Colonization and Immune Responses in Mice to *Helicobacter pylori* Expressing Different Lewis Antigens

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ABSTRACT: BACKGROUND AND AIMS: A mouse model was established to compare colonization by the *H. pylori* Sydney strain SS1 with several clinical isolates expressing different Lewis antigens on their surface. In addition, both humoral and cell mediated immune responses were determined for different *H. pylori* strains. **METHODS:** Mice were inoculated intragastrically separately with the Sydney strain as well as with five clinical isolates of *H. pylori* expressing different Lewis (Le) antigen phenotypes. Colonization of the mouse stomach by the bacteria was monitored from two to fourteen weeks post inoculation by four independent methods namely, urease, PCR (using *Cag*A primers), bacterial culture and histology. Antibody titers and cellular immune responses were monitored by ELISA and antigen stimulation test respectively. **RESULTS:** Different degrees of colonization were observed in C57, CD1 and Balb/c mice inoculated with *H. pylori* strain SS1 (Le^x, Le^y) and clinical isolates UA948 (Le^a,Le^x), UA861 (α-glucosyl polyLacNAc), UA1258 (Le^y), UA802 (Le^y) and UA1264 (no Le antigen) starting from week two post inoculation. All three mice strains mounted high immune responses against different *H. pylori* antigens. Treatment of mice with vancomycin prior to inoculation has no effect either on colonization of the stomach or the immune response of the mice. Histological evaluation established colonization after 10 weeks post inoculation but not gastritis. **CONCLUSIONS:** Stomach of mice can be colonized consistently, with *H. pylori* strain SS1, and colonization was also achieved with all clinical isolates that were not mouse adapted. These strains could be detected more consistently by PCR in the early stages, then by culture only after 8 – 10 weeks. In our study, $Lewis^x$ expressing bacterial strain (UA948) failed to colonize Balb/c mice, whereas the Le^y expressing strain (UA1258) did not colonize C57/BL6 mice.

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Abbreviations: BHI- Brain Heart Infusion, BSA- Bovine Serum Albumin, CFU- Colony Forming Unit, ELISA-Enzyme-Linked Immunosorbent Assay, FBS- Fetal Bovine Serum, HRPO- Horseradish Peroxidase, PCR- Polymerase Chain Reaction, PBST- Phosphate Buffer Saline with 0.1% Twen-20, TMB- 3, 3', 5, 5' - Tetramethylbenzidine.

INTRODUCTION

The role of *H. pylori* in the development of peptic ulcers has become increasingly clear since it was first reported in 1983(1). It is now recognized as the cause of chronic gastritis in humans, one of the major casual factor in peptic ulcer disease and is associated with an increased risk of gastric adenocarcinoma or gastric lymphoma(2-4). *H. pylori* is a spiral or curved Gram-negative microaerophilic bacterium which colonizes the human gastric mucosa. The organism is found in the mucus layer and adheres to the surface epithelium of the stomach(4).

To study and develop novel therapeutic and/or preventive agents, animal models which are inexpensive, easily reproducible have been developed including the use of gnotobiotic piglets, dogs, monkeys, cats, mice, and rats(6-17). Problems associated with these models such as low infection rates, establishing germ-free environment coupled with difficulty of handling animals in large numbers hinders the optimization and standardization of the models(7). Work of Lee et al.(16) provided a mouse model of *H. pylori* infection and introduced the Sydney strain (SS1), a mouse adapted strain. The standardized Sydney strain gives consistent colonization, although the pathology following *H. pylori* infection does not precisely mimic human gastritis.

H. pylori express Lewis antigens on their surface as part of their lipopolysaccharide(18). We used immunoelectron microscopy to demonstrate the presence of Lewis X antigen (Galβ→4[Fucα1→3] GlcNAc) [Le^x] on the *H. pylori* surface(19). Lewis Y [Le^y] and other Lewis structures, Le^a and Le^b, have also been identified(20). Since gastric epithelial tissue also expresses Le^x, Le^y, Le^a and Le^b on its surface, *H. pylori* mimics these human antigens(21). It has been suggested that this molecular mimicry may relate to efficient colonization of gastric epithelial cells by *H. pylori* (22,23). The aim of the present study was to compare the ability of *H. pylori*, expressing different Lewis antigens, to colonize a mouse model. The humoral and cell mediated immune responses to *H. pylori* expressing different Lewis antigens were also assessed. All studies were performed during early phases of colonization.

MATERIALS AND METHODS

Bacterial Growth

A frozen *H. pylori* strain SS1 was initially obtained from A. Lee, University of New South Wales, Sydney, Australia and strains UA861, UA802, UA948, UA1258, UA955, UA1182 and UA1264 were from the *H. pylori* collection of clinical isolates maintained at the University of Alberta, Edmonton, Alberta, Canada (Table 1).

Table 1: Helicobacter pylori strains used in this study.

Strain	Lewis status	Reference for Lewis Antigen expression *
SS1	Le^x, Le^y	Monteiro et al., 2000(20)
UA802	Le^y	Wang et al., 1999(36)
UA861	$[LacNAc]_n$ | α-Glu	Monteiro et al, 1998(37)
UA948	Le^a, Le^x	Monteiro et al., 1998(38)
UA955	Le^x, Le^y, Le^b precursor	Monteiro et al., 1998(38)
UA1182	Le^x, Le^y	Monteiro and Rasko, unpublished
UA1258	Le^y	Monteiro and Rasko, unpublished
UA1264	No Le antigen	Monteiro and Rasko, unpublished

**Determined chemically as described in the cited reference.*

Strains were plated onto Brain Heart Infusion (BHI) agar plates supplemented with 5% defibrinated sheep blood and were incubated for 2 days as described previously(24). The colonies were removed from the plates, suspended in Brucella broth containing 5% Fetal Bovine Serum (FBS), 15µg/ml vancomycin and 15µg/ml ampicillin and incubated microaerobically (5% carbon dioxide, 10% hydrogen and 85% nitrogen) overnight at 37^0C with agitation on a rotary shaker at 100 rpm. The cultures were checked by phase contrast microscopy to ensure their purity, and were centrifuged at 10,000 rpm for 20 minutes and suspended in Brucella broth containing 30% glycerol to an OD660 of 1.

Inoculation of Mice

The C57/BL/6NCr1BR male mice, age 6 to 8 weeks, were obtained from Jackson Laboratory (Bar Harbor, Maine). The CD1 male mice, age 6 to 8 weeks, were obtained from Charles River Canada (St. Constant, Quebec) and the Balb/c male mice, age 6 to 8 weeks, were obtained locally from Health Sciences Laboratory Animals Services (HSLAS) of the University of Alberta, Edmonton. Animal treatment and care were carried out according to the guidelines of the HSLAS of the University of Alberta. All mice were fasted overnight before inoculation and prior to euthanasia. Mice were then given 10^8 colony-forming units (cfus) of *H. pylori* in 100µl of Brucella broth containing 30% glycerol through gastric gavage using a 22G gastric gavage needle. The same treatment was repeated after 3 and 5 days. Mouse stomach was removed and opened through the lesser curvature using sterile surgical instruments and cut into small pieces. Colonization of a given mouse was assessed from two to fourteen weeks post inoculation by urease test, bacterial culture, PCR and histology of stomach tissue. To study the effect of vancomycin on the colonization of *H.pylori* mouse stomach, C57 mice were treated with vancomycin solution in water at a concentration of 15µg/ml for 10 days. The mice were then orally inoculated with *H. pylori* SS1 strain. Similarly, untreated mice were also inoculated with the same strain.

Polymerase chain reaction (PCR)

DNA was isolated from stomach tissues by using QIAamp Tissue Kit (QIAGEN INC. Santa Clara, CA) following the manufacturer's protocol. For the PCR analysis, *H. pylori Cag*A primers (5'AGTAAGGAGAAACAATCA 3' and 5'AATAAGCCTTAGAGTCTTTTTGGAAATC 3') were used (25). PCR was performed at 94°C for 50 s, at 50°C for 45 s, and at 72°C for 1 min. in a Perkin-Elmer model 480 thermocycler. Each PCR product was subjected to electrophoresis on an agarose gel. Positive samples contained a 1.37kb band that was visualized under UV light.

Bacterial culture

Stomach samples from the inoculated mice were homogenized with PBS and a portion of the homogenates were placed on agar plates and incubated at 37°C under microaerobic conditions. The presence of *H. pylori* was checked after 3 to 5 days of incubation. The culture was identified as *H. pylori* based on morphology and production of urease(24).

Humoral immune response

Blood was collected from each mouse before sacrificing, and the antibody titer was measured by direct Enzyme-Linked Immunosorbent Assay (ELISA). Briefly, a 96 well microtiter plate (Nunc, Denmark) was coated with 1 µg/ml mixture of different strains of *H. pylori* whole bacterial antigens (see Table 1) and incubated overnight at 4°C. The plate was washed and incubated with 3% milk in PBS for 1 hr at room temperature to block nonspecific binding. The plate was washed three times with PBS containing 0.1% Tween-20 (PBST) and incubated with 100µl of the serum samples at a dilution of 1:100 for 1 hr at room temperature. After washing, 100µl of a 1:1000 dilution of goat anti-mouse IgG (whole molecule) HRPO conjugate or 1:16000 dilution of goat anti-mouse IgM (µ chain specific) peroxidase conjugate or 1:20000 dilution of goat anti-mouse IgA (α chain specific) peroxidase conjugate (Sigma, St. Louis, Mo.) were separately added and incubated for another 1 hr at room temperature. Following incubation, the plate was washed three times with PBS containing 0.1% Tween-20 (PBST) and 100µl of TMB peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD.) was added as a chromogen. Absorbance values at 405 nm were measured in a Vmax kinetic microplate reader (Molecular Device Corp., California, USA).

Cell mediated immune response

Spleens were removed from mice inoculated with *H. pylori* strain UA948, UA1258 and UA1264 following euthanasia and single cell suspension was prepared by grinding the tissue with a plunger on a cell strainer in the presence of 10 ml RPMI 1640 media (Gibco-BRL, Burlington, Ontario, Canada).The single cell suspension was centrifuged at ~500xg for 7 min and the cell pellet was resuspended in 5 ml of ACK lysing buffer (8.29 g NH_4Cl, 1 g $KHCO_3$ and 37.2 mg Na_2EDTA in 1 L of water adjusted to pH 7.4) to lyse erythrocytes and then incubated for 5 min at room temperature and washed three times with RPMI 1640 media. The cells were plated in 96-well microtiter plates (Costar, cambridge, MA) in triplicate at a concentration of 10^5 cells/well in RPMI 1640 media containing 10% FBS (Gibco-BRL) with or without antigen or mitogen. The cells were incubated for 5 days at 37 ^{0}C and 5% CO_2 and pulsed with 1µCi [^{3}H] thymidine (Amersham, Arlington Heights, IL) per well for 18 hrs of incubation. Cells were then harvested on to a Printed Filtermate A (glass fiber filter), size 90 X 120 nm (Wallac Oy, Turku, Finland) using a Harvester (Tomtec, Hamden, CT.). A Melt-on Scintillator Sheet size 73 X 109 nm (Wallac Oy, Turku, Finland) was melted on the filtermate, allowed to cool and sealed into a plastic cover. Thymidine incorporation was measured using a Microbeta ® Trilux Reader (Wallac, Turku, Finland). The stimulation index was calculated by dividing the CPM counts obtained from the antigen stimulated cells by the CPM counts obtained from the unstimulated cells.

Histology

At 10 week post inoculation, one-half stomach sample of a CD1 mouse and a control mouse stomach were fixed in 10% formalin and embedded in paraffin. Subsequently the samples were processed by standard histochemical techniques and hematoxylin, Eosin, Giemsa and Warthin Starry stained slides were prepared. The stained samples were examined for the presence or absence of *H. pylori* and/or other bacteria under a microscope(24).

RESULTS AND DISCUSSION

Colonization of mice by Strain SS1

In this study the ability of *H. pylori* to colonize C57 and CD1 mouse stomachs at a bacterial inoculum of 10^8 cfu was demonstrated. Colonization of the gastric mucosa was investigated by all of the three methods described above, i.e. urease test, PCR and bacterial culture and was subsequently confirmed by histology. Fasting the mice overnight prior to inoculation appear to facilitate colonization and allowed us to eliminate gastric food contents as a factor in this study. During fasting the acid secretion of the stomach is low. Since the bacteria are sensitive to acid in humans they presumably colonize the stomach during a time period when the stomach is hypochlorhydric which can occur during infection or poor nutrition(26). We found that the urease test is the most rapid detection method, however the sensitivity is low. Consistently positive urease test results were obtained after 4 weeks of inoculation in both C57 and CD1 mice (Table 2). Even though the colonization of the stomach by *H. pylori* was demonstrated at earlier stages by both the urease test and by PCR (Table 2 and Fig 1), the bacteria were not able to be cultured. Positive bacterial cultures were obtained only after 4 and 6 weeks of inoculation for CD1 and C57 mice respectively.

In case of Balb/c mice consistent colonization was not observed by any of the three methods (data not shown). A fourteen week colonization study of C57 mice by *H. pylori* SS1 strain was also performed. Ten mice were sacrificed at week 6, 10 and 14 and colonization was assessed as described above. In our experiments, colonization tended to decline after 10 weeks post inoculation (Fig 2).

Table 2: Time course of colonization of C57 and CD1 mice fed with *H. pylori* SS1 and other *H. pylori* clinical isolates.

H. pylori Strain	Mouse Strain	Number of weeks post inoculation														
		Urease test					PCR					Bacterial culture				
		2	4	6	8	10	2	4	6	8	10	2	4	6	8	10
SS1	C57	-	+	+	+	ND	-	+	+	+	ND	-	-	+	+	ND
SS1	CD1	+	+	+	+	ND	+	+	+	+	ND	-	+	+	+	ND
UA861	C57	-	+	-	+	+	+	-	+	+	+	-	-	-	+	+
UA802	C57	-	+	-	+	+	+	-	-	+	+	-	-	-	-	+
UA948	C57	-	+	-	+	+	+	-	+	+	+	-	-	-	-	+
ND=Not done																

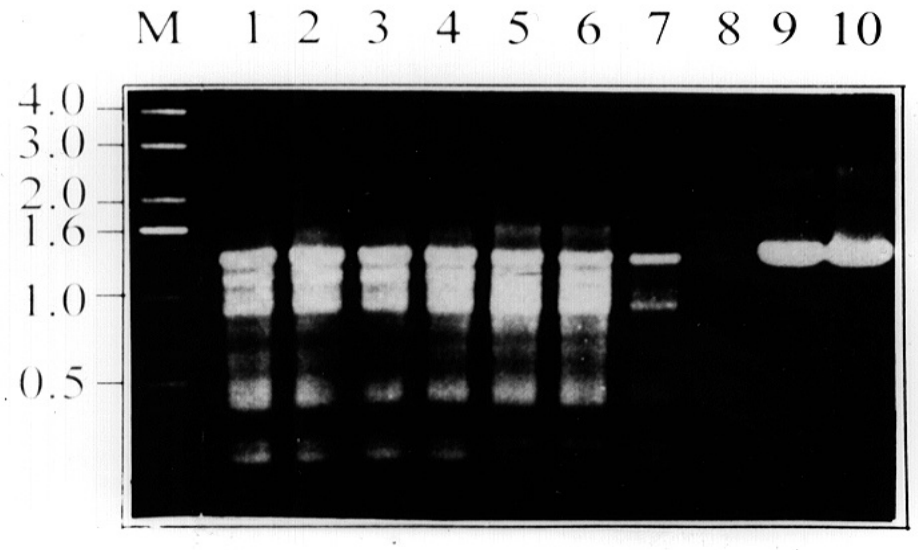

Figure 1: Agarose gel electrophoresis of PCR products from the *cagA* gene(25): Lane 1, 2, 3 and 4 show DNA from C57 mouse stomach at week 8 post inoculation; Lane 5, 6, 7, and 8 show DNA from CD1 mouse stomach at week 4 post inoculation and Lane 9 and 10 show PCR from DNA of *H. pylori* SS1 strain not used to infect mice.

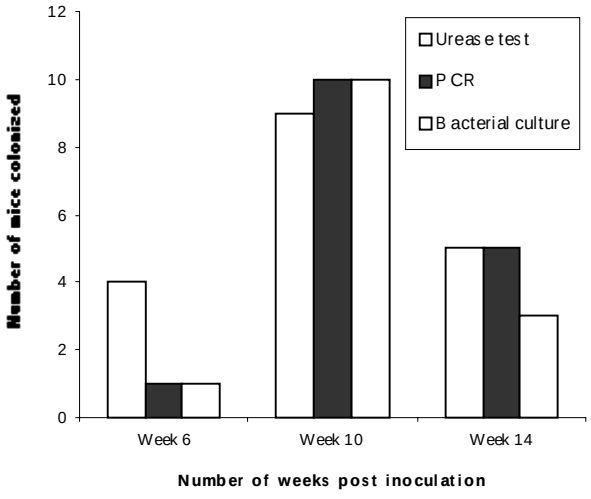

Figure 2: Colonization of C57 mice inoculated with SS1 strain.

Effect of vancomycin treatment

In our early mouse colonization experiments, we observed *Lactobacillus*-like rod shaped organisms along with *H. pylori* in histology. At least one report suggests that *Lactobacillus* can inhibit or interfere with *H. pylori* infection(27). Hence, we investigated if vancomycin could enhance colonization of the stomach by *H. pylori* since *Lactobacilli* were susceptable to this antibiotic. This would also allow us to study the colonization of one species without interference from the other. Vancomycin treatment of mice before inoculation showed no significant effect on colonization ability (data not shown). The pattern of colonization, as shown by the urease test, PCR and bacterial culture was similar in both the treated and untreated groups of mice. In both cases, 80% to 100% of the mice were colonized by the bacteria irrespective of the antibiotic treatment.

Colonization of mice by H. pylori clinical isolates

As shown previously by Lee and co-workers, we have observed consistent colonization of C57 mice by *Helicobacter pylori* SS1 strain(16). We further investigated to test if other clinical isolates expressing a variety of Lewis antigens can also colonize these mice. We inoculated them with UA948, UA802 and UA861 expressing known surface carbohydrate antigens, which had undergone many passages *in vitro*. All three strains showed positive urease test results after week 4 post inoculation. Variable PCR positivity was observed 2 weeks after inoculation. Positive bacterial cultures were, however, observed after week 8 (Table 2) consistent with our previous observations with SS1 strain. These results indicate that the mouse stomach can be successfully colonized by several *H. pylori* strains, with different Lewis blood group status. These isolates are not selected for colonization of the mouse stomach as SS1 has been. In another study the colonization of C57 mice inoculated with clinical isolates of *H. pylori* was compared with that of Balb/c mice at week 8 post inoculation (Table 3).

The PCR result showed that Balb/c mice were not colonized by *H. pylori* clinical isolate UA948 (Le^a, Le^x) whereas 80% colonization was observed in C57 mice. When these mice were inoculated with *H. pylori* UA1258 expressing Le^y surface antigen 80% colonization was observed with Balb/c mice but none in C57 mice. Inoculation of *H. pylori* UA1264, which does not express either of the complete Le^x or Le^y antigens, revealed 100% and 80% colonization of C57 and Balb/c mice respectively. In contrast, SS1 expresses Le^x and Le^y and colonizes both strains of mice(16). This interesting and complex colonization pattern of the Le^x, Le^y, and Le^0 expressing *H. pylori* in the various strains of mice cannot be easily rationalized. One previous report(28) documents that human oncofetal Lewis antigen (also known as stage-specific embryonic antigen [SSEA]) is highly immunogenic in Balb/c. Our experiments which showed no colonization of $Lewis^x$ expressing *H. pylori* suggesting that immune responses could partially explain the lack of colonization. An alternative explanation is that bacterial colonization bearing the SSEA could be dependent on the homotypic Le^x-Le^x or glycan-glycan interactions based on several observations(29). For example Le^x glycosphingolipid liposomes adhered to plastic plates coated with Le^x but not with other related carbohydrates. It is possible that the colonization pattern of *H. pylori* exhibiting molecular mimicry of various cell surface mammalian carbohydrate antigens is a function of the comparable glycan structures on gastric mucin and mucosal surfaces facilitating adhesion(30,31). However, it must be remembered that it is likely that UA1264 may produce precursor molecules, although neither the complete Le^x nor Le^y structures are present(32). In addition SS1 has been selected for colonization in the mouse model and other receptors may be of primary importance in this strain of *H. pylori*.

Table 3: Comparison of the colonization of C57 and Balb/c mice with different *H. pylori* clinical isolates at 8 weeks post inoculation evaluated by PCR.

Bacterial strain	Lewis States	C57 Mice	Balb/c Mice
UA948	Le^a, Le^x	4/5	0/5
UA 1258	Le^y	0/5	4/5
UA 1264	No Lewis* antigen	5/5	4/5

**No complete Lewis antigen synthesized, although precursor molecules may be produced.*

Humoral immune response

All three strains of mice showed significant immune responses to whole *H. pylori* antigens in sera two weeks after inoculation with strain SS1 (Figure 3). This immune response detected in terms of IgG levels increased with time. The IgM levels, on the other hand, were detectable in week 2 and remained at the same level. The control mice (week 0), however, showed significantly low antibody levels against *H. pylori* whole cell antigens both in terms of either IgM or IgG titers. The IgA antibody level of these mice was very low and nearly undetectable throughout the study period (data not shown) indicating that oral inoculation did not elicit serum IgA response in keeping with previous observations(33). There was no significant difference observed in the immune response between the C57 mice treated with vancomycin and those which were not. In both cases the response was shown to decrease after ten weeks (data not shown). Comparable immune responses in terms of IgG were observed when C57 mice were inoculated with strains UA861 (α-glucosyl polyLacNAc), UA802 (Le^y) and UA948 (Le^a and Le^x) (data not shown).

Cellular immune response to Lewis antigens

The cellular immune response of C57 and Balb/c mice was studied 8 weeks after oral inoculation with three different *H. pylori* strains, UA948 (Le^a, Le^x positive), UA1258 (Le^y positive) and UA1264 (no complete Lewis antigen expression). The spleen cells were stimulated with different concentrations of BSA, Synthetic Le^x trisaccharide-BSA (V- Labs, Covington, LA), a Le^x positive bacterial antigen, and a Le^y positive bacterial antigen depending on the nature of the antigen used for inoculation (Figure 4).

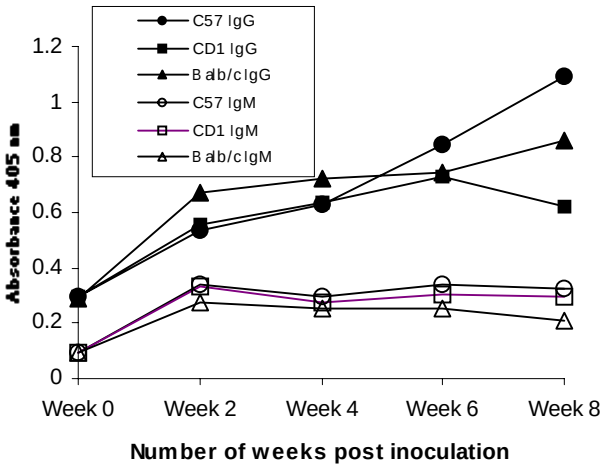

Figure 3: Serum IgM and IgG anti- *H. pylori* **antibody levels determined after mice were inoculated intragastrically with 10^8 cfu of the** *H. pylori* **SS1 strain. Mice were bled 2-8 weeks post inoculation and antibody levels estimated by ELISA.**

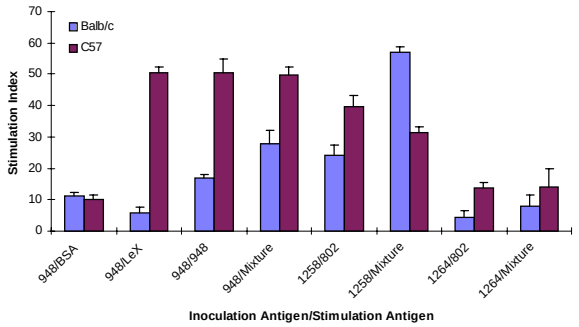

Figure 4: Comparison of cellular proliferation in C57 and Balb/c mice inoculated with *H. pylori*. **The clinical strains UA948, UA1258 and UA1264 were inoculated and spleenocytes stimulated with BSA, $Lewis^x$-BSA,** *H. pylori* **strains UA948 (Le^x), UA802 (Le^y), and a mixture consisting of UA948, UA802, UA955 (Le^x, Le^y and Le^b precursor), and UA1182 (Le^x, Le^y).**

All cells were also stimulated with an antigen mixture containing Le^x, Le^y, Le^a, and Le^b -precursor. The proliferative response of the spleen cell was found to be dose-dependent. In all cases maximal stimulation was observed at an antigen concentration of 1 µg/ml (data not shown). Proliferation also depends on the type of antigens used for colonization and stimulation. No significant degree of correlation was observed between colonization and cellular immune responses. Balb/c mice, for instance, were not colonized by Le^x positive bacteria after 8 weeks post inoculation. The stimulation index for these mice was found to be comparatively lower than that of C57 mice when stimulated with both Le^x positive bacterial antigen and Le^x - BSA. But when these mice were inoculated with a Le^y positive bacteria 80% colonization was observed. The stimulation index was also high when the cells were stimulated with a Le^y positive antigen and a mixture of antigens. The C57 mice on the other hand were 80% colonized by a Le^x positive bacterial antigen and showed a very high stimulation index. No colonization of C57 mice was observed when they were inoculated with a Le^y positive bacteria but a relatively high proliferation of the splenocytes was observed when stimulated with a similar antigen. Both mice strains (80% of Balb/c and 100% of C57) were colonized by the bacterial strain which does not express complete Lewis antigen. However, the proliferation of the spleen cell of these mice was found to be marginal.

Histology

Microscopic examination of the stomach section of inoculated mouse revealed tightly coiled organisms with the typical morphology of *H. pylori* colonizing the gastric glands of the test animal (Figure not included). There were no bacteria visible in the gastric glands of the control mouse by standard hematoxylin and eosin (H and E) or Giemsa or Warthin Stary strains (Figures not included). In contrast, numerous bacteria of at least two different types were clearly evident on both the H and E sections and also on the Giemsa and Warthin Stary stains of stomach tissue from the test animal. Both the longer tightly coiled spirals of *H. pylori* as well as the thicker rod shaped organism, probably *Lactobacillus*, were visible.

CONCLUSION

H. pylori colonized the gastric epithelium in mice, although the efficiency of colonization varied with the strain of mouse used and the type of Lewis antigens expressed. The PCR assay was the most sensitive indicator of colonization followed by the urease test. Culture positivity was demonstrable subsequently. Confirmation of the presence of *H. pylori* was shown by histology, although the sections also

show abundant rod shaped Lactobacillus-like bacteria. All *H. pylori* strains inoculated have shown significant humoral and cellular response after two weeks post inoculation. No correlation was observed between colonization ability of *H. pylori* and the type of Lewis antigen expressed by the bacterium that was used for inoculation. However, it must be noted that Lewis antigens are subject to antigenic variation(34) and may actually undergo phenotype changes in mice(35). The availability of stable *H. pylori* strains, which would not be subject to phase variation *in vivo*, would be a major advance enabling more detailed study of the role that Lewis antigens play in colonization.

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