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# ATTACHMENT OF *HELICOBACTER PYLORI* STRAINS TO HUMAN EPITHELIAL CELLS

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The aim of the study was to characterize several clinical isolates of *H. pylori* as regards the activity and specificity of their haemagglutinins and the involvement of surface sialic acid-specific and heparin-binding compounds in the adhesin of the bacteria to human epithelial cell lines. Although *H. pylori* strains caused haemagglutination (HA) of sheep erythrocytes, they differed markedly by activity and specificity. On the basis of haemagglutination inhibition study three types of *H. pylori* strains could be distinguished. The HA of Type I strains was inhibited with fetuin/mucin but not asialofetuin/asialomucin. The HA activity of Type II strains was inhibited with fetuin/mucin and asialofetuin/asialomucin. The HA of Type III strains was not influenced by any of these inhibitors. *In vitro*, *H. pylori* strains bound to the cells of human epithelial lines: HeLa, Kato-3, Ags. However, various compounds mediated the binding of *H. pylori* types distinguished by HA, to epithelial cells. The interaction of some of *H. pylori* strains with epithelial cells was mediated by bacterial sialic acid-binding compounds. The majority of *H. pylori* strains used heparin-binding surface compounds to attach to epithelial cells. Clinical *H. pylori* strains differ by the compounds used in adhesin to epithelial cell lines, however, this process also depends on the expression of appropriate receptors on the host cells.

Key words: *Helicobacter pylori, epithelial cells, adhesion, bacterial sialic acid-/heparin-binding compounds*

## INTRODUCTION

Most of bacterial pathogens enter the man organism through the mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts. The initial event of colonization and invasion is the adherence of microorganisms to the epithelial cells of the mucosal surfaces (1). The initiation of the immune response of the host against bacterial pathogens is also preceded by attachment of the bacteria to the host cells: dendritic celyl-9-0-alls and macrophages (2, 3).

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There is still little known on the initial step of colonization on the gastric mucosa by *H. pylori* bacteria, a gastric pathogen causing peptic ulcer diseases (4). Adherence of *H. pylori* bacteria, to gastric mucin and gastric epithelial cells has been suggested to initiate the infection (5). Identification of *H. pylori* adhesins would be an important step for establishing the specificity of bacteria-host interactions. Several *H. pylori* surface compounds have been proposed to play a role of adhesins (6). Interactions between possible *H. pylori* cell surface adhesion molecules and receptors on the host cells have often been screened by haemagglutination assay (7—10), since erythrocytes and epithelial cells commonly share similar glycoconjugate structures. It has been established that *H. pylori* strains express surface haemagglutinins with different specificity (7, 10). The inhibition of haemagglutinating activity of *H. pylori* bacteria by lectins suggested that the bacterium binds specifically to carbohydrate erythrocyte membrane compounds (10). It has been reported that attachment of *H. pylori* to gastric epithelium could be mediated by fibrillar adhesin which binds specifically to a host molecule, presumably a glycoprotein, containing N-acetyl-neuraminyl-α(2, 3)-lactose (NL). The gene hpaA, encoding the adhesin subunit protein HpA has been cloned and sequenced, and within HpA the NL-binding motif of the adhesin has been identified (12, 13). Several studies proved that *H. pylori* haemagglutinins have sialic acid-specific receptors (7, 10, 14). Binding of *H. pylori* strains to gastric mucin (5), cholesterol (15), Lewis $^{b}$antigen (16), extracellular matrix proteins (17, 18), and gangliosyde $GM_3$ (19) has also been established. Moreover, it has been shown that majority of *H. pylori* strains produce surface proteins binding sulphated glycosaminoglycans (GaGs) such as heparan sulphate (20, 21), and that they differ in the expression of these constituents. Several reports have stated that *H. pylori* bind to different cell lines: HeLa, Hep-2, INT 407, Y-1 and Kato-3 (22).

Recently we have found that interactions: sialic acid — bacterial sialic acid — specific haemagglutinins and heparin — bacterial heparin binding proteins are important for the direct non-opsonic phagocytosis of *H. pylori* bacteria by macrophages (23—25). We showed that *in vitro* a deposition of sialic acid containing compounds and heparin on bacterial surface made the bacteria resistant to the phagocytosis. The process of *H. pylori* ingestion was also inhibited by vitronectin in a presence of complement. It has been suggested that such effect might partly be a result of the interaction between macrophage glycosaminoglycans and bacterial heparin binding proteins (25).

In this study we asked the question as to whether bacterial structures binding heparin and sialic acids mediate attachment of *H. pylori* bacteria to human epithelial cells. Three different human cell lines: HeLa, Kato-3 and THP-1 were used for the study. The *H. pylori* strains were screened for

haemagglutinating activity towards sheep erythrocytes (SRBC), and the specificity of bacterial haemagglutinins was estimated by inhibition of *H. pylori* driven haemagglutination of SRBC with fetuin and mucin containing sialic acids, and with asialofetuin or asialomucin which do not contain such compounds. The attachment of *H. pylori* bacteria to human epithelial cell monolayers fixed with formaldehyde was estimated by enzyme linked immunosorbent assay (ELISA) with antibodies against *H. pylori* bacteria. The specificity of bacteria — epithelial cell interactions was determined on the basis of binding inhibition assay in which the bacteria treated with sialic acid-(fetuin, mucin) and non-sialic acid compounds (asialofetuin, asialomucin), and heparin have been used.

## MATERIAL AND METHODS

### *Bacterial strains and culture conditions*

The *H. pylori* reference strains 17874 and 17875 were obtained from the Culture Collection, University of Gothenburg (CCUG), Gothenburg, Sweden. *H. pylori* strain G33 was obtained from Dr Natale Figura, IRIS, Siena, Italy. *H. pylori* strain 25 a clinical isolate from adenocarcinoma was obtained from Prof. T. Wadström, Lund University, Sweden. The other *H. pylori* strains were clinical isolates from dyspeptic children being under the care of Mother and Child Health Center, Lodz, Poland (2 strains), and from dyspeptic adults being under the care of K. Jonscher's Hospital, Lodz, Poland (2 strains). The *H. pylori* strain 17874 and 17875 were used as standard strains expressing sialic acid-specific- and non-sialic acid-specific haemagglutinins respectively (7). Additionally, strain 25 demonstrated a comparatively strong and strain 17874 comparatively weak heparin-binding activity (20). The bacteria were stored at −70°C in Tryptic Soy Broth containing 15% glycerol. Before being used in experiments the bacteria were cultured for 48h at 37°C in microaerophilic conditions on blood agar containing 10% heat inactivated fetal calf serum.

### *Erythrocytes*

Sheep erythrocytes (SRBC) collected on sodium citrate as anticoagulant were washed three times in phosphate buffered saline, pH 7.2 (PBS).The 1% (v/v) solution of SRBC in PBS was used for haemagglutination and haemagglutination inhibition assay.

### *Haemagglutination assay (HA)* (26)

Bacterial cells were harvested from agar plates, washed 3 times in PBS and resuspended in the same buffer to $1 \times 10^9$ cells/ml. Serial dilutions of bacterial cell suspensions in PBS (50 l/well) were prepared in the wells of 96-well U-shaped microplates (Falcon, Becton Dickinson, California), and mixed with equal volume of 1% suspension of SRBC in PBS. Haemagglutinating activity of the bacteria was expressed in haemagglutinating units (HAU), after 18 h incubation of the plates at room temperature. One HAU was defined as a minimal number of the bacteria causing complete agglutination of erythrocytes.

## *Haemagglutination inhibition assay (HAI)* (26)

Three HAU of the bacteria in 50 µl PBS were mixed with 30 µl of inhibitors (3mg/ml PBS) and incubated for 1 h at room temperature under agitation. Incubation of the plates was continued after addition of 50 µl 1% SRBC to the wells. The inhibitors used were: fetuin and asialofetuin from fetal calf serum, mucine Type I-S and asialomucin from bovine submaxillary glands, all from Sigma, St. Louis, US.

## *Cell lines*

Three different human cell lines from European Cell Culture Collection (ECACC, Salisbury Wiltshire, UK) were used for the study: HeLa cells-epithelial like cells, Kato-3 cells — derived from metastasis of gastric carcinoma and Ags cells — derived from adenocarcinoma of the stomach. The cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine and 50 µg/ml gentamicin, all from ICN Pharmaceuticals, Costa Mesa, US (complete medium), for 3—4 days at 37°C in a humidified 5% $CO_2$ atmosphere. Before being used in adhesion assay, $1 \times 10^5$ cells suspended in 200 µl of complete medium were placed into the wells of 96-well flat bottom microplates (Falcon), and allowed to settle within 18 h (37°C, 5% $CO_2$).

## *Adhesion assay* (27, 28)

The culture medium was aspirated from the wells and the cells were fixed for 30 min at 37°C with 4% glutaraldehyde in PBS, and washed 4 times with PBS containing 0.05% Tween 20 (PBS-T). The excess of binding sites on the plastic was blocked for 18 h at 4°C with 1% bovine serum albumine (BSA) in PBS-T. The wells were emptied, washed twice with PBS-T then filled with 100 µl of bacterial cell suspensions in PBS ($1 \times 10^8$/ml) and incubated for 1 h at 37°C. The unbound bacteria were washed out with PBS-T and the number of attached bacteria was estimated by enzyme linked immunosorbent assay (ELISA) with anti-*H. pylori* antibodies. In adhesion/inhibition assay the bacteria ($1 \times 10^8$) incubated for 1 h at 37°C in PBS containing 1 mg/ml fetuin, asialofetuin, mucin, asialomucin or heparin ammonium salt from porcine intestinal mucosa (Sigma) were used. The bacteria were washed once with PBS, adjusted to $1 \times 10^8$ cells/ml and supplied to the wells containing epithelial cell monolayers as previously described.

## *Estimation of attached bacteria by ELISA*

Attachment of bacteria to epithelial cells was determined by modified ELISA assay (29). The monolayers of epithelial cells with attached bacteria were incubated for 5 min with 3% $H_2O_2$ in order to inhibit the activity of endogenous peroxidase. After 3 washes with PBS-T the cells were incubated for 1 h at 37°C with 100 µl of IgG fraction (10 µg/ml in PBS-T), separated from rabbit anti-*H. pylori* serum towards 17874 strain on protein G-Sepharose as recommended by the manufacturer (Pharmacia LKB Biotechnology, Uppsala, Sweden). After 3 washes with PBS-T the plates were incubated for 1 h at 37°C with 100 µl/well of peroxidase-conjugated goat anti-rabbit IgG antibody (Dakopatts, Alvsjo, Denmark), di 1:1000 in PBS/BSA/T containing 0.1% normal goat serum. The plates were washed and solution containing 1mg/ml o-phenylenediamine hydrochloride-OPD (Sigma) in citric phosphate buffer pH 5.0 and 0.5 µl/ml 30% $H_2O_2$ was distributed into the wells of microplates (100 µl/well). The colour was developed at room temperature in dark for 30 min. The reaction was stopped with 0.5 M citric acid and optical density units (OD) were estimated at 450 nm wave length in ELISA reader (Labsystem multiskan Plus, Turku, Finland). The attachment of the bacteria to epithelial cells was expressed in relative optical

density units. The controls used in ELISA assay were: the wells containing the cells incubated with primary or secondary antibody and OPD, and the wells containing the cells incubated only with OPD. The OD values for the controls were $0.070 \pm 0.010$, $0.080 \pm 0.010$ and $0.076 \pm 0.015$ respectively. Before using the bacteria in adhesion study the reaction of *H. pylori* bacteria with rabbit antibody against the reference *H. pylori* strain 17874 was examined by ELISA on microplates coated with whole bacterial cell antigen. The *H. pylori* strains reacting identically with anti-*H. pylori* antibody were selected for adhesion study. The ability of *H. pylori* bacteria to bind to epithelial cells (binding %) was calculated on the basis of OD units in the wells containing a total number of the bacteria at the beginning of the assay (100%) and OD values in the wells containing the bacteria remaining after washing out of unbound microbes after 1 h incubation at 37°C. The binding inhibition was calculated by comparison of OD values in the wells containing untreated bacteria (100% binding) with OD values in the wells containing the bacteria treated with different inhibitors. The experiments were repeated four times. In all experiments three wells for each type of bacteria/cell interaction were used. Data are expressed as mean values $\pm$ standard deviation (SD). Statistical significance was determined by Student's *t* test.

## RESULTS

All *H. pylori* strains agglutinated sheep erythrocytes. However, they varied by haemagglutinating intensity. On the basis of haemagglutinating units four *H. pylori* strains were classified as causing strong haemagglutination of SRBC (HAU were in the range $5.0 \times 10^5$—$1.25 \times 10^7$ of bacterial cells), the other four *H. pylori* strains showed weak haemagglutinating activity towards erythrocytes (HAU were in the range $2.5 \times 10^7 - 5.0 \times 10^7$ of bacterial cells). In adhesion assay there was no correlation between the intensity of *H. pylori*-driven erythrocyte haemagglutination and binding of the bacteria to human epithelial cells (data not shown).

On the basis of the inhibition of bacteria-induced haemagglutination of SRBC by sialoglycoconjugates such as fetuin and mucin, the *H. pylori* strains used for the study could be divided into three groups: Type I strains, producing sialic acid-dependent haemagglutinins — *H. pylori* - driven HA of SRBC was inhibited by fetuin and mucin but not by asialofetuin and asialomucin (four strains); Type II strains, producing haemagglutinins expressing mixed specificity — *H. pylori* - driven HA of SRBC was inhibited by fetuin, mucin but also by asialofetuin and asialomucin (2 strains); Type III strains, producing non-sialic acid-dependent haemagglutinins — *H. pylori* - driven HA of SRBC was not inhibited by any of inhibitors used (2 strains). Such division was based on the scheme proposed by Lelwala-Guruge *et al.* (7). All *H. pylori* strains expressing sialic acid-dependent haemagglutinins bound to HeLa cells in a higher number than other *H. pylori* strains (60—70 % of binding) (*Fig. 1*). Two of four *H. pylori* strains expressing a sialic acid-dependent haemagglutinating activity attached in a higher number also to Kato-3 cells (70—80% of binding). Two other Type I *H. pylori* strains attached to Kato-3 cells similarly as *H. pylori* strains representing Type II and Type III

haemagglutinating pattern (40—50% of binding). The exception was one Type III strain which attached to this cell line by 60%. There was no difference in binding to human Ags cells of *H. pylori* bacteria differing as regards the specificity of haemagglutinins. For all *H. pylori* strains the binding of bacterial cells to Ags cells was in the range 40—50%.

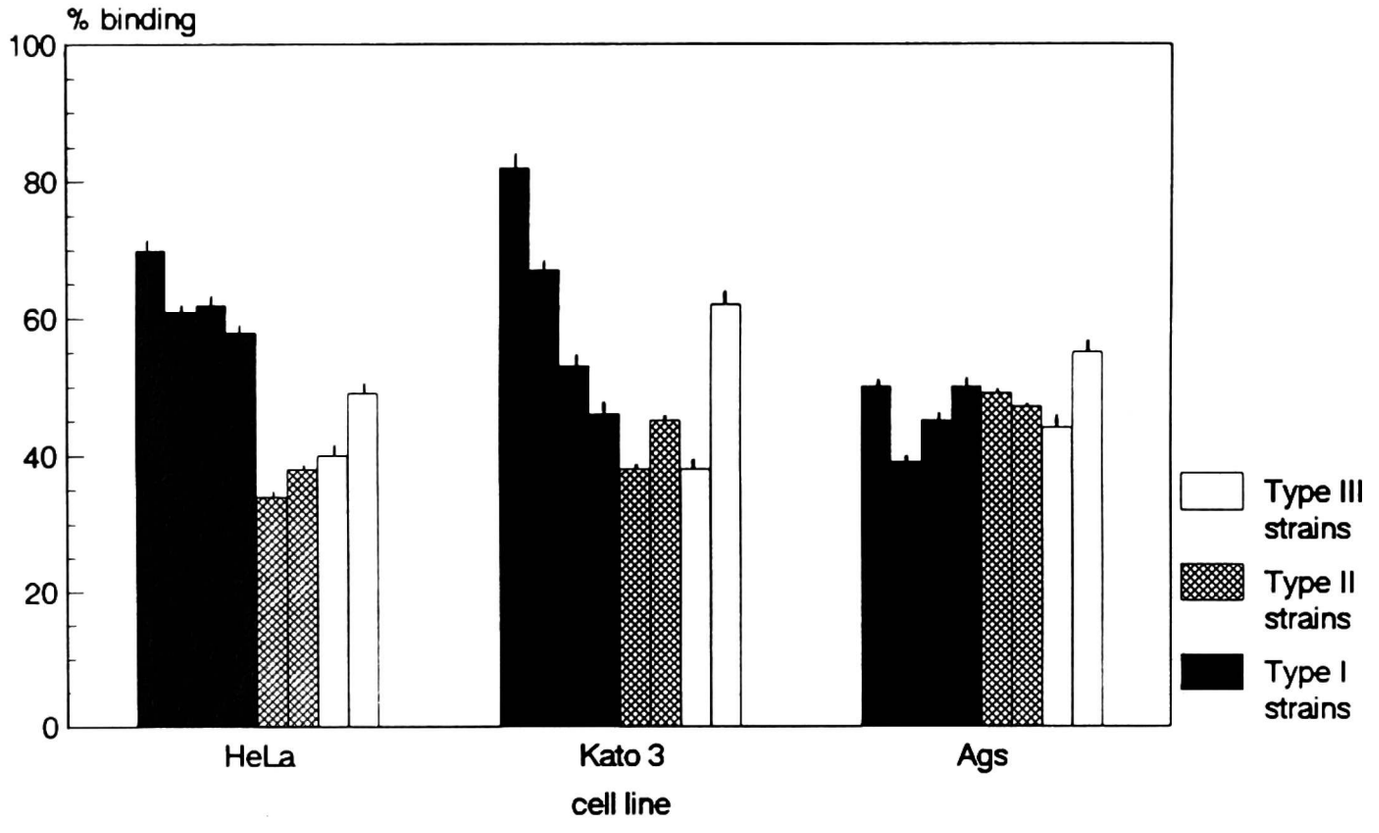


*Fig. 1.* Binding to human epithelial cells of *H. pylori* bacteria differing in the specificity of haemagglutinins. The *H. pylori* strains were tested for haemagglutinating activity (HA) towards sheep erythrocytes (SRBC), and on the basis of haemagglutination inhibition they were divited into three types: Type I strains — *H. pylori*-driven HA of SRBC was inhibited with fetuin/mucin, but not with asialofetuin/asialomucin (4 strains, black bars); Type II strains — HA was inhibited with fetuin/mucin but also with asialofetuin/asialomucin (2 strains, crossed bars); Type III strains — HA was not inhibited by any of these inhibitors (2 strains, empty bars). The ability to bind to human epithelial cells of *H. pylori* bacteria distinguished by HA was determined.

The involvement of bacterial surface sialic acid-dependent haemagglutinins and heparin-binding compounds in adhesion of *H. pylori* bacteria to human epithelial cells was investigated by blocking bacterial ligands recognized by epithelial cell receptors containing sialic acid residues or heparan sulphate. *Fig. 2.* shows a decrease in binding of fetuin and mucin treated *H. pylori* Type I strains to HeLa cells (inhibition of binding by 40—50%), Kato-3 cells (inhibition of binding by 20—40%), and Ags cells (inhibition of binding by 25—40%). The preincubation of those strains with asialofetuin or asialomucin only slightly reduced the binding of the bacteria to three different epithelial cell lines (inhibition of binding by 4—8%). A great variation in the binding to three

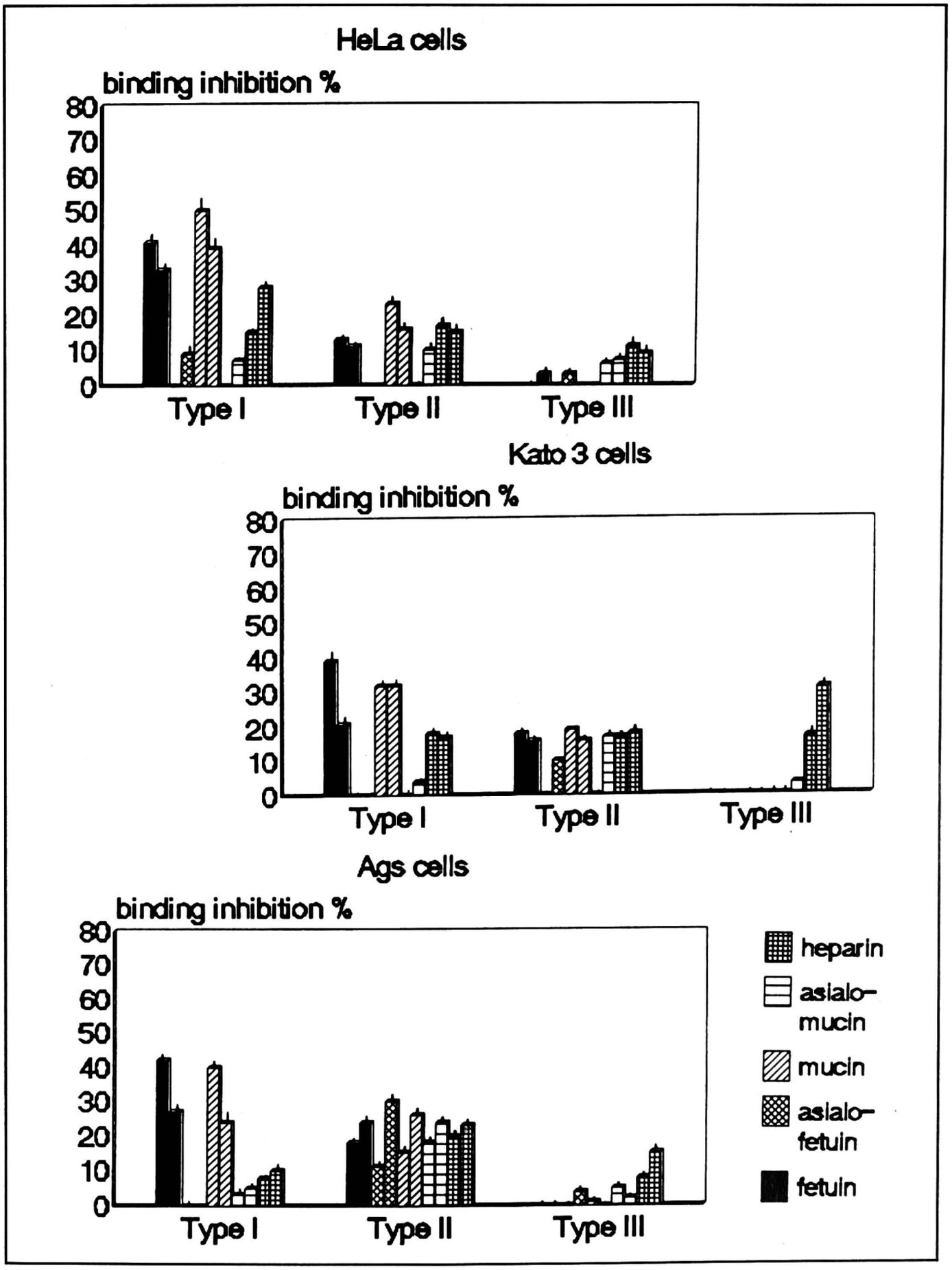


*Fig. 2.* The role of sialic acid-specific haemagglutinins and heparin-binding proteins in adhesion of *H. pylori* bacteria to human epithelial cells. Before being used in adhesion study the *H. pylori* bacteria distinguished by haemagglutination inhibition as Type I strains (*H.pylori*-driven HA of SRBC was inhibited by fetuin/mucin but not by asialofetuin/asialomucin, 2 strains), Type II strains (HA was inhibited by fetuin/mucin and asialofetuin/asialomucin; 2 strains), and Type III strains (HA was not influenced by any of these inhibitors), were preincubated with fetuin, asialofetuin, mucin, asialomucin or heparin.

human cell lines of Type II *H. pylori* strains treated with sialic acid-rich inhibitors versus non-sialic acid inhibitors was observed. The binding of fetuin and mucin treated bacteria (two strains) to HeLa cells and Kato-3 cells was inhibited by 15—20% and 20% respectively. The binding of one of two *H. pylori* strains from this group to HeLa cells was inhibited by asialomucin (15%) and to Kato-3 cells by asialofetuin and asialomucin (10—20%). The binding of Type II *H. pylori* strains to Ags cells was not inhibited by the inhibitors tested. In contrast to Type I and Type II strains, the preincubation of Type III *H. pylori* strains with fetuin, asialofetuin, mucin or asialomucin caused no or very slight reduction in the binding of the bacteria to HeLa, Kato-3 and Ags cells. Moreover, *Fig. 2.* shows the decrease in binding to three cell lines of all eight *H. pylori* strains treated with heparin (inhibition of binding by 10—30%).

## DISCUSSION

The mucosal surfaces and underlying epithelial cells consist of important defence towards invading microorganisms. However, many of pathogens develop mechanisms which allow them to brake such barriers and colonize the mucosa. The adherence of bacteria to the host cells depends on specific recognition system between bacterial ligands and specific receptors in the membranes of tissue cells (1). The sulphated glycoconjugates that are present on the surface of virtually all epithelial cells can act as receptors for many microorganisms. The interaction with heparan sulphate has been found crucial for initiation of many viral (30,31) and parasitic (32) infections. It has been demonstrated that binding of mycobacteria to epithelial cells but not to macrophages could be specifically inhibited by sulphated carbohydrates (33). The heparin-binding 28-kD protein was purified from culture supernatants and cell extracts of *Mycobacterium bovis* and *Mycobacterium tuberculosis.* This protein promoted the agglutination of rabbit erythrocytes and induced mycobacterial aggregation. Recently, it has been reported that the heparan sulphate proteoglycans mediate the attachment and entry to the host professional and non professional phagocytes of *Listeria monocytogenes* an intracellular pathogen (34). The listerial surface protein ActA has been suggested to function as ligand for heparan sulphate. The interaction heparin-bacterial heparin-binding proteins were proved to be important for the direct non-opsonic phagocytosis of *H. pylori* bacteria by macrophages (23—25). In the present study we have demonstrated that *H. pylori* bacteria also use heparin-binding activity in their attachment to human epithelial cells. This was determined by the inhibition of binding of several *H. pylori* strains to human epithelial cells by blocking of heparin-binding sites on the surface of bacteria with heparin. The binding of all eight *H. pylori* strains to three

different cell lines was decreased by pretreatment of bacterial cells with heparin. However, the inhibition of binding with heparin was never complete indicating that the interaction of *H. pylori* bacteria with epithelial cells could be simultaneously mediated by other bacterial cell surface compounds. Moreover, we were not able or in the case of few strains we could only slightly block the *H. pylori*-driven erythrocyte agglutination with heparin (data not shown). This may suggest that haemagglutinins produced by *H. pylori* bacteria have no or very weak heparin-binding activity. In the light of our results, the *H. pylori* strains use heparin-binding compounds to attach to the host cells. However, Kawaski *et al.* (35) reported that cell surface glycosaminoglycans are not involved in the adherence of *H. pylori* to Hs 198.St human gastric cells, Hs 746T human gastric adenocarcinoma cells, or HeLa cells. The removal of glycosaminoglycans by prior treatment of the cells with heparinases and preincubation of the bacteria with heparin sulphate, heparin or chondroitin/dermatan sulphate had no effect on adherence. This discrepancy may reflect the variable expression of sulphated glycoconjugates on the surface of different epithelial cell lines. It is also possible that in our model the pretreatment of cell monolayers with formaldehyde caused better exposure of cell surface compounds important for an interaction of bacteria with the host cells. Moreover, the *H. pylori* reference strain 17874 (CCUG) which was the same as *H. pylori* strain 11637 (NCTC) has been demonstrated to express low heparin-binding activity (20, 21). The analysis of our previous data led to a conclusion that the adhesion to macrophages of *H. pylori* strain 25 expressing strong heparin-binding activity was mainly determined by heparin-binding structures while the adhesion of *H. pylori* 17874 bacteria mainly depended on the expression of sialic acid-dependent haemagglutinins. Moreover, we showed that surface haemagglutinins and heparin-binding proteoglycans may mask each other to some degree (23 ). It is worth mentioning that the expression of some glycolipid-binding putative surface adhesins of *H. pylori* depends on the composition of culture medium and growth conditions (6).

Several bacterial pathogens which colonize epithelial cells are known to possess surface associated haemagglutinins. In general, surface adhesins of Gram negative bacteria are divided into two classes: D-mannose sensitive and D-mannose insensitive haemagglutinins (1). The heterogeneity of *H. pylori* strains as regards the expression and specificity of surface haemagglutinins has been demonstrated (7, 10, 12, 14), and we have recently shown that sialic acid-specific haemagglutinins mediate the interactions of *H. pylori* bacteria with human and murine polymorphonuclear leukocytes and macrophages (23,25). It has been reported that N-acetyl-9-0-acetylneuraminic acid is used by influenza C virus as recognition site on cell surface receptor (35). Also *Moraxella catarralis* which is one of the major pathogens of respiratory tract attach to pharyngeal epithelial cells via a sialic acid component (37). Our

present findings indicate that the adhesion of some *H. pylori* strains to human epithelial cells could be mediated by bacterial compounds recognizing sialic acid residues. Firstly, we showed that *H. pylori* strains expressed a sialic acid-dependent- and non-sialic acid-dependent or mixed haemagglutinating activity. Secondly, we established that *H. pylori* strains expressing sialic acid-dependent but not sialic acid-independent haemagglutinins bound to HeLa and Kato-3 cells in a higher number than other *H. pylori* strains. The inhibition of binding to epithelial cells of *H. pylori* bacteria producing sialic acid-dependent haemagglutinatinins by fetuin and mucin but not by asialomucin and asialofetuin suggested that haemagglutinins with sialic acid-specificity might play a role of adhesins initiating the interaction of bacteria with human epithelial cells. Moreover, inability to inhibit the attachment of *H. pylori* strains expressing non-sialic acid-dependent haemagglutinins with any of inhibitors suggested that those bacteria interacted with the epithelial cells *via* compounds lacking sialic acids. The complexity of adhesion process was confirmed by unsuccessful blocking of attachment to epithelial cells of majority of *H. pylori* strains tested by a single inhibitor and by the fact that the interaction of *H. pylori* bacteria with epithelial cell lines depended on the cell type. It seems, that various cell lines differ as regards the expression of surface compounds important for binding of *H. pylori* bacteria.

In conclusion, this study shows that the adhesion of *H. pylori* bacteria to human epithelial cells is a cooperative process involving both bacterial and cell surface compounds. Evidently, an interaction: sialic acid-bacterial sialic acid-binding haemagglutinins and heparin-bacterial heparin-binding compounds seems to be effective way for the initial adherence stage of *H. pylori* bacteria to the epithelial cells cultured *in vitro*. It is probable that *in vivo* the same interactions may allow the bacteria to colonize the gastric mucosa. Moreover, the described variation in *H. pylori* bacterial surface structures mediating their adhesion to the cells of epithelial cell lines may suggest a similar variation in the bacterial adhesion to stomach mucosa.

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