Adherence of *Helicobacter pylori* to human gastric epithelial cells *in vitro*

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Summary. Gram-negative spiral organisms, currently referred to as *Helicobacter pylori*, are associated with primary gastritis and duodenal ulceration. The organisms colonise gastric mucus and adhere to epithelial cells of inflamed antra. To further examine the binding of *H. pylori* to human gastric epithelial cells, we developed and characterised an in-vitro bacterial adherence assay. Scanning electronmicroscopy suggested that spiral-shaped bacteria were adherent to the surface of KATO-III cells which were derived from a human gastric adenocarcinoma. Transmission electronmicroscopy confirmed the attachment of *H. pylori* to these epithelial cells in tissue culture. Some bacteria were adherent to intact microvilli, others were closely adherent to the plasma membrane in regions where microvilli were effaced. In studies with radiolabelled *H. pylori*, adherence to epithelial cells in tissue culture contrasted with minimal binding of bacteria to polystyrene wells alone. Incubation of bacteria with gastric cells at 4°C significantly reduced adherence of *H. pylori*. We conclude that adherence of *H. pylori* to gastric epithelial cells in tissue culture involved “attachment and effacement mechanisms”. This assay could serve as a suitable in-vitro model for the study of the bacterial adhesins and host receptors which mediate attachment of *H. pylori* to gastric epithelial cell surfaces.

Introduction

Gram-negative, spiral shaped organisms, now known as *Helicobacter pylori* (previously *Campylobacter pylori*)¹ have recently been associated with both antral gastritis and peptic ulcer in adults^{2,3} and children.^{4,5} Histological examination of diseased antral mucosa has demonstrated the presence of these bacteria within the gastric mucus layer and at the surface of gastric epithelial cells.⁶ Ultrastructural changes on the apical plasma membrane of gastric epithelial cells to which bacteria have adhered include loss of microvilli and the development of adherence pedestals.^{7,8} Bacterial attachment to adherence pedestals is also present in a ferret model of *H. pylori*-associated disease.⁹ These changes appear morphologically identical to the attaching and effacing adherence phenomena which have been observed in the small intestine and colon in enteropathogenic *Escherichia coli* infection in animals.¹⁰

Binding of *H. pylori* to HEp-2 and INT-407 epithelial cells in tissue culture was described recently.¹¹ However, characterisation of binding by *H. pylori* to gastric epithelial cells *in vitro* has not been reported. Such an adherence assay could be used to identify the

adhesins expressed by *H. pylori* and could serve as a model to determine the mechanism of bacterial adherence to gastric epithelial cells. Therefore, we examined the adherence of *H. pylori* to KATO-III cells, an epithelial cell line derived from a human gastric adenocarcinoma.¹² Binding of *H. pylori* to KATO-III cells was examined by scanning and transmission electronmicroscopy (SEM and TEM) and by a quantitative adherence assay with radio-labelled bacteria.

Materials and methods

Bacteria and growth conditions

H. pylori strains LC-3 and LC-11 were isolated originally from the antral mucosae of two children with primary gastritis and associated duodenal ulcer.^{5,13} Strains were stored at –70°C, as described previously,¹⁴ until used in these studies. Brucella Broth (Gibco Laboratories, Madison, WI, USA) containing trimethoprim (Sigma Chemicals, St. Louis, MO, USA) 5 mg/L and vancomycin (Sigma) 10 mg/L was supplemented with fetal bovine serum (Boknek Laboratories, Ontario, Canada) 10% for the culture of *H. pylori*.^{14,15} After rapid thawing, 0.5 ml of stored *H. pylori* culture was mixed with 11 ml of supplemented Brucella broth in a 250-ml Ehrlenmeyer flask. The flask was closed

with a loosely fitted screw cap and placed inside an incubation jar which was then evacuated and flushed through with a gas mixture containing CO₂ 10%, O₂ 5%, N₂ 85%. The sealed jar was then placed on a rotary shaker and incubated at 37°C with shaking at 100 rpm. After 24 h, 1 ml of bacterial culture was transferred to fresh medium and re-cultured. The absence of bacterial contaminants was confirmed by subculture of broth on blood-agar plates incubated in air at 37°C. The presence of *H. pylori* was confirmed by a positive urease reaction (Christensen's urea broth) and by inspection of bacterial morphology by phase-contrast microscopy.^{14, 16}

H. pylori produced smooth, translucent colonies on supplemented Brucella agar. To improve the accuracy of viable counts (cfu), tetrazolium salts (4 mg/L) were added into the agar medium, as described previously.¹⁷ After incubation for 5 days in micro-aerophilic conditions, *H. pylori* produced red colonies on this medium.

Growth studies with *H. pylori*

The growth curve for *H. pylori* in the broth cultures was constructed from sequential measurements of optical densities of cultures at 600 nm and from quantitation of viable bacteria by colony counts after incubation for 6, 12, 18, 24, 30 and 36 h. Viable counts were determined by inoculating dilutions of broth in triplicate on to Brucella agar with tetrazolium salts. Colonies were counted after incubation of plates for 5 days at 37°C in micro-aerophilic conditions.

Radiolabelling of *H. pylori*

Tritium-labelled (8.3 µCi/ml) or ¹⁴C-labelled (1.7 µCi/ml) proline and histidine (New England Nuclear, Boston, MA, USA), were added to the growth medium at the time of inoculation with bacteria. *H. pylori* strain LC-3 was used for all radiolabelling studies. Incubation conditions were identical to those described above. When ¹⁴C-labelled proline and histidine were used, gases which were released into the culture jar were absorbed in Protosol (New England Nuclear). After incubation for 18 h, bacteria were pelleted by centrifugation at 3000 *g* for 15 min, washed three times in sterile phosphate-buffered saline (PBS), and resuspended in PBS to an optical density of 0.35 at 600 nm. Incorporation of radioactive label into the bacteria was determined by counting disintegrations/min (dpm) in a β-scintillation counter (Model LS 7500, Beckman Instruments, Palo Alto, CA, USA).

Adherence assay

KATO-III cells, obtained from the American Type Culture Collection, Rockville, MD, are gastric epithelial cells originally derived from a human gastric adenocarcinoma.¹² The cells were grown in RPMI-

1640 medium (Flow Laboratories, McLean, VA, USA) supplemented with heat-inactivated fetal bovine serum (Bocknek Laboratories) 20% v/v, penicillin 100 IU/ml and streptomycin 0.1 mg/ml (Flow Laboratories). Cells were cultured in polystyrene tissue-culture flasks in an atmosphere of CO₂ 5% in air and 98% humidity at 37°C. Confluent growth was obtained when flasks were incubated for 8–10 days, changing the growth medium at least once every 3 days. Cells were counted by microscopy on a haemocytometer slide with phase-contrast microscopy; *c.* 10⁶ cells/ml were initially attached to the polystyrene and *c.* 10⁴ cells/ml remained in suspension.

In studies of bacterial adherence, KATO-III cells were subcultured and grown in 12-well tissue-culture cluster dishes. After 8–10 days, the number of gastric epithelial cells adherent to polystyrene was approximately 2 × 10⁶ cells/well. For electronmicroscopy, the KATO-III cell monolayers were washed twice with Hanks's Balanced Salts Solution (HBSS; Flow Laboratories). *H. pylori* cells grown in supplemented Brucella broth for 18 h were resuspended in RPMI-1640 without antibiotics (pH 7.6), and 0.5 ml of medium containing 1 × 10⁸ cfu was then added to each well. Cluster dishes were incubated in micro-aerophilic conditions at 37°C for 3.5 h. After incubation, wells were washed twice with HBSS to remove non-adherent bacteria. The KATO-III cells and adherent bacteria were then removed from the wells with a rubber scraper. Samples were prepared for SEM and TEM, as described previously.¹⁸

For quantitation of *H. pylori* adherence to KATO-III cells, 10⁸ ³H-labelled bacteria were resuspended in 1 ml of RPMI-1640 (pH 7.6) without antibiotics and added to the wells of a cluster dish. Non-specific binding of bacteria to polystyrene was examined by incubation of an equal number of radiolabelled *H. pylori* in wells that did not contain KATO-III cells. The cluster dishes were incubated in micro-aerophilic conditions for 3.5 h at 37°C. Non-adherent bacteria were then removed by six washes with 1 ml of Dulbecco's PBS (Flow Laboratories). To remove adherent *H. pylori* and KATO-III cells from the wells, trypsin (Flow Laboratories) 0.25% w/v was added and incubated at 37°C for 10 min. The number of bacteria adherent to KATO-III cells and polystyrene surfaces was determined by counting dpm in 0.1-ml samples collected from each well after trypsinisation. Viability of KATO-III cells was determined at the beginning and end of the assays by trypan blue exclusion.

Adherence assays were also performed at 25°C and 4°C to determine whether binding of *H. pylori* was temperature-dependent. The effect of decreasing the pH of the assay medium on bacterial attachment was examined by measurement of adherence of *H. pylori* to KATO-III cells at pH 5.0 and 2.0. To examine the effects of inhibition of bacterial protein synthesis on adherence, erythromycin lactobionate (Abbott Laboratories, Montreal, Quebec, Canada) was added to bacterial suspensions at sub-inhibitory concentrations

(0.1 and 1 mg/L) and at an inhibitory concentration (10 mg/L) 15 min before incubation with KATO-III cells. The minimum inhibitory concentration (MIC) of erythromycin for *H. pylori* strain LC-3 grown in broth culture was 5 mg/L.

Statistical analysis

Results are expressed as mean and SEM. Differences between groups were calculated by the two-tailed unpaired Student's *t* test.¹⁹

Results

Growth of *H. pylori*

As shown in fig. 1, turbidity and viable counts of *H. pylori* cultures reached a maximum within 18 h. After 18 h, clumping of bacteria and a decrease in motility were observed by phase-contrast microscopy. Numbers of viable bacteria also declined precipitously (fig. 1). Optical densities and viable counts of cultures were similar for a fresh isolate (strain LC-11) and an isolate (strain LC-3) that had been stored at -70°C for 1.5 years after 18 h in supplemented Brucella broth. Viable bacterial counts indicated that cultures contained 2×10^8 cfu/ml at 18 h. Growth of *H. pylori* in Brucella broth was reproducible when cultures were passaged serially after incubation for 15–18 h. Bacteria had the spiral morphology typical of *H. pylori* seen in the human antrum *in vivo*.

Incorporation of radiolabelled amino acids

Incorporation of radioactive label into bacteria was determined after 18 h because the number of viable organisms and optical density in broth cultures was maximal at this time. Maximum incorporation of radiolabelled proline and histidine by *H. pylori* occurred after incubation for 18 h and label was retained by the organisms until the bacteria reached stationary phase. The growth curve for *H. pylori* in broth

supplemented with proline was similar to that obtained without supplementation. The average incorporation of ^3H -proline was 10^{-3} dpm/bacterium. During the 3.5-h period used in the adherence assays, there was minimal elution of label from bacteria into the tissue-culture medium ($<5\%$ of incorporated ^3H). Under the conditions of these tests, tritiated amino acids were found to be more suitable for use in subsequent experiments because *H. pylori* released $^{14}\text{CO}_2$ when ^{14}C substrates were added to culture media.

Adherence of *H. pylori* to KATO-III cells

SEM suggested that attachment of spiral-shaped bacteria to the surface of KATO-III cells had occurred (fig. 2). TEM confirmed that *H. pylori* were adherent to KATO-III cells. Some bacteria were adherent to intact microvilli while others were closely bound to the plasma membrane of the gastric epithelial cells (fig. 3). Microvilli were absent at sites of close attachment and the plasma membrane of KATO-III cells was raised to form cup-like projections referred to as "pedestals" (fig. 3).

As shown in fig. 4, studies with radiolabelled *H. pylori* also confirmed their adherence to KATO-III cells (20 198 SEM 1434 dpm). In contrast, there was only minimal binding of organisms to polystyrene surfaces alone (738 SEM 103 dpm, $p < 0.00001$). Serial plating to quantitate the number of viable adherent organisms bound to KATO-III cells demonstrated that there were $(3.5 \times 10^3) - (4 \times 10^5)$ cfu/well. These numbers were less than the 10^7 bacteria/well expected from calculations based upon estimations of dpm.

Binding of *H. pylori* to KATO-III cells was greater at 37°C than at either room temperature or 4°C (fig. 4). The pH of assay medium (pH 7.6) remained stable at each of three ambient temperatures tested. At pH 5, the number of *H. pylori* adhering to KATO-III cells was not reduced even though $>75\%$ of gastric epithelial cells were no longer viable at the end of the incubation period. This figure contrasts with the finding that $>90\%$ of the epithelial cells remained

Fig. 1. Growth curve of *H. pylori*, strain LC-3. Optical density (○) (at 600 nm) and colony-forming units (●) were determined every 6 h for 36 h.

Fig. 2. Scanning electronmicrograph of *H. pylori* strain LC-3 after incubation with KATO-III cells for 3.5 h at 37°C.

viable at the end of the 3.5-h incubation period when the pH of the assay medium was 7.6. In acidic conditions (pH 2.0), bacterial adherence to KATO-III cells was significantly reduced (9298 SEM 255 dpm, $p < 0.001$); <10% of gastric epithelial cells were viable after 3.5 h under these conditions.

Sub-inhibitory concentrations of erythromycin enhanced the binding of *H. pylori* to KATO-III cells—at 0.1 mg/L, 39 752 SEM 4030 dpm and at 1 mg/L, 34 419 SEM 6248 dpm. An inhibitory concentration of erythromycin (10 mg/L) did not affect adherence of the organisms (25 824 SEM 2470 dpm).

Discussion

Adherence of pathogenic bacteria to target cells is an important step in the pathogenesis of many bacterial diseases.²⁰ For example, following attachment of organisms to gut mucosal surfaces, host tissues are exposed to higher concentrations of bacterial enterotoxins.²¹ Adherence is also important for entry of organisms into epithelial cells.²² However, little is

known about the factors that promote attachment to and colonisation of the gastric mucosa by the newly-recognised pathogen *H. pylori*. Histological examination of biopsy specimens from the antrum of human stomach has revealed *H. pylori* within gastric mucus and adherent to the apical membranes of gastric epithelial cells.⁸ Depletion of microvilli and disruption of cytoskeletal filaments were observed at points of bacterial attachment. These adherence pedestals on the cell surface appeared morphologically identical to lesions observed in the small and large intestines in association with enteropathogenic *E. coli* infections.^{10, 23}

In the present study, we have demonstrated attachment of *H. pylori* to epithelial cells of gastric origin *in vitro*. Adherence of *H. pylori* to KATO-III cells was morphologically identical to the *in-vivo* observations reported previously.⁶⁻⁸ Clinical isolates of *H. pylori* bind to, and mediate mannose-resistant haemagglutination of, erythrocytes derived from man and from several animal species.²⁴⁻²⁶ Evans *et al.*²⁷ characterised a surface-associated haemagglutinin which is expressed by *H. pylori* as a fibrillar adhesin. *In-vitro* studies showed that *H. pylori* also bind to HEP-2 and Intestine-407 human epithelial cell lines¹¹ as well as Y-1 murine adrenal cells²⁸ in tissue culture. Although these established cell lines are not derived from gastric epithelium, TEM has demonstrated that *H. pylori* adheres closely to the surface of plasma membranes.

In bacterial adherence assays, adherent bacteria can be counted by microscopy, live adherent bacteria can be quantitated by surface viable counts and attached labelled bacteria determined by measurements of radioactivity. As *H. pylori* requires incubation for ≥ 5 days to achieve adequate growth on agar media, a rapid and reproducible method for quantitation of bacterial adherence with radiolabelled organisms was employed in most assays in this study. Due to the fastidious growth requirements of *H. pylori in vitro*, differences between numbers of adherent bacteria as estimated by measurement of dpm and viable counts can probably be accounted for by a decrease in numbers of viable organisms with time.

Fig. 3. Transmission electronmicrograph demonstrating adherence of *H. pylori* to KATO-III cells after incubation for 3.5 h (panel A, $\times 7200$). Some bacteria are adherent to microvilli on KATO-III cells (arrows), others (arrowheads) are attached to pedestals in regions where microvilli are disrupted (panel B, $\times 10\,300$).

Fig. 4. Adherence (dpm 10^3 /well) of *H. pylori* strain LC-3 to KATO-III cells (□) and polystyrene surfaces (■) at 4°C, 25°C and 37°C, bar, SEM.

Minimal binding of radiolabelled bacteria to plastic alone and the negligible loss of radioisotope from *H. pylori* both indicated that free radioisotope was not an important confounding variable in these experiments.

Adherence of *H. pylori* to tissue-culture cells was significantly reduced at extreme acidity when compared to the binding of bacteria at pH 5.0 or pH 7.6. The reduction in bacterial binding to KATO-III cells at pH 2.0 was probably related to reduced viability of organisms since Itoh *et al.*²⁹ showed that *H. pylori* does not survive below pH 2.5. Nevertheless, since < 10% of gastric epithelial cells were viable at this pH value, it is possible that receptors for binding of organisms were chemically modified in a strong acid environment. However, because the micro-environ-

ment in which *H. pylori* resides *in vivo* is likely to be close to neutral pH further in-vitro characterisation of bacterial adherence was performed at pH 7.6. In addition, adherence of *H. pylori* to KATO-III cells was a temperature-dependent process that was inhibited at 4°C. Inhibition of *H. pylori* binding at 4°C suggested that continuous bacterial protein synthesis was required for adherence.

Previous studies have shown that the expression and normal function of bacterial adhesins are variably inhibited or promoted by incubation of bacteria with subinhibitory concentrations of antibiotics.³⁰ Therefore, the *H. pylori* adherence assay was performed in the presence of subinhibitory concentrations of erythromycin. Although ultrastructural changes, such as incomplete cell-wall development, have been observed when *H. pylori* is grown in inhibitory concentrations of erythromycin,³¹ our results indicated that subinhibitory concentrations of erythromycin enhanced the binding of *H. pylori* to epithelial cells *in vitro*. Increased bacterial attachment to eukaryotic cells in the presence of sublethal antibiotic concentrations has been demonstrated previously for other human pathogens including *E. coli*, *Enterococcus faecalis* and *Streptococcus sanguis*.³⁰ The mechanism underlying enhanced binding of these organisms in the presence of certain antibiotics is not understood.³⁰

The similarities observed between the binding of *H. pylori* to tissue culture cells and the adherence of the bacteria *in vivo* indicates that the adherence assay described will serve as a useful in-vitro model for investigations aimed at defining bacterial adhesin(s) and host receptor(s) which mediate attachment of *H. pylori* to eukaryotic cell surfaces.

P.S. is the recipient of a Career Scientist Award from the Ontario Ministry of Health.

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