anti-Le^b mAbs on *H. pylori* whole-cell samples were unsuccessful, and it was assumed that these antibodies did not react under Western blot conditions (Monteiro *et al.*, 1998). However, Holgersson & Löfling (2006) have shown recently that the detection of Le^b antigens on glycoconjugates can be achieved with anti-Le^b mAbs in a Western blot format when a chemiluminescent substrate is utilized. By adopting a similar approach, we were able to demonstrate the recognition of Le^b epitope by anti-Le^b mAbs under Western blot conditions in 6/9 *H. pylori* isolates tested. Three Le^b-containing *H. pylori* isolates, 049CL, 104CL and 240CL, did not react in the Western blot format but were positive for the presence of Le^b antigen in WCE (Table 1). It is possible that in these three strains Le^b epitope is linked directly to the core region of LPS and is conformationally inaccessible. The silver-staining pattern of Le^b-containing *H. pylori* strain 240CL (Fig. 2a, panel iv) was consistent with the presence of a high-molecular-mass non-Le structure, as this strain had a low content of Le^y and Le^x antigens according to the WCE

results and tested negative for Le^y and Le^x antigens by Western blotting (Fig. 2b, panel iv, and Fig. 2c, panel iv). The presence of the O-chain in two other Le^b-containing strains, 049CL (Fig. 2a, panel ii) and 104CL (Fig. 2a, panel iii), was confirmed by silver staining although the signal was faint. Only one of these strains, 049CL, tested positive for the presence of Le^y antigen by Western blotting (Fig. 2c, panel ii). It is plausible that O-chain polysaccharide in the LPS of these strains is composed of non-fucosylated or partially fucosylated type 2 *N*-acetylglucosamine repeating units capped by Le^y and/or Le^x antigens (Fig. 1). These structures were previously found to be co-expressed with type 1 Le^b antigens in *H. pylori* LPS from Asian hosts (Monteiro *et al.*, 2000). Further structural studies are needed to confirm these findings.

Silver staining and Western blotting with anti-Le^b mAbs revealed significant variations in the O-chain length of Le^b-containing isolates (Fig. 3). In *H. pylori* strains 016CL, 099CL and 109CL, the Le^b epitope was associated with short O-chains, whilst in strains 048CL and 153CL, the Le^b structure was capping longer O-chains. One strain, 026CL, appeared to produce a very long O-chain capped by Le^b antigen (Fig. 3).

Previous studies have addressed the distribution of Le antigens in *H. pylori* isolates from different geographical regions, including North America, Europe and Asia. These investigations have identified type 2 Le^x and Le^y antigens as the most frequently encountered antigens in *H. pylori* LPS (Simoons-Smit *et al.*, 1996; Wirth *et al.*, 1996). Recently, expression of type 1 Le^a and Le^b antigens in LPS from Asian *H. pylori* isolates has been demonstrated, either solely or in combination with type 2 Le and/or *N*-acetylglucosamine epitopes (Monteiro *et al.*, 2000). The tendency for expression of type 1 Le blood group antigens in *H. pylori* LPS from Asian hosts compared with Western populations is not well understood but is thought to be related to the host Le phenotype (Wirth *et al.*, 1997).

We were able to demonstrate the presence of Le^b antigen in the LPS of *H. pylori* strains from Chile by two methods, WCE and Western blotting. The results of the Western blot analysis suggested further structural diversity among type 1 Le^b antigen-containing *H. pylori* strains associated with the presence of capping Le^b epitopes on either short or long O-chain LPSs. To our knowledge, this is the first instance in which this blood group epitope has been identified in *H. pylori* strains from Latin America. Previous studies of *H. pylori* isolates obtained from various geographical regions have shown type 1 Le^b antigen expression in 13 % of isolates (Wirth *et al.*, 1996). Of these, only three strains were from Latin America (Peru), and a correlation between geographical origin and observed Le antigen type was not reported.

Our study examined the correlation between CagA expression and Le status. *H. pylori* strains isolated from both symptomatic and asymptomatic patients were examined. In a previous study by Cover *et al.* (1995), the

Fig. 2. Silver-staining and Western blots of proteinase K-treated whole cells of *H. pylori*. (a) Silver-stained 12.5% SDS-polyacrylamide gels. *H. pylori* strain 26695 was used as a positive control; 002 denotes strain 002CL, etc. (b, c) Corresponding Western blots using anti-Le^x mAb at a 1:150 dilution (b) and anti-Le^y mAb at a 1:500 dilution (c).

proportion of CagA⁺ *H. pylori* isolates from duodenal ulcer patients was found to be 87.5% based on combined bacteriological and serological testing. Wirth *et al.* (1996) found that *cagA* was expressed by 97% of isolates, of which 75.7% were CagA⁺ as determined by WCE. In the present study, we observed a higher number of CagA⁺ strains. No correlation between the absence of CagA expression and Le antigen type was found.

It has been shown that, whilst *H. pylori* infection is common among the Chilean adult population, seroconversion occurs early in life and is related directly to socioeconomic status (Hopkins *et al.*, 1993). Increased seroprevalence of *H. pylori* in children of lower socioeconomic groups has been reported in other Latin American countries, such as Brazil (Oliveira *et al.*, 1994), Peru (Klein *et al.*, 1994), Colombia (Goodman *et al.*, 1996)

Fig. 3. Silver-stained 12.5% SDS-polyacrylamide gels (a) and Western blots (b) of proteinase K-treated whole cells of *H. pylori* Le^b-containing isolates. The Western blot used anti-Le^b mAb at a 1:100 dilution. Std, Le^b-hexasaccharide-BSA used as a positive control; SS1, *H. pylori* strain SS1 used as a negative control; 016 denotes 016CL, etc. (see Methods for details).

and Mexico (Castillo-Rojas *et al.*, 2004). Consumption of uncooked vegetables and shellfish has been found to correlate with *H. pylori* seropositivity, as have modes of vegetable washing and swimming near contaminated beaches (Hopkins *et al.*, 1993). Evaluation of the Le antigen distribution in *H. pylori* isolates from Chile could be applied to general epidemiological and population studies to assess the effectiveness of *H. pylori* prevention and eradication strategies.

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