Influence of soluble haemagglutinins on adherence of *Helicobacter pylori* to HEP-2 cells

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Summary. In a study of six laboratory strains of *Helicobacter pylori*, two different modes of bacterial adherence to HEP-2 cells were found. Electron microscopy revealed that strains known to possess soluble haemagglutinin adhered intimately to the cell surfaces, with cupping of the plasma membrane and coalescence of glycocalyxes at sites of attachment. Strains of *H. pylori* without soluble haemagglutinin also attached, but did not induce membrane cupping or show glycocalyx fusion. Light microscopy did not distinguish between these patterns of adherence. Bacterial attachment was unaffected by pre-treatment of HEP-2 cells with neuraminidase. Exposure of the bacteria to trypsin or to colloidal bismuth subcitrate (CBS) before being added to HEP-2 cells markedly impaired bacterial adherence. This effect of CBS may contribute to the known efficacy of bismuth therapy in patients with *H. pylori*-related gastritis.

Introduction

Active chronic gastritis and peptic ulcer disease are strongly associated with colonisation of the gastric mucosa by curved or spiral bacteria.^{1–3} These organisms, previously known as *Campylobacter pylori*, have been re-designated as *Helicobacter pylori*.⁴ *H. pylori* displays a preferential affinity for epithelium of the gastric antrum, attaching to mucus-secreting cells through adherence pedestals.^{2, 5–7} These attachment sites are similar to those formed by enteropathogenic *Escherichia coli* on intestinal epithelium.⁸ The ability of bacterial pathogens to adhere to mucosal surfaces and so avoid expulsive mechanisms such as peristalsis, is crucial for colonisation⁹ and it seems likely that adherence of *H. pylori* in the stomach is a pre-requisite for establishment and persistence of infection.

H. pylori can adhere to a wide range of cell types *in vitro* including mouse Y-1 adrenal cells,¹⁰ human buccal epithelial cells,¹¹ HEP-2 and Intestinal 407 cells,¹² and to HeLa cells.¹³ Presumably there are special factors that effectively restrict its adherence behaviour *in vivo*; but *in-vitro* models can be useful for elucidating the modes and mechanisms of interaction between bacterial ligands and surface membrane receptors.¹⁴ The ability of bacterial ligands to bind with receptors on various species of erythrocytes has led to haemagglutination being employed for the detection of such ligands, or haemagglutinins. It has

identified the ligands as colonisation factors of *E. coli*,¹⁵ *Vibrio cholerae*¹⁶ and *Aeromonas* spp.¹⁷ For *H. pylori* there have been varying reports of haemagglutination. Evans *et al.*¹⁸ reported that all strains agglutinated strongly all species of erythrocytes tested; but others have described different patterns of haemagglutination with some strains failing to agglutinate any of the erythrocyte species tested.^{19,20}

A possible explanation emerged with the finding²¹ that *H. pylori* strains may possess both cell-associated and soluble haemagglutinins, and that the latter is sometimes absent, possibly having been lost in the course of repeated passage. Of two standard passaged strains of *H. pylori*, NCTC 11637 and NCTC 11638, the former regularly produced soluble haemagglutinin whereas the latter did not. An early subculture of NCTC 11638 however, which had been lyophilised 1 month after initial isolation in 1982, had retained the ability to produce soluble haemagglutinin. Other similarly paired and individual strains, with or without soluble haemagglutinins, were identified.²¹

In the present study we have used some of these strains to investigate the influence of soluble haemagglutinin on the adherence of *H. pylori in vitro*, and also the behaviour of the bacterial glycocalyx in this process.⁴ We report also the effects of several treatments that might modify adhesion, including exposure of the organisms to colloidal bismuth subcitrate (CBS).

Materials and methods

Bacterial strains

Six strains of *H. pylori* were studied. Four, here designated Q4P, R107P, Q3E and Q3P, were isolated

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from gastric endoscopic biopsy specimens from patients with gastritis at the Royal Perth Hospital. Strains Q4P (NCTC 11637) and R107P have undergone repeated passage in our laboratory, whereas strains Q3E and Q3P comprise, respectively, the 'early' and 'repeatedly-passaged' forms of strain NCTC 11638. Strains designated CP2 and 512 were kindly supplied by Dr Wee Tee (Fairfield Hospital, Melbourne); they too had originated as human gastritis-related isolates and had been passaged repeatedly. Erythrocyte agglutination assay²¹ showed that all six strains possessed cell-associated haemagglutinin. Strains Q4P, R107P and Q3E also produced a soluble haemagglutinin whereas strains Q3P, CP2 and 512 consistently failed to do so. All were preserved by lyophilisation, or at -70°C in peptone water (Oxoid) 1% w/v with glycerol 25% v/v. Stock cultures were maintained on solid medium for no longer than 1 month.

Media and reagents

Cultures were maintained on Brain Heart Infusion Agar (Oxoid), with horse blood lysed with saponin, and incubated in a micro-aerophilic atmosphere containing CO_2 8%, O_2 5%, H_2 7% and N_2 80%.²¹ Phosphate-buffered saline (PBS) at pH 7.2 was used to wash the bacterial suspensions.

Adherence assay

Bacteria were inoculated on to lysed blood agar and harvested into PBS after 2–3 days to give a concentration equivalent to McFarland standard No. 4—c. $(2-4) \times 10^9$ cfu/ml. HEP-2 cells, obtained from CSL Laboratories Melbourne, were grown in Earle's Modified Eagles Medium supplemented with fetal bovine serum 10% v/v and glutamine 1% w/v with penicillin 50 IU/ml, neomycin 60 mg/ml and streptomycin 100 IU/ml, and buffered with sodium bicarbonate solution. Culture plates with 24 wells containing coverslips were inoculated with 1 ml of cell suspension and incubated for 24 h to obtain semi-confluent monolayers. Monolayers were washed three times with PBS and 1 ml of Eagles Medium added, without antibiotics. Bacterial suspension (50 μl) was added to each monolayer and the plates were then incubated at 37°C in an atmosphere containing CO_2 5% v/v for periods of up to 1 h. The monolayers were washed three times with PBS, fixed in methanol 70% v/v for 10 min and stained with Giemsa stain 10% v/v or by the Warthin-Starry silver impregnation method with Light Green counterstain.^{22,23} Giemsa-stained specimens were mounted in DPX (BDH, Kilsyth, Victoria) and the Warthin-Starry preparations in 'Entellan' (Merck, Darmstadt, Germany) and examined by light microscopy. At least four microscopic fields, comprising at least 200 cells, were assessed, and the numbers of cells with and without visibly adherent bacteria were counted. Results were expressed as the percent-

age of cells with attached bacteria and also, where feasible, as the mean number of bacteria/cell. All experiments were performed in duplicate and with parallel controls.

Treatment of bacteria and cells prior to adherence assays

Trypsin (Sigma) (1 ml) at a concentration of 1 mg/ml in PBS was added to 0.25 ml of bacterial suspension of strains Q4P, R107P, Q3E and Q3P, which were then incubated at 37°C for 30 min. After centrifugation (10 000g for 1 min) the organisms were washed twice with PBS and resuspended in PBS to a concentration equivalent to McFarland standard No. 4; 50 μl of this was added to HEP-2 cell monolayers and the adherence assay was done as described.

Neuraminidase from *Clostridium perfringens* (Sigma) was prepared in PBS at concentrations of 1 unit and 2 units/ml; 0.5 ml was added to HEP-2 cell monolayers and incubated at 37°C for 10 min. The cells were washed twice with PBS before use with strains Q4P, Q3E and Q3P.

Fetuin and mannose. Fetuin (Sigma) at 2 mg/ml in PBS, or mannose at 10 mg/ml, were added to an equal volume of the various bacterial suspensions and incubated at room temperature for 20 min.

n-Octylglucose (NOG). Bacteria of strains Q4P, Q3E and Q3P were suspended in 1 ml of NOG (Sigma) 0.5% w/v and incubated for 20 min at room temperature. Suspensions were then centrifuged at 10 000g for 1 min and washed twice with PBS before use.

Mucin. Bacteria of strains Q4P, Q3E and Q3P were suspended in porcine mucin (Sigma) 2% w/v in PBS, and left on ice for 10 min.

Colloidal bismuth subcitrate (CBS) (De-Nol[®]: Gist-Brocades, The Netherlands) was made up in PBS and mixed with suspensions of strains Q4P and Q3P, to give a final concentration of 1 mg/ml. Mixtures were incubated at room temperature for 30 min.

Electronmicroscopy

Thin sectioning was used to examine the adherence to HEP-2 cells by all six strains of *H. pylori* and also the effects of trypsin, NOG and CBS treatments (of selected strains as above), and neuraminidase pretreatment of the HEP-2 cell monolayers. The coverslip monolayers were fixed *in situ* by immersion in glutaraldehyde 2.5% v/v in 0.16 M cacodylate buffer, pH 7.2, for 24 h at room temperature. After washing with fresh buffer solution the cell sheets were dislodged from the glass with a rubber pusher and incubated, for stabilisation of the glycocalyxes, in a solution of tannic acid (Sigma) 1% w/v in 0.16 M cacodylate buffer, pH 7.2, for 24 h at 4°C . Subsequent processing comprised post-fixation with aqueous osmium tetroxide 1% w/v, dehydration and infiltration with epoxy resin. Duplicate specimens were processed similarly but omitting tannic acid treatment. Thin sections on

naked copper grids were viewed with a Philips EM 410 (HM) electronmicroscope, after staining with uranyl acetate and lead citrate.

Results

Adherence to HEp-2 cells as seen by light microscopy

With all strains of *H. pylori* except strain Q4P, light microscopy of monolayers incubated for 5 min showed bacteria adhering to only about 10% (range 5–15%) of HEp-2 cells. Strain Q4P exhibited more avid adherence with some 60% of cells showing attached bacteria. In all monolayers, the intercellular spaces remained virtually free of visible organisms. Adherence levels increased with incubation time. With all strains after 30 min, 100% of HEp-2 cells had adherent bacteria (fig. 1). The mean number of organisms/cell after 30 min was higher (at almost 50/cell) for strain Q4P than for the other strains. In most monolayers the heaping of abundant organisms over some individual cells made counting difficult. No differences between strains, as to their precise mode of attachment to the cell surfaces, were evident by light microscopy.

Electronmicroscopy

Electronmicroscopy revealed two different modes of interaction between the bacteria and the HEp-2 cells, which were distinctive and consistently strain-related. Strains Q4P, R107P and Q3E gave rise to profiles of intimate contact, with shallow cup-like indentations of the cell surface reminiscent of that seen at adherence pedestals *in vivo* on the gastric mucosa (figs. 2 and 3A). After tannic acid processing for polysaccharide stabilisation, the sites of adherence by these strains of *H. pylori* showed focal blending, or fusion, of the contiguous bacterial and epithelial glycocalyxes (fig. 3B). In contrast, strains Q3P, CP2 and 512 failed to develop the same intimacy of adherence; although coming into close apposition with the cell surface (figs. 4 and 5A) they failed to induce the cupping of plasma membranes, or to exhibit coalescence of the glycocalyxes despite the bacterial glycocalyx appearing no less well developed than in the other strains (fig. 5B).

Effect of pre-treatment on adherence

Pre-incubation of the bacteria with trypsin resulted in a marked impairment of adherence by all four of

Fig. 1. HEp-2 cell monolayer with adhering *H. pylori* strain Q3P, 30 min after addition of the bacterial suspension. Warthin-Starry silver stain, $\times 1000$.

Fig. 2. Electronmicrograph of thin section of HEp-2 cell monolayer inoculated with *H. pylori* strain Q4P and showing organisms heaped and adhering intimately to the cell surface. Bar = 500 nm.

Fig. 3. Electronmicrographs of thin sections showing sites of intimate adherence by *H. pylori* strain Q4P to the HEP-2 cell surface. A shows the appearance after conventional processing, with bacteria embraced in cup-like indentations of the cell membrane. In B, a specimen processed with tannic acid, the glycocalyx appears to be involved in the pedestal-like attachment. Bars = 500 nm.

the *H. pylori* strains tested; <5% of HEP-2 cells showed any adherent organisms after 30 min, compared to 100% in the paired controls. Attached bacteria were too few, in all specimens, for adequate visualisation of attachment by electronmicroscopy of thin sections.

Addition of fetuin or mannose had no detectable influence on adherence behaviour of the various strains; appearances were indistinguishable from their respective controls.

Treatment of *H. pylori* strains Q4P and Q3E with NOG resulted in a marked reduction in the numbers of cells with adhering bacteria. In control preparations, without NOG, 100% of HEP-2 cells showed attached organisms after 30 min. With NOG, <1% of cells for strain Q3E and <50% in the case of strain Q4P had any attached bacteria. Adherence of strain Q3P, in contrast, appeared to be quite unaffected by the pre-incubation with NOG. Electronmicroscopy of NOG-treated bacteria revealed a severe ultrastructural disruption affecting the interior of the organisms, with a resulting distortion of their shape. However, the microbial cell walls and the glycocalyx were largely preserved and the different modes of adherence between strains Q4P and Q3P were still apparent.

In the presence of porcine gastric mucin, adherence by all strains of *H. pylori* was enhanced. This was not quantifiable in terms of HEP-2 cell numbers with attached organisms as virtually 100% levels were achieved in the parallel control preparations after incubation for 30 min, but it was evident in terms of mean numbers of bacteria/cell. For example, with addition of mucin the mean number of organisms/cell for strains Q3E and Q3P rose to 30 (range 20–50)

compared with control values of 15 (range 10–30). For strain Q4P the mean number of organisms adhering increased to 75/cell from a control value on this occasion of 50/cell.

Pre-incubation of strains Q4P and Q3P with CBS resulted in *c.* a 10-fold reduction of bacterial adherence to the HEP-2 cells. For strain Q3P, 11% of cells had adherent organisms after 30 min, with only 3–5 organisms/cell, compared to 100% of cells and a mean of 45 organisms/cell in the untreated control. For strain Q4P, about 50% of cells had adherent organisms (5–20 organisms/cell) compared to the control in which all cells showed 50–70 adherent bacteria. Electronmicroscopy revealed no obvious change in the modes of adherence exhibited by the two strains of *H. pylori* apart from a marked reduction in the frequency with which sites of cell-bacteria contact were visualised by thin sectioning.

Exposure of HEP-2 cells to neuraminidase had no visible effect on the subsequent bacterial adherence behaviour of any of the strains tested. In particular, from electronmicroscopy it was evident that the qualitatively distinct modes of adherence shown by strains Q4P and Q3P were not modified by neuraminidase treatment of the target HEP-2 cells.

Discussion

This study of six laboratory strains of *H. pylori* has revealed significant differences with regard to the avidity and precise mode of their adherence *in vitro* to an epithelial cell line. One group of strains, comprising Q4P, R107P and Q3E, regularly showed a propensity

Fig. 4. Electronmicrograph of thin section of a HEp-2 cell monolayer showing close but not intimate adherence of *H. pylori* strain Q3P, 30 min after addition of the bacterial suspension. Bar = 500 nm.

for intimate attachment to the epithelial plasma membrane with coalescence of the glycocalyxes, and also cupping profiles as reported in other *in-vitro* systems.^{10,12} The phenomenon seems analogous to adherence pedestal formation on the gastric mucosa in infected patients.^{2, 5-7, 24-26}

The other strains (Q3P, CP2 and 512) failed to adhere so intimately, lying close to the cell surface but without cupping or glycocalyx fusion. We found the bacterial glycocalyx to be similarly developed in both groups and possession of cell-associated haemagglutinin is also common to both.²¹ However, those strains of *H. pylori* in the first group also produced soluble haemagglutinin whereas the others did not. The findings suggest that this property may indeed promote the intimate mode of adherence. Soluble haemagglutinin production could thus be similarly important in promoting colonisation of gastric epithelium *in vivo* and be a significant factor in pathogenesis of *H. pylori* infection. At the same time, adherence behaviour of this organism *in vivo* is clearly selective, and is largely restricted to the gastric mucus-secreting cell type. *In vitro*, however, it will adhere to a variety of cell lines,¹¹ suggesting that additional factors limiting bacterial behaviour in the human stomach may be lacking in cell-culture systems.

The reason why only some strains of *H. pylori* should yield soluble haemagglutinin merits further study and a comparison of strain Q3E (adhering intimately, and a producer of soluble haemagglutinin) with Q3P (failing to adhere intimately, and lacking soluble haemagglutinin) may be significant. This pair of strains consist, respectively, of a salvaged early subculture and a repeatedly-passaged strain of the same original isolate (NCTC 11638). Our findings

Fig. 5. Electronmicrographs of thin sections showing adherence sites of *H. pylori* strain Q3P, for comparison with fig. 3. **A** shows the appearance after conventional processing, and **B**, with tannic acid, revealing the bacterial and epithelial glycocalyxes. Bars = 500 nm.

support a previous suggestion that soluble haemagglutinins may have been reduced or entirely lost by some strains during passage.²¹ Nevertheless, the properties of other laboratory strains (e.g., Q4P, identical to NCTC 11637) testify that such loss is by no means an invariable outcome of repeated passage.

Evans *et al.*¹⁸ reported that the haemagglutinin of *H. pylori* could be extracted by NOG. Soluble haemagglutinin is also a feature of the strains described by Evans *et al.*,^{18, 21} therefore, we attempted in this study to elute the soluble haemagglutinin with NOG to examine the effect on bacterial adherence. By light microscopy, adherence of the strains producing soluble haemagglutinin (Q4P and Q3E) was seen to have decreased, whereas that of a strain not possessing soluble haemagglutinin (Q3P) was unchanged. However, electronmicroscopy revealed that the treatment with NOG caused considerable damage to the internal bacterial structure, although the cell wall appeared to remain intact. Viability of the NOG-treated specimens was markedly affected. Thus, although our findings do suggest different effects of NOG on the two types of strains, the severe morphological damage to the organisms invalidated a comparison related to production of soluble haemagglutinin alone.

We found bacterial adherence to be impaired significantly by treatment with trypsin, suggesting that protein moieties are concerned in the mechanisms of adherence. Addition of fetuin or mannose had no effect, at least as monitored by light microscopy, in agreement with the findings of Leunk *et al.*²⁷ In the presence of porcine gastric mucin there was a modest, but consistent, enhancement of adherence to HEP-2 cells by all the strains of *H. pylori* tested. This apparently reflects the influence of some as yet uncharacterised promoting factor, or factors, present in mucins of gastric origin.

Incubation of *H. pylori* with CBS resulted in a

substantially decreased adherence to HEP-2 cells by strains Q4P and Q3P; that is, by strains with and without soluble haemagglutinin. Clinically, CBS has an important role in the therapeutic approach to *H. pylori*-related gastritis^{3, 5, 26} but its mode of action has remained unclear. Bactericidal activity against *H. pylori* has been demonstrated *in vitro*, but was of delayed onset with a lag period of several hours.²⁸ However, in patients undergoing endoscopic biopsy, most of the colonising bacteria had already dislodged from the mucosa and were degenerating within 90 min of the initial dosage with CBS.²⁶ Interference with bacterial adherence was suggested as a possible mode of action. The present findings are in keeping with this suggestion, but precise mechanisms underlying CBS impairment of *H. pylori* adherence have yet to be determined.

In a previous study,²¹ pre-incubation of erythrocytes with neuraminidase effectively abolished the haemagglutinating activity of soluble haemagglutinin produced by strains Q4P and Q3E, but not that of the cell-associated haemagglutinins. However, we found here that neuraminidase treatment of HEP-2 cells did not significantly influence subsequent adherence by strains Q4P, Q3E or Q3P of *H. pylori*, either quantitatively or (at the ultrastructural level) by modifying the pattern of intimate adherence typically shown by the two soluble haemagglutinin-producing strains. This disparity in the blocking role of neuraminidase suggests that the phenomena may involve different membrane receptors, and supports the view that soluble haemagglutinin of *H. pylori* is polyvalent.²¹ A novel *H. pylori*-binding glyceroglycolipid was recently extracted by Lingwood *et al.*²⁹ from gastric mucosa, human erythrocytes and also HEP-2 cells. It was proposed as the receptor for *H. pylori* common to all three situations, but has yet to be characterised in structural terms.

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