BACTERIAL PATHOGENICITY

Involvement of the heparan sulphate-binding proteins of *Helicobacter pylori* in its adherence to HeLa S3 and Kato III cell lines

MARIA A. GUZMAN-MURILLO, EDUARDO RUIZ-BUSTOS, BOW HO* and FELIPE ASCENCIO†

Center for Biological Research, Marine Pathology Unit, La Paz, BCS 23000, Mexico and *Department of Microbiology, National University of Singapore, Singapore 117597

To determine whether *Helicobacter pylori* heparan sulphate-binding proteins (HSBPs) are involved in the adherence of *H. pylori* to HeLa and Kato III cells, monolayers were pre-incubated with various preparations and concentrations of *H. pylori* HSBPs at 37°C, washed and then challenged with bacteria. HSBPs did not prevent but enhanced *H. pylori* adherence. However, challenging cultured cells with *H. pylori* previously incubated with rabbit anti-HSBP IgG resulted in significant inhibition of bacterial adherence. These data demonstrate that the extracellular HSBP plays an important role in promoting *H. pylori* attachment to Kato III and HeLa S3 cells, that adhesion of *H. pylori* to Kato III and HeLa S3 cells is promoted by the presence of the 71.5-kDa extracellular HSBP and that rabbit polyclonal antibodies against this HSBP can inhibit adhesion of *H. pylori* to the cultured cell lines and detach cell-bound *H. pylori*.

Introduction

Epidemiological studies have provided evidence of a correlation between the presence of *Helicobacter pylori* in the gastric antrum and duodenal ulcers [1, 2]. Furthermore, epidemiological data sustain the hypothesis of an association between infection with *H. pylori* and the development of various gastric cancers [3]. Adhesion of *H. pylori* to human gastric mucosa is an important step in *H. pylori* colonisation. Thus, in developing strategies against *H. pylori* infection, it is important to analyse the adhesion mechanisms of this pathogen [4]. Cell adhesion in conjunction with the production of surface urease, lipase and toxic proteins may induce rapid destruction of epithelial cells, exposing subepithelial tissue and extracellular matrix. Bacteria can attach to the basement membrane by high affinity binding *via* collagen IV and laminin [5]. Laminin and collagen IV help to bind epithelial cells to the basal lamina and fibronectin helps to bind the matrix macromolecules and connective tissue on the

opposite side [6]. Several researchers have proposed that *H. pylori* recognises other cell receptors, such as those glycoconjugates exposed on HeLa, HEp-2, Vero and Kato III cells lines [7–10], or sialic acid-rich glycoproteins [11]. *H. pylori* also binds sulphated glycolipids [12], sulphomucins [13] and sulphated glycosaminoglycans such as those exposed on Kato III and Hu Tu-80 cells [14–16]. The high affinity of *H. pylori* for these molecules suggests that cell surface and extracellular matrix heparan sulphate are likely to be targets for *H. pylori* at an early stage of colonisation of the gastric mucosa.

Binding of *H. pylori* to all these host molecules may be also a strategy of the bacteria to prevent attack by phagocytes and immune reactions. Such phenomena may in part explain why *H. pylori* is a slow pathogen [17, 18] that can survive in the gastric mucosa for decades in patients with chronic type B gastritis. Chmiela *et al.* [19] demonstrated that *H. pylori* also used heparan sulphate-binding activity in its adhesion to murine peritoneal macrophages.

The aims of the present study were to investigate whether heparan sulphate-binding proteins (HSBPs) of *H. pylori* are involved in the adhesion of the bacteria to HeLa S3 and to Kato III cell lines, and whether bacterial adhesion can be blocked with polyclonal antibodies raised against a 71.5-kDa extracellular HSBP from *H. pylori*.

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†Corresponding author: Dr F. Ascencio (e-mail: micv4@nus.edu.sg). Present address: Department of Microbiology, National University of Singapore, MD4/A, Science Drive 2, Singapore 117597.

Materials and methods

Bacteria and growth conditions

H. pylori strain 25 was obtained from Professor T. Wadström (Department of Medical Microbiology, University of Lund, Sweden). This strain was isolated from a patient with duodenal ulcer disease. Identification was based on Gram's staining, cell morphology and positive reactions for catalase, oxidase and urease activity, and then confirmed with the API ZYM kit (bioMérieux, France) [20]. Further analysis indicated that *H. pylori* strain 25 binds heparan sulphate [14] and heparin-dependent growth factors [21] and that it is Ure⁺ and Cag⁺ [22]. *H. pylori* strains associated with gastro-oesophageal reflux disease (GERD) and duodenal ulcers and cancer-associated strains were isolated at the Department of Microbiology of the National University of Singapore from gastric biopsy samples obtained at the National University Hospital, Singapore, and identified as described above. *H. pylori* strain 51932 (CagA⁻-VacA⁻, Ure⁺, non-cytotoxic) was obtained from the American Type Culture Collection (Mannassas, VA, USA) and *H. pylori* strain 51110 (a Ure⁻ mutant, Cag⁺-VacA⁺ and cytotoxic strain, generated from the reference *H. pylori* strain 49503, also from ATCC) was from Professor B. J. Marshall (Department of Microbiology, University of Western Australia, Perth, Australia). The strains were cultured on GAB-Camp agar supplemented with lysed human blood (80°C, 20 min) 8.5%, inactivated horse serum (56°C, 30 min) 10%, cysteine hydrochloride 0.05%, IsoVitaleX 0.35% and the following antibiotics: vancomycin (6 µg/ml), nalidixic acid (20 µg/ml) and ketoconazole (3 µg/ml), and incubated at 37°C for 2–3 days in micro-aerobic conditions (O₂ 5%, CO₂ 10%, N₂ 85%) [23]. For the isolation of the extracellular HSBPs, *H. pylori* strain 25 was cultured at 37°C (in the same micro-aerobic conditions as above) in Brucella broth containing fetal calf serum 10% and supplemented with antibiotics. Stock cultures of *H. pylori* were stored at -80°C in trypticase soy broth (TSB) containing glycerol 15%.

For cell adhesion studies, *H. pylori* strains were grown on GAB-Camp agar as described above, harvested and washed twice in phosphate-buffered saline (PBS). Bacterial cell suspensions were adjusted to a density of 10⁹ cfu/ml and then labelled with n-biotin as described previously [24].

The mouse-adapted *H. pylori* strain 25 was generated by repeated *in-vivo* passage, cultured as described above, through oral administration of 10⁹ cells/ml (0.5 ml/mouse) in BALB/c mice [25]. After 10 days of such treatment, mice were killed by cervical vertebral dislocation under light anaesthesia; stomach and small intestine were excised and homogenised in PBS. Then, 250 µl of this suspension were cultured on GAB-Camp agar supplemented with human blood 8.5% and horse serum 10% and the following antibiotics: vancomycin (100 µg/ml), amphotericin B

(50 µg/ml) and nalidixic acid (10.7 µg/ml). Plates were incubated for 48 h and *H. pylori* colonies were identified by morphology and confirmed by a positive rapid urease reaction. The *H. pylori* strain was passaged through mouse gut up to eight times before use in the experiments.

HeLa S3 and Kato III cell lines

HeLa S3 cells, (derived from a human epithelioid carcinoma) and Kato III cells (a gastric adenocarcinoma cell line) were obtained from ATCC. The Kato III and HeLa S3 cells were grown in 75-cm² tissue-culture flasks with RPMI-1640 medium containing gentamicin 40 µg/ml, 2 mM L-glutamine, and supplemented with fetal calf serum (FCS) 15%. The tissue-culture flasks were incubated at 37°C (humidity 95% and CO₂ 5%). The cells were washed with PBS without Mg²⁺ and Ca²⁺, pH 7.2, and then treated with trypsin 0.25% in Hanks's modified balanced salts solution for 5 min at 37°C to release the cells from the plastic surface. The trypsinised cells were resuspended in fresh medium and then seeded into new tissue-culture flasks. The cells were transferred approximately every 3 days for HeLa S3 and every 5 days for Kato III cells.

For cell adhesion studies, 100 µl (4 × 10⁷ cells/ml) of the Kato III and HeLa S3 cell suspensions in RPMI 1640 medium containing FCS 15% were seeded on to 96-well tissue-culture plates and incubated at 37°C until the formation of a semi-confluent cell monolayer.

Isolation of H. pylori HSBPs

Supernates from *H. pylori* strain 25 cultures, grown as described above, were precipitated with ammonium sulphate 60–80% saturation. The precipitated proteins were centrifuged (18 000 rpm for 30 min at 5°C), resuspended in distilled water and then dialysed against 0.01 M ammonium bicarbonate.

The protein fraction was subjected to a modified heparin affinity-chromatography procedure with a 5-ml Heparin Hi-trap Column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.1 M sodium acetate buffer, pH 5.0 [26]. Adsorbed proteins were eluted with an NaCl gradient (0–2 M) over 30 min at the same flow rate (1 ml/min) and 1-ml fractions were collected. Fractions were dialysed extensively against 0.01 M ammonium bicarbonate and concentrated. SDS-PAGE and Western blot analysis of the chromatographed HSBP preparation, with horseradish peroxidase labelled heparan sulphate as a probe, indicated the co-purification of three major protein bands of 71.5, 66.2 and 11.3 kDa [26]. The first two proteins were sequenced at their amino-terminal region, and it was found that the 71.5-kDa protein is a *H. pylori* protein that has not been described before [26], and this protein was further purified by SDS-PAGE to

produce rabbit polyclonal antibodies and used in the blocking experiments described below.

Horseradish peroxidase-labelling of HSBPs and heparan sulphate

Chromatographically purified HSBPs (as described above) and heparan sulphate partially oxidised with 0.1 M NaIO₄ for 1 h at 22°C were conjugated with horseradish peroxidase as described previously [24]. Briefly, 0.2 ml of 0.1 M NaIO₄ was mixed with 4 mg of horseradish peroxidase. After incubation for 20 min at 22°C, horseradish peroxidase was dialysed against 1 mM sodium acetate buffer, pH 4.4, at 4°C for 24 h. This solution was then mixed with 1 mg of heparan sulphate or HSBP in 0.01 M Na₂CO₃ buffer, pH 4.4, and incubated at 22°C for 4 h. The reaction was stopped by adding 100 µl of 0.1 M NaBH₄. Horseradish peroxidase labelled protein solutions were adjusted to 50% v/v with glycerol and stored at -20°C until use.

Binding of horseradish peroxidase-labelled HSBPs to H. pylori cells

The binding assay was done in Eppendorf tubes as described previously for binding of horseradish peroxidase-labelled lactoferrin to *H. pylori* [27]. Briefly, 100 µl of an *H. pylori* suspension (10⁹ cells) grown on GAB-Camp agar were mixed with 100 µl of horseradish peroxidase-labelled HSBPs (stock solution diluted from 300 to 1 ng/p 100 µl) in 0.15 M sodium phosphate buffer (PBS) pH 7.2 at 37°C for 1 h. After the addition of 2 ml of ice-cold PBS containing Tween-20 0.05% v/v (PBS-Tween), the mixtures were centrifuged (4500 g, 15 min, 4°C) and the cell pellets were suspended in 100 µl of horseradish peroxidase substrate solution (containing *o*-phenylenediamine-HCl 1 mg/ml in 50 mM sodium citrate buffer, pH 5.0), follow by incubation for 30 min at 22°C. The reaction was stopped by adding 100 µl of 2 M H₂SO₄ and the absorbance was measured at 495 nm.

Enzyme-linked, biotin-streptavidin bacterial adhesion assay

Semi-confluent cell monolayers (0.5–1.0 × 10⁵ cells/well) grown on 96-well tissue-culture plates were washed three times in RPMI-1640 medium without antibiotics and incubated for 1 h at 25°C with a suspension of biotin-labelled *H. pylori* cells in RPMI-1640 medium without antibiotics (10⁹ cfu/ml). After incubation, plates were washed three times with PBS-Tween-20 to remove non-adhering bacteria. Then, 100 µl of horseradish peroxidase-conjugated streptavidin (Boehringer Mannheim, GmbH, Germany; diluted 1 in 2000 in PBS) were added to each well and the plates were incubated for 90 min at 25°C. After washing the plates three times with PBS-Tween, 100 µl of *o*-phenylenediamine-HCl were added to each well, and the plates were incubated for further 20 min

at 22°C in the dark. The reaction was stopped by the addition of 100 µl of 2 M H₂SO₄ and colour development was measured at 495 nm. Results of adhesion of biotin-labelled *H. pylori* to HeLa S3 and Kato III cell lines are expressed in optical density (OD) units.

Other adhesion studies were done with semi-confluent cell monolayers previously fixed with formaldehyde 0.5% in 0.1 M phosphate buffer (pH 7.2) for 30 min at 37°C. After washing five times with PBS-Tween, 200 µl of bovine serum albumin (BSA) 3% were added to each well. After incubation for 1 h at 37°C, plates were washed three times with PBS-Tween and used for the enzyme-linked, biotin-streptavidin bacterial adhesion assay as described above.

Inhibition assays were done by incubating *H. pylori* suspensions (100 µl containing 10⁹ cells) at 37°C for 1 h with 100 µl of rabbit polyclonal antibodies raised against the 71.5-kDa *H. pylori* HSBP (previously diluted in PBS), and with non-immune rabbit serum. Pre-incubated bacterial suspensions were added to the HeLa S3 and Kato III cell monolayers and the enzyme-linked, biotin-streptavidin bacterial adhesion assay was performed as described above.

Similarly, before the enzyme-linked, biotin-streptavidin bacterial adhesion assay, HeLa S3 and Kato III cell monolayers were pre-incubated for 1 h at 37°C with soluble HSBP adjusted to different concentrations (from 1 mg to 1 µg/ml) in PBS.

Glycosidase treatment

Formaldehyde-fixed cell monolayers were treated with several glycosidases. *Bacteroides fragilis* keratanase (endo-β-galactosidase) (6 mU/ml) digestion was done at 37°C for 24 h in 0.05 M sodium acetate buffer (pH 5.8) containing BSA 0.2 mg/ml. *Escherichia coli* β-galactosidase (1 U/ml) digestion was done in 0.1 M sodium citrate buffer (pH 4.3) and the reaction was run at 37°C for 16 h. *Bacillus stearothermophilus* α-glucosidase (0.5 mg/ml) digestion was done in 0.2 M sodium acetate buffer (pH 5) at 37°C for 16 h. *Clostridium perfringens* type V neuraminidase (5 U/ml) treatment of cells was done at 37°C for 16 h in 50 mM sodium acetate buffer (pH 5.5) containing 4 mM CaCl₂, and BSA 0.1 mg. *Flavobacterium heparinum* heparinase I (0.1 U/ml), *Proteus vulgaris* chondroitinase ABC (0.1 U/ml) and bovine testes hyaluronidase type VI-S (1 U/ml) digestion were done at 37°C for 24 h in 0.05 M sodium acetate buffer (pH 5.8) containing BSA 0.2 mg/ml. After each treatment, the monolayers were washed three times with PBS and blocked with BSA 3% for 1 h at 37°C. After washing with PBS-Tween, plates were used for the enzyme-linked, biotin-streptavidin bacterial adhesion assay as described above.

Detachment

To measure detachment, *H. pylori* was first allowed to bind to HeLa S3 and Kato III semi-confluent monolayers for 90 min at 37°C as described above. After washing the 96-well tissue-culture plates with PBS-Tween to remove non-adhering bacteria, rabbit polyclonal antibodies against the 71.5-kDa HSBP and non-immune rabbit serum were added to the 96-well tissue-culture plates in 100 µl of PBS at different concentrations. Plates were washed with PBS-Tween after incubation for 60 min at 22°C, and residual bacteria were quantified as described above.

Antisera

To raise antisera against the 71.5-kDa extracellular *H. pylori* HSBP [26], c. 30 µg of HSBP protein band was isolated from a SDS-PAGE preparation, emulsified with Freund's adjuvant and injected intramuscularly in rabbits three times at intervals of 2 weeks. Antiserum was collected 2 weeks after the final antigen dose. Immunoblotting and ELISA assays were performed to ensure that the antiserum contained antibodies that recognised the 71.5-kDa extracellular *H. pylori* HSBP (data not shown).

Statistical analysis

Student's *t* test was used to assess the significance of differences between means in binding and inhibition assays. Comparisons involving more than two groups were performed with ANOVA. Values are expressed as means (SD) of at least three experiments.

Results

Effect of repetitive passage of H. pylori through the BALB/c intestinal tract on adherence to HeLa S3 and Kato III cell lines

Because of interest in the development of HSBP-based vaccines against *H. pylori* infections, and the possibility of using the BALB/c mouse to study *H. pylori* adhesion and colonisation factors *in vivo*, this study aimed to evaluate whether repetitive passage of *H. pylori* through the BALB/c gut enhanced its ability to adhere to both live semi-confluent monolayers (Fig. 1a and b) and formaldehyde-fixed cell monolayers (Fig. 1c and d) of HeLa S3 and Kato III cells. Both the *H. pylori* wild strain (Fig. 1a and c) and the corresponding mouse-adapted strain (Fig. 1b and d) adhered to HeLa S3 and Kato III cells in a similar manner. The dose-response adherence of *H. pylori* 25 and its correspond-

Fig. 1. Effect of repetitive passage of *H. pylori* through the BALB/c mouse intestinal tract on its adherence to HeLa S3 (■, □) and Kato III (▲, △) cell lines. (a) and (c) show the adherence of *H. pylori* strain 25 (■, ▲) to live and formaldehyde-fixed cells, respectively. (b) and (d) show the adherence of the mouse-adapted *H. pylori* strain 25 (□, △) to live and formaldehyde-fixed cells, respectively. Each point represents the mean of three experiments; bars indicate SD.

ing mouse-adapted strain to HeLa S3 and Kato III cells were determined. *H. pylori* strain 25 showed a tendency to adhere more to Kato III cells (Fig. 2a), whereas the mouse-adapted *H. pylori* strain adhered more to HeLa S3 cells (Fig. 2b). In both cases, there was a dose-dependent effect on bacterial adhesion to HeLa S3 and Kato III cell lines.

Evidence of the HSBPs from H. pylori participating in adherence to HeLa S3 and Kato III cells

A series of studies was conducted to determine whether the HSBPs are important for the adhesion of *H. pylori* to HeLa S3 and Kato III cells. Because the HSBP is an extracellular lectin, the study aimed to determine whether its presence in the medium surrounding *H. pylori* could be an element for cell adhesion. First, the question as to whether soluble HSBP can be bound to *H. pylori* cells was addressed. *H. pylori* strain 25 and its corresponding mouse-adapted variant were incubated with increasing concentrations of horseradish peroxidase labelled HSBP. Fig. 3 shows the dose-response binding kinetics of horseradish peroxidase labelled HSBP to *H. pylori* cells.

The next step was to determine whether soluble HSBP could promote adhesion of *H. pylori* to HeLa S3 and Kato III cells. Bacterial cells were incubated with soluble HSBP before the adhesion assay. HSBP enhanced the adhesion of *H. pylori* strain 25 (Fig. 4a) and its corresponding mouse-adapted variant (Fig. 4b) to HeLa S3 and Kato III cells.

A similar assay was performed with glycosidase-treated HeLa S3 and Kato III cells (Table 1). Treatment of HeLa S3 cells with neuraminidase, chondroitinase, keratanase and galactosidase before the adherence assay significantly reduced ($p \leq 0.005$) adherence of *H. pylori* strain 25 (Table 1). However, when the same

Fig. 3. Binding of horseradish peroxidase-labelled HSBP to *H. pylori* strain 25 (●) and its corresponding mouse-adapted variant (○). Different concentrations of horseradish peroxidase-labelled HSBP were incubated with the *H. pylori* cells and adherence was assessed by the same procedure as used to evaluate the adherence of bacteria to cultured cells outlined in *Materials and methods*. Each point represents the mean of three experiments.

glycosidase treatment was done in the presence of extracellular HSBP, a distinct pattern was observed; there was no promotion but rather inhibition of *H. pylori* adherence to HeLa S3 cells (Table 1). An analogous pattern was observed in the adhesion of *H. pylori* to glycosidase-treated Kato III cells (Table 1). Pre-treatment of Kato III cells with neuraminidase and glucosidase resulted in a reduction of *H. pylori* adherence (Table 1). Furthermore, adhesion of *H. pylori*

Fig. 2. Dose-course kinetics of *H. pylori* strain 25 (a) and its corresponding mouse-adapted variant (b) adhesion to HeLa S3 (■, □) and Kato III (▲, △) cell monolayers. Bacteria input, OD₅₄₀ of 1.0, 1×10^8 cfu/ml. Each point represents the mean of three experiments; bars indicate SD.

Fig. 4. Effect of the presence of soluble HSBP in the adhesion assay mixture on the adhesion of *H. pylori* strain 25 (a), and its mouse-adapted variant (b), to HeLa S3 (■, □) and Kato III (▲, △) cell monolayers. Each point represents the mean of three experiments; bars indicate SD.

strain 25 to glycosidase-treated Kato III cells in the presence of extracellular HSBP was diminished, especially in those Kato III cells treated with keratanase and chondroitinase (Table 1).

However, in the adhesion experiments with the mouse-adapted *H. pylori* strain and glycosidase-treated HeLa and Kato III cells, promotion of bacterial adhesion was observed, especially to neuraminidase-treated Kato III cells (Table 1). Nevertheless, most of the glycosidase treatment of HeLa S3 and Kato III cells also resulted in an inhibition of cell binding by the mouse-adapted *H. pylori* variant (Table 1).

The next question to be addressed was whether antibodies raised against HSBPs could interrupt adherence of *H. pylori* to HeLa S3 and Kato III cell monolayers. The *H. pylori* cells were first incubated in the presence of different dilutions of antibodies, and then added to the HeLa S3 and Kato III cell monolayers. Antibodies raised against the 71.5-kDa HSBP [26] inhibited the adhesion of both *H. pylori* strain 25 (Fig. 5a) and its corresponding mouse-adapted variant (Fig. 5b) to the HeLa S3 and Kato III cells. The study also evaluated whether the rabbit polyclonal

Table 1. Adhesion of *H. pylori* strain 25 and its corresponding mouse-adapted variant to glycosidase-treated HeLa S3 and Kato III cell monolayers in presence or absence of soluble HSBP

<i>H. pylori</i> strain and cell monolayer presentation	Adhesion of <i>H. pylori</i> measured as OD ₄₉₅ (SD) to cell monolayers treated with							
	No treatment	Keratanase	Galatosidase	Glucosidase	Neuraminidase	Heparitinase	Chondroitinase	Hyaluronidase
<i>H. pylori</i> strain 25								
Glycosidase-treated HeLa	0.603 (0.030)	0.533 (0.016)	0.539 (0.020)	0.533 (0.015)	0.512 (0.007)	0.561 (0.007)	0.523 (0.015)	0.567 (0.011)
Glycosidase-treated HeLa + HSBP	0.556 (0.032)	0.437 (0.044)	0.479 (0.012)	0.524 (0.024)	0.497 (0.007)	0.513 (0.015)	0.458 (0.007)	0.523 (0.012)
Glycosidase-treated Kato	0.686 (0.028)	0.696 (0.037)	0.886 (0.030)	0.634 (0.026)	0.589 (0.009)	0.639 (0.012)	0.636 (0.035)	0.672 (0.018)
Glycosidase-treated Kato + HSBP	0.640 (0.027)	0.554 (0.032)	0.574 (0.006)	0.573 (0.003)	0.571 (0.004)	0.532 (0.043)	0.555 (0.023)	0.602 (0.03)
Mouse-adapted <i>H. pylori</i>								
Glycosidase-treated HeLa	0.458 (0.009)	0.441 (0.043)	0.422 (0.010)	0.456 (0.031)	0.485 (0.050)	0.434 (0.006)	0.399 (0.004)	0.433 (0.012)
Glycosidase-treated HeLa + HSBP	0.482 (0.050)	0.363 (0.013)	0.414 (0.058)	0.478 (0.067)	0.473 (0.015)	0.406 (0.039)	0.379 (0.027)	0.386 (0.015)
Glycosidase-treated Kato	0.598 (0.002)	0.501 (0.015)	0.508 (0.015)	0.481 (0.011)	0.556 (0.021)	0.474 (0.018)	0.481 (0.014)	0.514 (0.06)
Glycosidase-treated Kato + HSBP	0.468 (0.005)	0.406 (0.023)	0.445 (0.015)	0.426 (0.011)	0.534 (0.017)	0.416 (0.014)	0.429 (0.007)	0.427 (0.009)

Each point represents the mean (SD) of three tests ($p \leq 0.005$).

Fig. 5. Blocking of *H. pylori* adherence with anti-HSBP. Rabbit antiserum against a 71.5-kDa HSBP from *H. pylori* was used to block adherence of *H. pylori* strain 25 (a), and its corresponding mouse-adapted variant (b), to HeLa S3 (■, □) and to Kato III cell monolayers (▲, △). Each point represents the mean of three experiments; bars indicate SD.

Table 2. Blocking of adhesion of *H. pylori* strains to HeLa S3 and Kato III cell monolayers after pre-incubation of bacterial cells with rabbit anti-serum (diluted 1 in 10 v/v) against a 71.5-kDa HSBP

<i>H. pylori</i> strain	Percentage blocking of adherence of <i>H. pylori</i> to	
	Kato III cells	HeLa S3 cells
GERD-associated strains		
20	80	80
72	65	54
91	70	51
125	82	71
152	59	55
185	37	41
193	63	61
238	62	59
291	80	70
Gastric cancer-associated strain		
1117	72	48
Duodenal ulcer-associated strains		
1126	72	49
RH-54	50	68
Reference strains		
51110 (ure ⁻ mutant from ATCC 49503)	67	77
ATCC 51932	48	59

antibodies raised against the 71.5-kDa HSBP could block the adhesion of different *H. pylori* strains to HeLa S3 and Kato III cell monolayers. Table 2 shows that the anti-HSBP blocked the adherence of *H. pylori* to differing extents depending on the strain. There was no inhibition in similar studies with non-immune rabbit serum (data not shown).

A separate experiment aimed to determine whether the antibodies raised against the 71.5-kDa HSBP could detach *H. pylori* bound to HeLa S3 and Kato III cells. Fig. 6a and 6b shows that there was some *H. pylori* detachment; however, it was not as great as the results for inhibition of adherence. Rabbit non-immune sera did not displace cell-bound *H. pylori* strain 25 or its corresponding mouse-adapted variant (data not shown).

Discussion

Many studies of *H. pylori* adhesion *in vitro* have shown binding of the bacteria to various cell lines in tissue culture, including primary cultures of human gastric epithelial cells [7, 9, 10, 16]. However, the mechanisms by which *H. pylori* adheres to various human gastric carcinoma cells with differing affinity are not fully clear. It is possible that the affinity of *H. pylori* for gastric carcinoma cells in terms of adhesion might be due to different surface structures based upon tumour characteristics [28]. Recent studies indicate that *H. pylori* adherence to cultured gastric epithelial cells is associated with several cellular events, including re-organisation of the host cell's actin, tyrosine phosphorylation of a 145-kDa protein and cytokine release [29].

Fig. 6. Partial detachment of bound bacteria by rabbit antiserum against a 71.5-kDa HSBP from *H. pylori* strain 25 (a) and the mouse-adapted *H. pylori* variant (b) were incubated with HeLa S3 (■, □) and Kato III (▲, △) cell monolayers for 1 h, and unbound bacteria were removed by three washes with PBS-Tween 20, and further incubated for 1 h with the indicated serum dilutions. Each point represents the mean of three experiments; bars indicate SD.

Osaki *et al.* [28] suggested the possibility that haemagglutinins and other surface components may be co-operatively involved in adhesion of *H. pylori* to cultured cells. It has also been reported that Lewis^b antigen and other fucosylated blood group antigens function as receptors for *H. pylori* attachment to fixed gastric tissue [30]; however, binding to Kato III cells is independent of Lewis^b antigen [15, 31]. Previous studies in this laboratory showed that the ability of *H. pylori* to bind heparan sulphate was not correlated with haemagglutination activity (unpublished data). *H. pylori* urease has been shown to bind gastric mucin, heparin and related heparinoids [32]. However, urease does not play a role in the adherence of *H. pylori* to gastric cells [33]. A 20-kDa haemagglutinin with affinity for N-acetylneuraminyl-lactose in mammalian cells in culture has been identified as a putative colonisation factor for *H. pylori* [34]. Fauchere and Blaser [35] described a 15-kDa antigen in *H. pylori* bacterial extracts that adhered to both neuraminidase-treated and native HeLa cells.

Namavar *et al.* [36] identified a 16-kDa OMP from *H. pylori* with affinity for sulphated carbohydrates of

mucin and for Lewis x blood group antigen. Odenbreit *et al.* [37] identified a novel genetic locus, the *alpAB* operon, which encodes two closely homologous integral outer-membrane proteins (OMPs) directly involved in adhesion of *H. pylori* to Kato III cells and to human gastric tissues. They also found that the pattern of AlpAB-dependent adherence of *H. pylori* to gastric epithelial surfaces showed a clear difference from the BabA2-mediated adherence to Lewis^b antigen, suggesting that a different receptor is involved.

It has been demonstrated that *H. pylori* binds to heparan sulphate and to heparin-dependent acidic and basic fibroblast growth factors [14, 21]. Utt and Wadström [38] found that *H. pylori* expressed a number of OMPs with affinity for heparin, and the authors of the present study recently found that besides a 45-kDa OMP HSBP, *H. pylori* produces three major extracellular HSBPs, one with a mol. wt of 66.2 kDa and a pI of 5.4, and another with a mol. wt of 71.5 kDa and a pI of 5.0 [26].

Two series of experiments serve to highlight the importance of the extracellular HSBPs for adhesion of *H. pylori* to Kato III and HeLa cells. First, studies of adhesion of *H. pylori* to cultured cells in the presence of extracellular HSBP showed enhancement of bacterial attachment to both Kato III and HeLa cells. Second, binding inhibition studies with rabbit polyclonal antibodies against the extracellular 71.5-kDa HSBP of *H. pylori*, but not with non-immune rabbit serum, resulted in blockade of adhesion of *H. pylori* to cultured cells and detachment of cell-bound *H. pylori* from both HeLa and Kato III cell lines. These findings are important, as monoclonal antibodies to Le^a and Le^b did not inhibit adherence of *H. pylori* to Kato III cells [31] and because the in-vitro demonstration that anti-HSBP can prevent adherence as well as induce detachment of cell-bound *H. pylori*, suggests that an HSBP-based vaccine preparation may be of value for protection against and eradication of *H. pylori* infections, as has been suggested recently [22]. It may be true that bacterial adherence is comprised of multiple non-covalent contact points, each in dynamic equilibrium. In principle, therefore, molecules capable of competitively inhibiting adhesion–ligand interaction will not only prevent binding, but also disrupt established adherence [16].

Treatment of cultured cells with glycosidases and endoglycolytic enzymes altered the binding of *H. pylori* strain 25 and its corresponding mouse-adapted variant to both HeLa S3 and Kato III cell lines (Table 1). Extracellular HSBP did not promote adhesion of mouse-adapted *H. pylori* to glycosidase-treated cultured cells, except in binding to neuraminidase-treated Kato III, where there was an enhancement of bacterial adhesion, and to glucosidase- and neuraminidase-treated HeLa S3 cells, where a tendency to promote bacterial adherence was observed. As reported by

Simon *et al.* [16], glycosidase treatment of Kato III cells resulted in altered bacterial binding but, most importantly, enzymic digestion with glycosidases suggests that *H. pylori* adhesion to gastric epithelial cells may rely on more than one adhesin.

Based on the report of Simon *et al.* [16] that the sensitivity of *H. pylori* to 3' sialyl-lactose (NeuAc α 2-3Gal β 1-4Glc; 3'SL) inhibition of binding to gastrointestinal epithelial cells diminished with increasing passage number, the present study evaluated whether the HSBP has any effect on the adhesion of a recently mouse-adapted *H. pylori* strain. Both *H. pylori* strain 25 and its corresponding mouse-adapted variant showed similar adhesion patterns to HeLa S3 and Kato III cell lines, which suggests that the adhesion activity of *H. pylori* strain 25 to both HeLa S3 and Kato III cell lines is not altered by serial passage.

Duensing *et al.* [39] proposed a novel strategic mimicry pathogenicity model based on heparan sulphate glycosaminoglycan-directed recruitment of mammalian host proteins to compromise the host's immune system. According to this model, bacterial pathogens need direct cell contact with a host-soluble glycosaminoglycan. The glycosaminoglycans bound at the bacterial cell surface subsequently serve as universal binding sites for any mammalian heparin-binding protein, without producing separate receptors for each protein. The repertoire of surface-bound proteins may affect various aspects of microbial virulence and host defence systems, such as chemotaxis, tissue invasion, tissue integrity and immunological responses [39].

However, if the fact that heparan sulphate glycosaminoglycan is exposed only during wound healing [40, 41] is taken into consideration, it would be an advantage for the bacteria to coat itself with the heparan sulphate glycosaminoglycan. A possible mechanism to achieve this would be the production of competitive extracellular HSBP. Producing an extracellular HSBP would enable the pathogen to search for soluble heparan sulphate glycosaminoglycan and then bring it to the bacterial cell for further recruitment of mammalian host heparin-binding proteins such as chemokines, growth factors, extracellular matrix proteins, enzymes and cytokines. Furthermore, because heparan sulphates are common constituents of the cell surface and the extracellular matrix [42], especially exposed during wound healing, they may serve as additional receptors for bacterial attachment. It has recently been reported by Hirno *et al.* [43] that heparin and other sulphated oligosaccharides can block the binding of *H. pylori* to immobilised sulphated mucins. The HSBPs from *H. pylori* may be involved in different biological processes, and not only in the adhesion of the bacteria to sulphated proteoglycans, as is the case for heparan sulphate, heparin, or heparin-dependent fibroblast growth factors. Thus it is possible that HSBPs may also be involved in the adhesion of the bacteria to

sulphomucins [13] and to sulphated glycolipids [12] exposed on the human gastric mucosal cells.

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References

- Forman D, Newell DG, Fullerton F *et al.* Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. *BMJ* 1991; **302**: 1302–1305.
- Moss S, Calam J. *Helicobacter pylori* and peptic ulcers; the present position. *Gut* 1992; **33**: 289–292.
- Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, Isaacson PG. *Helicobacter pylori*-associated gastric and primary B-cell gastric lymphoma. *Lancet* 1991; **338**: 1175–1176.
- Evans DG, Evans DJ. Adhesion properties of *Helicobacter pylori*. *Methods Enzymol* 1995; **253**: 336–360.
- Trust TJ, Doig P, Emödy L, Kienle Z, Wadström T, O' Toole P. High-affinity binding of the basement membrane proteins collagen type IV and laminin to the gastric pathogen *Helicobacter pylori*. *Infect Immun* 1991; **59**: 4398–4404.
- Simon-Assman P, Keding M, Haffén K. Immunocytochemical localization of extracellular-matrix proteins in relation to rat intestinal morphogenesis. *Differentiation* 1986; **32**: 59–66.
- Clyne M, Drumm B. Adherence of *Helicobacter pylori* to primary human gastrointestinal cells. *Infect Immun* 1993; **61**: 4051–4057.
- Lingwood CA, Wasfy G, Han H, Huesca M. Receptor affinity purification of a lipid-binding adhesin from *Helicobacter pylori*. *Infect Immun* 1993; **61**: 2474–2478.
- Wadström T, Ascencio F, Ljungh Å *et al.* *Helicobacter pylori* adhesins. *Eur J Gastroenterol Hepatol* 1993; **5 Suppl 2**: S12–S15.
- Wadström T, Hirno S, Borén S. Biochemical aspects of *Helicobacter pylori* colonization of the human gastric mucosa. *Aliment Pharmacol Ther* 1996; **10 suppl 1**: 17–27.
- Evans DG, Evans DJ, Graham DY. Adherence and internalization of *Helicobacter pylori* by Hep-2 cells. *Gastroenterology* 1992; **102**: 1557–1567.
- Saitoh T, Natomi H, Zhao W *et al.* Identification of glycolipid receptor for *Helicobacter pylori* by TLC-immunostaining. *FEBS Lett* 1991; **282**: 385–387.
- Slomiany BL, Piotrowski J, Slomiany A. Effect of sucralfate on the degradation of human gastric mucus by *Helicobacter pylori* protease and lipases. *Am J Gastroenterol* 1992; **87**: 595–599.
- Ascencio F, Fransson L-Å, Wadström T. Affinity of the gastric pathogen *Helicobacter pylori* for the N-sulphated glycosaminoglycan heparan sulphate. *J Med Microbiol* 1993; **38**: 240–244.
- Kamisago S, Iwamori M, Tai T, Mitamura K, Yazaki Y, Sugano K. Role of sulfatides in adhesion of *Helicobacter pylori* to gastric cancer cells. *Infect Immun* 1996; **64**: 624–628.
- Simon PM, Goode PL, Mobasser A, Zopf D. Inhibition of *Helicobacter pylori* binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. *Infect Immun* 1997; **65**: 750–757.
- Borén T, Falk P, Roth KA, Larson G, Normark S. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. *Science* 1993; **262**: 1892–1895.
- Valkonen KH, Ringner M, Ljungh Å, Wadström T. High-affinity binding of laminin by *Helicobacter pylori*: evidence for a lectin-like interaction. *FEMS Immunol Med Microbiol* 1993; **7**: 29–37.
- Chmiela M, Paziak-Domanska B, Rudnicka W, Wadström T. The role of heparan sulphate-binding activity of *Helicobacter pylori* bacteria in their adhesion to murine macrophages. *APMIS* 1995; **103**: 469–474.
- Kung JSL, Ho B, Chan SH. Biotyping of *Campylobacter pylori*. *J Med Microbiol* 1989; **29**: 203–206.
- Ascencio F, Hansson HA, Larm O, Wadström T. *Helicobacter pylori* interacts with heparin and heparin-dependent growth factors. *FEMS Immunol Med Microbiol* 1995; **12**: 265–272.

22. Ruiz-Bustos E, Sierra-Beltran A, Romero MJ, Rodriguez-Jaramillo C, Ascencio F. Protection of BALB/c mice against experimental *Helicobacter pylori* infection by oral immunisation with *H. pylori* heparan sulphate-binding proteins coupled to cholera toxin β -subunit. *J Med Microbiol* 2000; **49**: 535–541.
23. Soltesz V, Schalen C, Mardh P-A. New selective medium for *Campylobacter pylori*. In: *Proceedings of the Fourth International Workshop on Campylobacter infection*. Kaijser B, Falsen E (eds) Gothenborg, Sweden, 1988: 433–436.
24. Harlow E, Lane DP. Antibodies. A laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory. 1988: 319–358.
25. Marchetti M, Aricó B, Burroni D, Figura N, Rappuoli R, Ghiara P. Development of a mouse model of *Helicobacter pylori* infection that mimics human diseases. *Science* 1995; **267**: 1655–1658.
26. Ruiz-Bustos E, Ochoa JL, Wadström T, Ascencio F. Isolation and characterization of putative adhesins from *Helicobacter pylori* with affinity for heparan sulphate proteoglycan. *J Med Microbiol* (in press).
27. Amini H-R, Ascencio F, Ruiz-Bustos E, Romero MJ, Wadström T. Cryptic domains of a 60-kDa heat shock protein of *Helicobacter pylori* bound to bovine lactoferrin. *FEMS Immunol Med Microbiol* 1996; **16**: 247–255.
28. Osaki T, Yamaguchi H, Taguchi H, Kumada J, Ogata S, Kamiya S. Studies on the relationship between adhesive activity and haemagglutination by *Helicobacter pylori*. *J Med Microbiol* 1997; **46**: 117–121.
29. Segal ED, Lange C, Covacci C, Tompkins LS, Falkow S. Induction of host signal transduction pathways by *Helicobacter pylori*. *Proc Natl Acad Sci USA* 1997; **94**: 7595–7599.
30. Su B, Hellström PM, Rubio C, Çelik J, Granström M, Normark S. Type I *Helicobacter pylori* shows Lewis^b-independent adherence to gastric cells requiring de novo protein synthesis in both host and bacteria. *J Infect Dis* 1998; **178**: 1379–1390.
31. Clyne M, Drumm B. Absence of effect of Lewis A and Lewis B expression on adherence of *Helicobacter pylori* to human gastric cells. *Gastroenterology* 1997; **113**: 72–80.
32. Icatlo FC, Kuroki M, Kobayashi C *et al*. Affinity purification of *Helicobacter pylori* urease. Relevance to gastric mucin adherence by urease protein. *J Biol Chem* 1998; **273**: 18130–18138.
33. Clyne M, Drumm B. The urease enzyme of *Helicobacter pylori* does not function as an adhesin. *Infect Immun* 1996; **64**: 2817–2820.
34. Evans DG, Evans DJ, Moulds JJ, Graham DY. N-acetylneuraminylactose-binding fibrillar hemagglutinin of *Campylobacter pylori*: a putative colonization factor antigen. *Infect Immun* 1998; **56**: 2896–2906.
35. Fauchere J-L, Blaser MJ. Adherence of *Helicobacter pylori* cells and their surface components to HeLa cell membranes. *Microb Pathog* 1990; **9**: 427–439.
36. Namavar F, Sparrius M, Veerman ECI, Appelmelk BJ, Vandenbroucke-Grauls CMJE. Neutrophil-activating protein mediates adhesion of *Helicobacter pylori* to sulfated carbohydrates on high-molecular-weight salivary mucin. *Infect Immun* 1998; **66**: 444–447.
37. Odenbreit S, Till M, Hofreuter D, Faller G, Haas R. Genetic and functional characterization of the alpAB gene locus essential for the adhesion of *Helicobacter pylori* to human gastric tissue. *Mol Microbiol* 1999; **31**: 1537–1548.
38. Utt M, Wadström T. Identification of heparan sulphate binding surface proteins of *Helicobacter pylori*: inhibition of heparan sulphate binding with sulphated carbohydrate polymers. *J Med Microbiol* 1997; **46**: 541–546.
39. Duensing TD, Wing JS, van Putten JPM. Sulfated polysaccharide-directed recruitment of mammalian host proteins: a novel strategy in microbial pathogenesis. *Infect Immun* 1999; **67**: 4463–4468.
40. Andriessen MP, van den Born J, Latijnhouwers MA, Bergers M, van de Kerkof PC, Schalkwijk J. Basal membrane heparan sulphate proteoglycan expression during wound healing in human skin. *J Pathol* 1997; **183**: 264–271.
41. Okasala O, Salo T, Tammin R *et al*. Expression of proteoglycans and hyaluronan during wound healing. *J Histochem Cytochem* 1995; **43**: 125–135.
42. Stringer SE, Gallagher JT. Heparan sulphate. *Int J Biochem Cell Biol* 1997; **29**: 709–714.
43. Hirno S, Artursson E, Puu G, Wadström T, Nilsson B. *Helicobacter pylori* interactions with human gastric mucin studied with a resonant mirror biosensor. *J Microbiol Methods* 1999; **37**: 177–182.