BACTERIAL PATHOGENICITY

Studies on the relationship between adhesive activity and haemagglutination by *Helicobacter pylori*

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The adhesion of *Helicobacter pylori* to gastric carcinoma cells (MKN45, KatoIII and MKN28) and Intestine-407 cells was tested by flow cytometric analysis. The mean adhesion rates of *H. pylori* strains to MKN45, KatoIII and Intestine-407 cells were 90.5, 42.7 and 15.1%, respectively. There was no statistical correlation between the adhesion rates to MKN45 cells and haemagglutination (HA) activity of *H. pylori* strains, although *H. pylori* strains with high HA activity with human type O erythrocytes tended to adhere effectively to MKN45 cells. No correlation between adhesion and production of vacuolating toxin was observed.

Introduction

Helicobacter pylori is a curved spiral gram-negative bacterium that is implicated as a gastroduodenal pathogen in man. It is now recognised as an aetiological agent of chronic active (type B) gastritis and peptic ulcer disease [1–3]. In addition, there is an epidemiological association with the incidence of gastric carcinoma [4, 5].

There are many reports on the adhesion of *H. pylori* to various cultured epithelial cells by microscopical analysis [6–9]. Flow cytometry with cultured cells was demonstrated to be a sensitive and objective method to evaluate adhesion of *H. pylori* [10–12]. The haemagglutinin of *H. pylori* has been implicated as an adhesin or colonisation factor antigen [13–17].

Several virulence factors of *H. pylori* – urease, flagella, adhesin and vacuolating toxin (VT) – are recognised. Furthermore, a subset of strains also produce VT, which induces cellular vacuolation in a number of different epithelial cell lines *in vitro* [18–20], and VT is thought to be important for virulence of *H. pylori* in a mouse model of disease [21, 22]. Although cytotoxin associated gene product (CagA) has been implicated in the production of cytokines by gastric epithelial cells after *H. pylori* infection, it was reported recently that *picAB* genes upstream to the

cagA gene are responsible for the production of cytokines by gastric epithelial cells [23].

This study examined adhesion of *H. pylori* to various cultured cells. Moreover, to study strain diversity, the adhesion rate of *H. pylori* strains was compared with their haemagglutination activity (HA) and production of vacuolating toxin (VT).

Materials and methods

Bacterial strains and growth conditions

H. pylori TK and KR strains were isolated from gastric biopsy samples of patients as described previously [24]. *H. pylori* NCTC 11638 was kindly provided by Dr T. Itoh (Tokyo Metropolitan Research Laboratory of Public Health). All strains were stored at –80°C until used in this study. For the assay of HA and adhesion, they were cultured on Brain Heart Infusion (BHI) Agar (Difco) containing defibrinated horse blood 5% (v/v) under micro-aerophilic conditions (O₂ 5%, CO₂ 10%, N₂ 85%) for 3–4 d at 37°C. These cultures were harvested in Hanks's Balanced Salts Solution (Gibco BRL, Gaithersburg, USA) containing gelatin 0.1% (HGS).

Cell culture

Human gastric carcinoma cells, MKN45, MKN28 and KATOIII, were obtained from the Japanese Cancer Research Resources Bank. The human intestinal cell

line, Intestine (Int)-407 was kindly provided by Dr Kanamori (Kyorin University). They were grown at 37°C in RPMI-1640 or kanamycin-free Eagle's MEM (Gibco) containing fetal calf serum (FCS) 10%, in an atmosphere containing CO₂ 5%. For the adhesion assay, cells were harvested from a flask by scraping with a sterile cell scraper (Falcon, NJ, USA), and centrifugation at 200 *g* for 5 min followed by resuspension in HGS.

Adhesion assay

Adhesion was assayed by the method of Dunn *et al.* [8]. The epithelial cells (5×10^6 cells) and *H. pylori* (5×10^8 – 1×10^9) were mixed in a sample tube (1 ml of HGS) followed by incubation at 37°C for 1 h with gentle shaking. Non-adherent bacteria were removed by centrifugation at 1000 rpm for 10 min on to 9 ml of sucrose 15% solution. Cells were washed once with HGS and treated with anti-*H. pylori* hyper-immune rabbit serum diluted 1 in 1000 with HGS for 30 min on ice. The cells were washed twice with HGS and stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (Capel, Durham, USA) diluted 1 in 20 with HGS for 30 min on ice. The cells were then washed twice with HGS and resuspended in 300 µl of PBS for the flow cytometric analysis. The EPICS-CS flow cytometer (Coulter Electronics, Hialeah, USA) was used for the measurement of bacteria adhering to the epithelial cells. The machine was gated to include single cells and to exclude not only cell debris but also non-adherent aggregated bacteria by means of a dot-plot display of forward-angle light scatter (FLS) and 90LS. Fluorescence data were acquired in log mode on a 256-channel scale. The mean fluorescence channel was calculated by an analysis of >5000 individual cells. The results were expressed as the percentage of fluorescent cells (positive percent) calculated from the fluorescence frequency distribution histograms. The threshold of cells without adherent bacteria was established for each experiment, which was stained with subsequent two-step FITC-conjugated *H. pylori* antibody staining as described above.

Haemagglutination assay

The haemagglutination assay was by the method of Kobayashi *et al.* [6]. *H. pylori* harvested from BHI agar plates containing horse blood 5% were washed once and suspended in PBS. Samples (100 µl); *c.* 10^8 cfu/ml) of the bacterial suspension to be tested were serially diluted in two-fold steps with 100 µl of PBS in a round-bottomed microtitration plate and 100 µl of erythrocytes (bovine, chicken, guinea-pig, horse, human, rabbit and sheep), 1% suspension in PBS, was added to each well and mixed. The plate was left at room temperature for 3 h. The titre was defined as the highest dilution showing visible haemagglutination.

Vacuolating toxin assay

Vacuolating toxin titre of culture filtrates of *H. pylori* was determined as described previously [20]. *H. pylori* was cultured in micro-aerophilic conditions with BHI broth containing FCS 5% for 6 days. The culture supernate to be tested was collected and was serially diluted in two-fold steps with RPMI-1640. Rabbit kidney (RK-13) cells were treated with the culture filtrates (50 µl) of *H. pylori*. After incubation for 24 h a morphological change of cytoplasmic vacuolation was observed microscopically. The titre was the highest dilution of the filtrate inducing vacuolation of >10% of cells.

Results

Adhesion of H. pylori to various cell lines

Adhesion of *H. pylori* to MKN45, KatoIII, MKN28 and Int-407 cells was tested by flow cytometry. The percentage (adhesion rate) of the fluorescence-positive cells was calculated according to the threshold between control cells which had not been exposed to *H. pylori* and positively stained cells with adherent *H. pylori*. The adhesion rates of *H. pylori* strain NCTC 11638 to MKN45, KatoIII, MKN28 and Int-407 cells were 87, 59, 3 and 14%, respectively (Fig. 1). During incubation with *H. pylori* strain NCTC 11638, many MKN28 cells were lysed and the adhesion rate of *H. pylori* was the lowest (3%). The mean adhesion rates of *H. pylori* clinical isolates to MKN45, KatoIII and Int-407 were 90.5% (39 strains tested), 42.7% (15 strains tested) and 15.1% (15 strains tested), respectively. The adhesion rate of *H. pylori* to MKN45 cells correlated with that for KatoIII cells ($r = 0.86$), but not that for Int-407 cells. The adhesion rate of 39 strains of *H. pylori* to MKN45 cells was between 57 and 98%, and the mean channel number of these histograms was between 132 and 209 (data not shown).

HA activity of H. pylori

Sixty-five strains of *H. pylori* were tested for HA activity with bovine, chicken, guinea-pig, horse, human, rabbit and sheep erythrocytes (Table 1). In the HA test with guinea-pig erythrocytes, it was shown that 63 strains (97%) of *H. pylori* were haemagglutination-positive and the mean titre ($2^{5.6}$) was the highest among the erythrocytes of seven species (Table 1). Of 65 *H. pylori* strains, 20 strains showed positive HA with erythrocytes of seven species. In contrast, only two strains showed negative HA with erythrocytes of the seven species tested. Seventeen *H. pylori* strains failed to haemagglutinate horse or bovine erythrocytes. No common characteristic profile in the HA test was demonstrated for the remaining 26 *H. pylori* strains (data not shown). There was no statistical correlation between HA titre and adhesive activity of strains of *H. pylori*. However, when the strains of *H. pylori* showing

Fig. 1. Flow cytometric analysis of adhesion of *H. pylori* to various cultured cells. **a**, MKN45; **b**, KatoIII; **c**, MKN28; **d**, Int-407; [□], histogram of control cells; [■], cells after incubation with *H. pylori*.

Table 1. Haemagglutination activity of *H. pylori* strains with erythrocytes of various animal species

Erythrocyte	HA positive strains (%) [*]	Mean HA titre (log ₂) [†]
Guinea-pig	97	5.6
Rabbit	95	3.6
Human‡	89	3.5
Sheep	88	3.0
Chicken	63	3.8
Horse	57	2.2
Bovine	57	2.5

^{*}Sixty-five strains of *H. pylori* were tested for HA.

[†]Mean HA titre of HA positive strains.

[‡]Blood type O.

high HA activity with human erythrocytes (titre 2⁵⁻⁷) were tested, adhesion to MKN45 cells was more intensive in the flow cytometric analysis with a mean adhesion rate of 92.4% (Table 2).

Table 2. HA activity and adhesive activity of *H. pylori* strains

HA titre (log ₂)	Number of strains tested	Mean (SD) adhesion rate (%)
0-1	8	78.9 (16.6)
2-4	21	87.9 (9.0)
5-7	10	92.4 (5.6)

Production of vacuolating toxin (VT)

Sixty-five strains of *H. pylori* were tested for production of VT; 32 strains (49%) were shown to be cytotoxic. There was no statistical correlation between VT titre and adhesion rate to MKN45 cells of 38 *H. pylori* strains (Table 3) tested for both. It was also shown that VT titre was not correlated with adhesion rate of *H. pylori* strains when KatoIII cells were used (data not shown).

Discussion

There have been many reports on the adhesion of *H. pylori* to various epithelial cells determined by microscopy [6-9]. In the present study, adhesion of *H. pylori*

Table 3. VT production and adhesiveness to MKN45 cells of *H. pylori* strains

Strain no.	VT titre (log ₂)	Adhesion rate (%)
TK1301	4	98.2
TK1308	4	96.4
TK1029	4	93.6
TK1076	3	97.4
TK1023	3	93.7
TK1302	2	98.4
TK1307	2	95.3
TK1135	2	94.1
TK1021	2	77.4
TK1057	2	68.6
TK1078	1	98.1
TK1313	1	97.7
KR2072	1	97.3
TK1147	1	96.8
TK1054	1	95.6
TK1042	1	95.5
KR2048	1	94.1
TK1102	1	93.7
TK1103	1	83.8
TK2070	0	96.3
TK1309	0	90.3
TK1063	-	97.6
TK1064	-	97.5
TK1030	-	95.5
TK1311	-	95.4
TK1003	-	94.9
TK1310	-	92.9
TK1028	-	91.1
NCTC11638	-	87.2
TK1025	-	86.6
TK1104	-	84.6
TK1008	-	84.4
TK1046	-	83.9
TK1079	-	74.8
TK1045	-	73.0
TK1022	-	65.4
TK1126	-	60.0
TK1036	-	57.4

to various gastric and intestinal epithelial cells was analysed by flow cytometry. The cells incubated with *H. pylori* were more intensively stained than control cells not incubated with bacteria. The mean adhesion rates of the tested strains of *H. pylori* to MKN45, KatoIII and Int-407 were 90.5, 42.7 and 15.1%, respectively. These results indicate that the MKN45 cell line is the most sensitive for adhesion of *H. pylori*. It was also shown by microscopy that many more bacteria attached to the cell surface of MKN45 than to other cells.

Many studies evaluating *H. pylori* adhesion *in vitro* have shown binding of the bacteria to various tissue culture cell lines, including KatoIII [10, 11, 25, 26], HEP-2 [26, 27] and Int-407 [28]. It has been reported that KatoIII is an ideal cell line for flow cytometric analysis as the cells grow in suspension and can be removed from the surface of the flask without the use of trypsin [10, 11]. Similarly, MKN45 cells grow both in suspension and on the surface of the flask, and it is not necessary to use proteolytic enzyme to remove the cells. Interestingly, a significant correlation was observed between the adhesion rates of *H. pylori* to MKN45 and to KatoIII, suggesting that a receptor for *H. pylori* on the cell surface of MKN45 was similar to that on KatoIII. Adhesion of *H. pylori* to MKN28 was not evaluable under the conditions used, because of cell lysis, indicating that MKN28 was not a suitable cell line for the adhesion assay by flow cytometry. The mechanism by which *H. pylori* adheres to various human gastric carcinoma cells with a different affinity is not clear. MKN45, KatoIII and MKN28 cell lines originated from poorly differentiated adenocarcinoma, signet cell carcinoma and well-differentiated tubular adenocarcinoma, respectively [29, 30]. Therefore, it is possible that the affinity of *H. pylori* to gastric carcinoma cells in terms of adhesion might be due to different surface structures based upon its tumour characteristics.

The adhesion rate of *H. pylori* to Int-407 was relatively low in comparison to MKN45 and KatoIII cells, suggesting that *H. pylori* has an affinity for gastric cells rather than intestinal cells. It is well known that *H. pylori* colonises the gastric epithelial cell surface and gastric metaplasia in the duodenum [31–34]. It was also reported that binding of *Helicobacter* spp. was greater to lipids derived from ferret antrum compared to colon in a solid-phase binding assay based on thin-layer chromatography [27].

Several investigators have confirmed the expression of HA by *H. pylori* [6, 11, 13–15]. Robinson *et al.* [15] reported a grouping of *H. pylori* strains according to erythrocyte HA of various animals. In their report, all except one of 14 strains showed HA with erythrocytes of guinea-pigs, man and sheep. Three groups were separated according to HA with chicken, bovine and

horse erythrocytes. Group 1 strains aggregate with chicken and bovine erythrocytes, but not with those of horse. Group 2a strains caused HA with chicken erythrocytes, but not with bovine or horse erythrocytes, and Group 2b strains were HA negative with chicken, bovine and horse erythrocytes. Similarly, in the present study, HA was less between *H. pylori* strains and chicken, horse and bovine erythrocytes than with guinea-pig, rabbit, human and sheep erythrocytes (Table 1). The heterogeneity of *H. pylori* strains with regard to the expression and specificity of surface haemagglutinins was demonstrated. It has been reported that among different species of erythrocyte, the sialic acid content is high in guinea-pig and human, but low in horse erythrocytes [35, 36]. Also, sialic acid as N-acetyl neuraminic acid is dominant in human erythrocytes, but sialic acid as an N-glycosyl derivative is dominant in horse erythrocytes [36]. The difference in the positivity of HA with erythrocytes of different species might be due to the quantity and quality of sialic acid located on erythrocytes.

HA by *H. pylori* was reported to be inhibited by pre-treatment of *H. pylori* with fetuin, a glycoprotein rich in sialylated oligosaccharides [11, 13, 15]. Furthermore, neuraminidase treatment of erythrocytes resulted in inhibition of HA [11, 13], indicating that the HA receptor on the erythrocytes contains sialylated oligosaccharides. We have reported previously that there is no significant inhibition in the adhesion rates of *H. pylori* to MKN45 cells by treatment with fetuin [12], in spite of the finding that HA was completely inhibited by fetuin. Other surface components on *H. pylori* have been reported to be associated with binding to cells. Chimiela *et al.* [37, 38] showed that *H. pylori* strains expressing sialic acid-independent haemagglutinin effectively attached to and were ingested by human polymorphonuclear leucocytes (PMNL), and that *H. pylori* strains expressing sialic acid-specific haemagglutinin effectively attached to, and were ingested by, human PMNL. It was also reported that sulphated glycosaminoglycans such as heparan sulphate bind to *H. pylori* with high affinity [39], suggesting the presence of heparan sulphate binding protein on the surface of *H. pylori*.

In this study, it was observed that the HA titre of *H. pylori* was not significantly correlated with adhesiveness to MKN45 cells, although the strains showing a high titre of HA tended to adhere to MKN45 cells with a high efficiency. These results suggest the possibility that the haemagglutinin and other surface components may be co-operatively involved in adhesion of *H. pylori* to cultured cells.

Among the virulence factors of *H. pylori*, VT appears to be important for the persistence of *H. pylori* and the occurrence of gastroduodenal diseases. It has been reported that gastric disease in an animal model was induced only by infection with VT-producing *H. pylori*

[21, 22]. Although this study showed that the ability of *H. pylori* to produce toxin was not correlated with adhesion to MKN45 cells, the direct effect of VT on the adhesion to MKN45 cells needs to be examined in detail.

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