Influence of Lewis Antigen Expression by *Helicobacter pylori* on Bacterial Internalization by Gastric Epithelial Cells

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The role of *Helicobacter pylori* lipopolysaccharide (LPS) Lewis antigens in infection is still not well known. We investigated the influence of Lewis antigen expression by *H. pylori* on its internalization by AGS cells and the epithelium of human gastric xenografts in nude mice using isogenic mutants in LPS biosynthetic genes. In vivo, colonization rates were unaffected by the change in *H. pylori* Lewis antigen expression, whereas the number of viable intracellular bacteria was significantly higher with wild-type *H. pylori* strains expressing Lewis antigens when compared to the isogenic mutants in both models. *H. pylori* strains expressing more Lewis X antigens (Le^x) were internalized at a higher rate than those expressing less Le^x, type II Lewis antigens (Le^a or Le^b) alone, or no Lewis antigens. Thus, Lewis antigens appear to be involved in the internalization of *H. pylori* by the gastric epithelium.

Helicobacter pylori is considered an extracellular pathogen but may reside inside gastric epithelial cells (2, 4, 11, 27). Adherence of *H. pylori* to epithelial cells may represent the first step in the process of colonization and endocytosis of this bacterium (3, 18, 24). *H. pylori* express Lewis antigens on their surface as part of their lipopolysaccharide (14). In Western populations most *H. pylori* strains express the type II glycoconjugate antigens, Lewis X (Le^x) and Le^y, whereas a small proportion express the type I glycoconjugates, Le^a and Le^b (15). Although no gastric Le^x-binding lectin has been identified, the detection of pedestal formation and the association of Le^x at this location suggests that Le^x-Le^x homotypic interaction between the eukaryotic cell Le^x and the *H. pylori* Le^x may be responsible for a Le^x-mediated adherence (26). It has been shown that the adherence of *H. pylori* strains expressing Le^x to cultured gastric epithelial cells was significantly inhibited by a monoclonal anti-Le^x (1, 17). Edwards et al. showed that, in contrast to a Le^x-positive parent strain, two strains that were unable to produce Le^x did not adhere to the epithelial cells of the gastric pits of human antral tissue sections (7). In patients with chronic gastritis, Heneghan et al. found a significant relationship between bacterial Le$^{x/y}$ expression and *H. pylori* colonization density (10). These data suggest that *H. pylori* Le^x antigens may play a role in colonization via a Lewis antigen-mediated adherence process. However, it has also been shown that *H. pylori* cells that do not express Lewis antigens but do express other complex carbohydrates may still colonize patients (23). Thus, the role of these antigens in colonization and internalization is unclear and requires further study. The goal

of the present work was to investigate the internalization of *H. pylori* in AGS cells and to determine if the internalization is affected by Lewis antigen expression. Additional studies of bacterial colonization and internalization were undertaken in vivo using a recently developed model of *H. pylori* infection using human gastric xenografts in nude mice (13) to complement the in vitro studies.

Strains used in this work and the corresponding genotypes and phenotypes are listed in Table 1. *H. pylori* strains UA948, 26695, and UA1111 express Le^a and Le^x, Le^x and Le^y, or Le^b and only trace amounts of Le^a, respectively (15, 21, 22). The difference in Lewis antigen expression in these wild-type strains reflects the interstrain variability of the fucosyltransferases (FucT) that are required in the synthesis of Lewis antigens (Fig. 1.). Isogenic mutants previously generated from the above-mentioned strains by insertional mutagenesis (chloramphenicol resistance gene cassette) of FucT genes were also studied: *H. pylori* UA948*fucTa$^-$* (no detectable Lewis antigen), *H. pylori* 26695*fucTa$^-$* (decreased amounts of both Le^x and Le^y), and *H. pylori* UA1111*fucT2$^-$* (Le^a) (21, 22; D. A. Rasko and D. E. Taylor, unpublished data). Strains were grown on Columbia agar supplemented with 10% horse blood or in brucella broth containing 5% fetal bovine serum under microaerobic conditions as previously described (13). For the mutants, the media were supplemented with chloramphenicol (20 µg/ml).

Internalization of *H. pylori* into cultured AGS cells (ATCC CRL 1739) was quantitated using a standard gentamicin internalization assay. AGS cells were grown in F-12 K medium (American Type Culture Collection, Manassas, Va.) containing 2 mM L-glutamine, sodium bicarbonate (1.5 g/liter), and 10% fetal bovine serum. Bacteria grown in broth were washed with F-12 K before inoculation. AGS cells (10^6 cells/well) were then incubated at 37°C with *H. pylori* suspensions in F-12 K for

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TABLE 1. *H. pylori* strains used in this study

Strain	Genotype	Lewis phenotype	Reference
UA948	Wild type	Le^a, Le^x	22
UA948*fucTa*$^{-a}$	α(1,3/4)*fucTa*::cat		22
26695	Wild type	Le^x, Le^y	15
26695*fucTa*$^{-b}$	α(1,4)*fucTa*::cat	(Le^x), (Le^y)	Rasko et al., unpublished
UA1111	Wild-type	Le^b, (Le^a)	21
UA1111*fucT2*$^{-c}$	*fucT2*::cat	Le^a	21

^{a} In this strain the inactivation of *fucTa* is responsible for the total loss of Lewis antigen expression as this gene encodes the only functional FucT enzyme which contains both α1,3 and α1,4 FucT activities.

^{b} In this strain the decrease of Le$^{x/y}$ expression is due to the fact that only one (α[1,3]*fucTa*) of the two genes encoding α(1,3)FucT is inactivated. Thus, the second enzyme (α[1,3]FucTb) is still functional but does not fucosylate the LPS to the same degree leading to a decrease in Le$^{x/y}$ expression.

^{c} In this strain the absence of Le^b production is due to the inactivation of *fucT2* that encodes the only functional α(1,2)FucT, involved in the conversion of Le^a to Le^b. Thus, a significant amount of Le^a is produced.

6 h at a multiplicity of infection of 100. Monolayers were washed three times, incubated for 1.5 h in the presence of gentamicin (50 μg/ml) to kill extracellular bacteria, and lysed using distilled water. The count of viable intracellular bacteria was determined by quantitative plating of serial dilutions of the lysates. The experiments performed in triplicate (10^6 cells/well) were repeated eight times for each strain, and the results were expressed as the mean percentage (± standard deviation) of the inoculum that was internalized in AGS cells.

Xenografts exhibiting human mature gastric epithelium and acidic secretion (pH range, 1.5 to 2) were obtained in nude mice as previously described (13). Prior to inoculation, one biopsy specimen was taken in each graft to determine the Lewis antigen status of the gastric mucosa. Immunostaining of

FIG. 1. Synthesis of Lewis antigens in *H. pylori* strains UA948, 26695, and UA1111 (15, 21, 22).

the biopsy samples was performed by a standard avidin-biotin-peroxidase technique using mouse monoclonal anti-Le^a, -Le^b, -Le^x, and -Le^y antibodies (Signet Pathology Systems Inc., Dedham, Mass.) (26). All of the biopsy specimens were positive for all four Lewis antigens with the exception of three grafts in which only Le^b, Le^x, and Le^y were detected. Bacterial inoculation was then performed as previously described (13). At 2, 4, and 8 weeks after inoculation, each graft was surgically opened, gastric juice was taken for pH determination, and mucus was sampled for qualitative culture onto blood agar. Three biopsy samples were then taken from adjacent sites in the antrum for quantitative culture and histology. At 8 weeks after inoculation, an additional biopsy sample was taken for electron microscopy. Biopsy specimens for culture were immediately placed in a semisolid agar transport medium (Portagerm pylori; bioMérieux) and weighed. One of these specimens was directly homogenized with an Ultra Turrax grinder (Labo-Moderne, Paris, France), and serial dilutions of the homogenate in sterile 0.9% NaCl were transferred onto blood agar plates. After 5 days of microaerobic incubation, bacterial counts were performed and expressed as CFU per gram of tissue. The second sample was used to determine the concentration of intracellular bacteria. This specimen was vigorously washed six times in broth, incubated for 2 h in the presence of gentamicin (100 μg/ml) to kill extracellular bacteria, washed six times, and then processed (29).

The expression of bacterial Lewis antigens was evaluated before inoculation and after 8 weeks of infection, testing isolates obtained from biopsy specimens treated with gentamicin (intracellular bacteria) and from mucus (extracellular bacteria). Lewis antigen expression was determined on bacteria grown in broth by an enzyme-linked immunosorbent assay previously described (26). For histological studies, specimens were processed by standard methods and stained with hematoxylin-eosin to assess the intensity of gastritis (13). For transmission electron microscopy, biopsy specimens were processed and examined as previously described (13).

Differences between means of internalization in AGS cells of wild-type strains and isogenic mutants were assessed by unpaired *t* test with Welch correction. Statistical analysis of the differences in colonization and internalization in gastric xenografts were performed using the Mann-Whitney test. A *P* of <0.05 was considered significant.

In vitro assays demonstrated that all six tested strains were able to penetrate in AGS cells. The level of internalization of *H. pylori* 26695 was comparable to that of *H. pylori* UA948 but significantly higher than that of each of the corresponding isogenic mutants (Fig. 2). Penetration rates of *H. pylori* UA948 and *H. pylori* 26695*fucTa*$^-$ were both significantly higher than those of *H. pylori* UA948*fucTa*$^-$, *H. pylori* UA1111, and *H. pylori* UA1111*fucT2*$^-$, for which no significant difference in intracellular uptake was observed.

All strains colonized all inoculated grafts for the 2-month period of the study. At any time period tested, culture of mucus and mucosa samples were positive for *H. pylori* only. The pH of the gastric juice measured at each time was increased to the range of 5 to 6.5. The levels of colonization determined by quantitative culture varied between 10^6 and 1.6 × 10^8 CFU/g of tissue. At any time, mutants colonized at levels comparable to those of wild type. In the three grafts with the
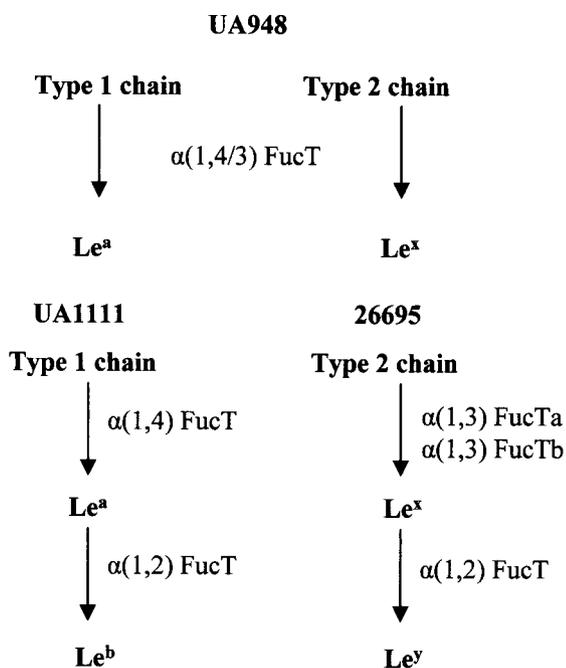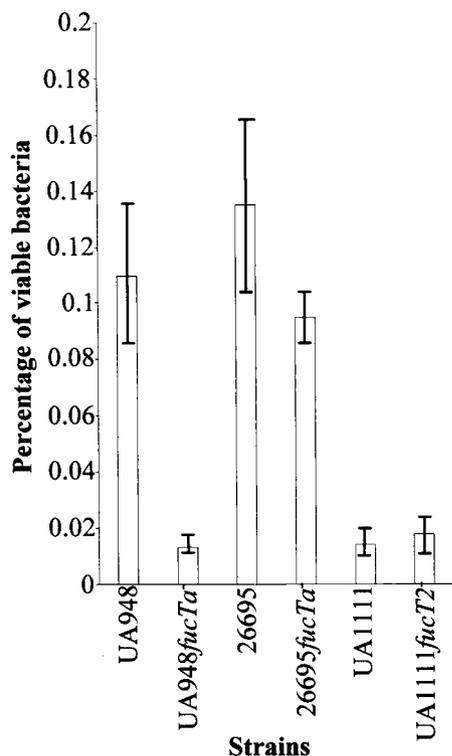
FIG. 2. Influence of Lewis antigen expression by *H. pylori* on its internalization by AGS cells. Viable bacterial uptake of wild-type strains UA948, 26695, and UA1111 was compared to that of isogenic mutants UA948*fucTa*⁻, 26695*fucTa*⁻, and UA1111*fucT2*⁻ using a gentamicin assay. Results are expressed as the percentage of the inoculum that was internalized (mean ± standard deviation [error bars]).

Le^b Le^x Le^y expression patterns, the colonization rates observed after inoculation of *H. pylori* strains UA948 ($n = 1$), UA1111 ($n = 1$), or UA1111*fucT2*⁻ ($n = 1$) were similar to those observed in the other grafts (Le^a Le^b Le^x Le^y) infected with the same strains at all time points. Intracellular *H. pylori* were detected in all infected xenografts at all times after challenge.

At all time points, *H. pylori* 26695 was significantly more internalized than all the other strains, including *H. pylori* 26695*fucTa*⁻ (Table 2). The internalization rate of *H. pylori* UA948 was higher than that of *H. pylori* UA948*fucTa*⁻ and

TABLE 2. Invasion of antral mucosa of human gastric xenografts after inoculation of *H. pylori* UA 948, UA 948*fucTa*⁻; UA 1111, UA 1111*fucT2*⁻; 26695 and 26695*fucTa*⁻

H. pylori strain (no. of xenografts)	Ratio of the no. of gentamicin-resistant CFU/g of tissue to the total number of CFU/g of tissue × 100[a] at:		
	2 wk	4 wk	8 wk
UA1111 (6)	0.50 (0.04–2.00)	0.10 (0.04–0.14)	0.50 (0.08–2.00)
UA1111*fucT2*⁻ (6)	0.40 (0.03–1.20)	0.10 (0.07–0.16)	0.45 (0.05–2.00)
UA948 (5)	0.48 (0.20–0.70)	0.70 (0.30–1.00)	0.66 (0.15–1.50)
UA948*fucTa*⁻ (5)	0.10 (0.04–0.50)	0.06 (0.03–0.09)	0.05 (0.01–0.10)
26695 (6)	3.14 (1.70–4.50)	1.30 (0.40–2.00)	1.90 (1.00–2.70)
26695*fucTa*⁻ (6)	0.20 (0.01–0.80)	0.09 (0.01–0.25)	0.21 (0.08–0.40)

[a] Values are shown as mean (range).

higher than those of all strains except *H. pylori* 26695 at 4 weeks. No difference in internalization was seen between the other strains tested. It is interesting that *H. pylori* UA948*fucTa*⁻ was found significantly less internalized than all the other strains at 8 weeks and does not express any of the complete Lewis antigens.

To determine whether the expression of Lewis antigens of *H. pylori* strains had changed during in vivo experiments, Lewis antigen typing was performed on each isolate obtained, after 8 weeks of infection, from the mucus and the mucosa (after incubation with gentamicin and cell lysis). For all strains tested and for all xenografts, no detectable change in the expression of Lewis antigens by *H. pylori* between inoculation and 2 months of infection was observed.

In inoculated grafts, erythematous areas were visible at the surface of the antrum at 2 weeks. These lesions were widespread from week 4 onwards. Histological examination of antral biopsy samples showed at any time a mild inflammation associated from week 4 with mild activity. These macroscopic and microscopic features were the same for all *H. pylori*-infected grafts. Electron microscopy studies confirmed the presence of both extracellular and intracellular bacteria in all biopsy specimens studied (Fig. 3). In all cases, numerous extracellular bacteria were seen within the mucous layer and often in close proximity to the surface of gastric epithelial cells. For each strain tested, bacteria were observed adhering to epithelial cells either through fibril-like strands or more intimate contact often associated with pedestal formations (Fig. 3A). In some cases, the outer membrane layer of the bacteria was apparently fused with the apical cell membrane associated with a nearly complete engulfment of the microorganism (Fig. 3B).

In this study, we have demonstrated for the first time that *H. pylori* internalization in human gastric epithelial cells may be influenced by Lewis antigen expression. *H. pylori* strains expressing Le^x were internalized to greater levels both in vitro and in vivo than isogenic mutants expressing this antigen at a lower level or not all. This suggests that Le^x may be involved in *H. pylori* internalization. However, the role of other carbohydrate antigens cannot be ruled out and needs to be further investigated by testing strains with various Lewis antigenic phenotypes. All *H. pylori* strains tested to date were able to invade human epithelial cells in vitro (8, 19, 28). This is in concordance with our in vitro and in vivo findings, and may be related to the fact that most *H. pylori* strains tested to date express Le^{x/y}.

It has been shown that the expression of Le^{x/y} by *H. pylori* is higher at pH 7 than at pH 5 (16). In humans, the antral juxtamucosal pH varies between 5 and almost 7 (9, 20). This may induce optimal expression of Le^x for adherence and subsequent internalization. Moreover, in xenografts, colonization was always related, as previously shown (13), to an increase of the gastric juice pH. This might also enhance the expression of Le^x and explain why no Le antigens phase variation was observed in isolates obtained from the mucus.

We could not detect any significant influence of the bacterial Lewis status on colonization. These results are similar to those recently obtained with mice by Takata et al. (25) with *H. pylori* 26695 and isogenic mutants expressing variable amounts of Le^{x/y}. Heneghan et al. (10) demonstrated that bacterial Le^x
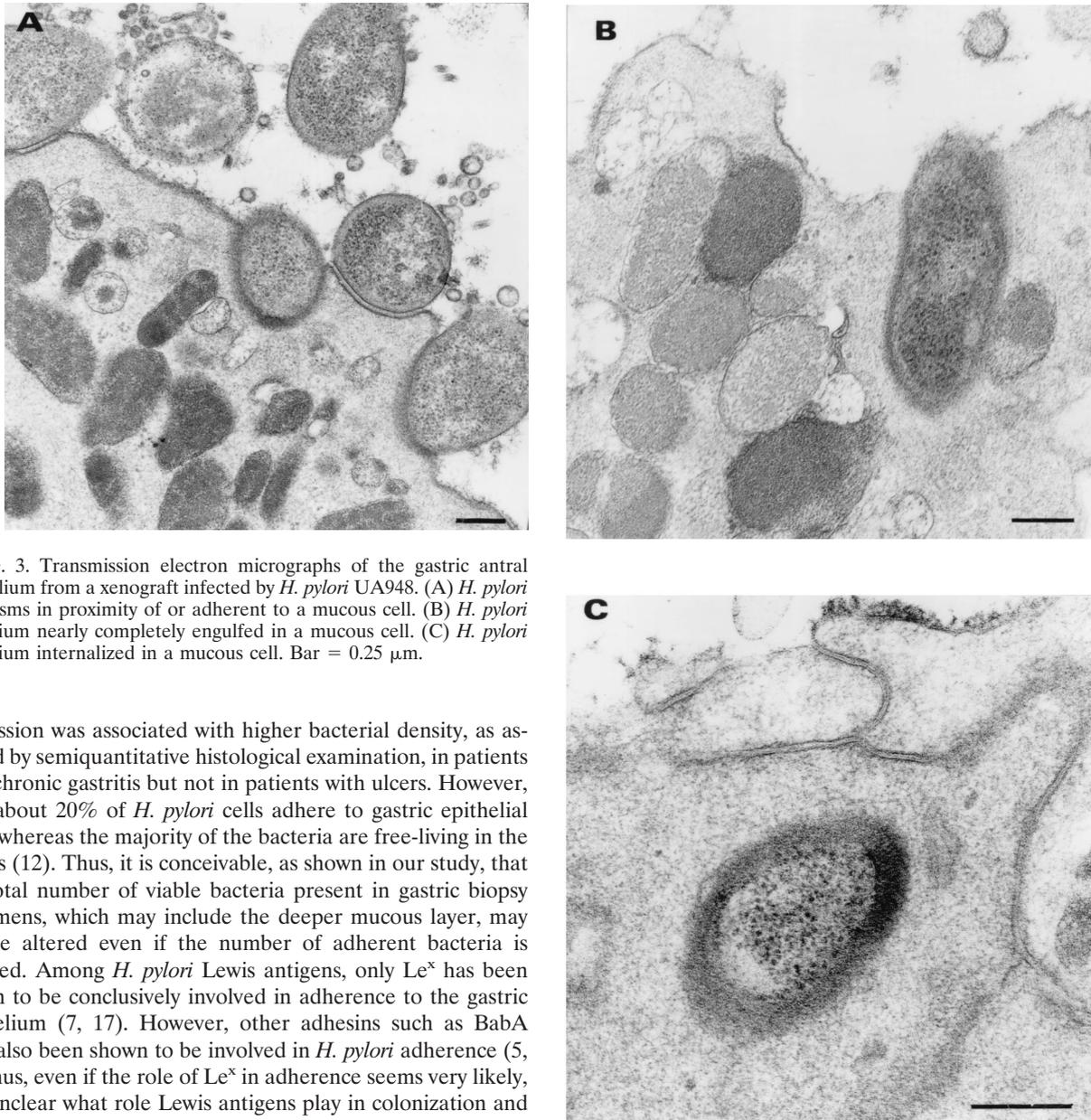
FIG. 3. Transmission electron micrographs of the gastric antral epithelium from a xenograft infected by *H. pylori* UA948. (A) *H. pylori* organisms in proximity of or adherent to a mucous cell. (B) *H. pylori* bacterium nearly completely engulfed in a mucous cell. (C) *H. pylori* bacterium internalized in a mucous cell. Bar = 0.25 µm.

expression was associated with higher bacterial density, as assessed by semiquantitative histological examination, in patients with chronic gastritis but not in patients with ulcers. However, only about 20% of *H. pylori* cells adhere to gastric epithelial cells, whereas the majority of the bacteria are free-living in the mucus (12). Thus, it is conceivable, as shown in our study, that the total number of viable bacteria present in gastric biopsy specimens, which may include the deeper mucous layer, may not be altered even if the number of adherent bacteria is reduced. Among *H. pylori* Lewis antigens, only Le^x has been shown to be conclusively involved in adherence to the gastric epithelium (7, 17). However, other adhesins such as BabA have also been shown to be involved in *H. pylori* adherence (5, 6). Thus, even if the role of Le^x in adherence seems very likely, it is unclear what role Lewis antigens play in colonization and internalization. Strains that do not express other adhesins or strains colonizing hosts that do not express the corresponding receptors may utilize Le^x antigens for their adherence. It appears as though *H. pylori* may require a number of different adhesins for colonization depending on the host bacteria interaction (1). We have demonstrated that Lewis antigens are involved with internalization of the bacteria. It is also conceivable that Le^x may increase the frequency or affinity of intimate contact between *H. pylori* and gastric epithelial cells without increasing the bacterial density of adherent bacteria or the colonization density. Moreover, more intimate contact may increase host cell responses and promote the subsequent *H. pylori* internalization. However, in the present study, transmission electron microscopy analyses showed that intimate contacts occurred in vivo between *H. pylori* and gastric cells with all strains tested, including *H. pylori* that did not internalize to a high degree (*H. pylori* 26695*fucTa*$^-$ and *H. pylori* UA948 *fucT2*$^-$). Further quantitative ultrastructural studies are nec-

essary to elucidate whether this phenomenon is less frequent with strains expressing Le^x than with isogenic mutants that do not express this antigen.

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Editor: V. J. DiRita