Affinity of the gastric pathogen *Helicobacter pylori* for the N-sulphated glycosaminoglycan heparan sulphate

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Summary. Binding of ^{125}I -heparan sulphate was a common property of *Helicobacter pylori* strains isolated from patients with gastroduodenal ulcer diseases. Binding was (i) saturable; (ii) reversible by the addition of unlabelled heparan sulphate and heparin; (iii) inhibited by unlabelled heparan sulphate, heparin, and heparin oligosaccharides but not by other glycosaminoglycans of comparable size (chondroitin sulphate and dermatan sulphate) or by highly glycosylated glycoproteins (hog gastric mucin and fetuin); (iv) reduced by heat treatment (80°C, 10 min) and exposure of the bacteria to pronase E, proteinase K, trypsin and chymotrypsin, but unaffected by treatment with pepsin and neuraminidase; and (v) time-, pH-, and ionic strength-dependent. Scatchard plot analysis of the binding data indicated the presence of one class of high-affinity receptor ($K_d = 9 \times 10^{-9} \text{ M}$) for heparan sulphate.

Introduction

Helicobacter pylori, previously called *Campylobacter pylori*,¹ is a highly motile organism which penetrates the gastric mucin layer and colonises the gastric epithelium or ectopic gastric tissues in other parts of the intestinal tract, such as gastric metaplasia in the duodenum.^{2,3} It has been reported^{4–7} recently to express sialic acid-specific agglutinating activity against erythrocytes of various animal species. The aim of this study was to examine the interaction between *H. pylori* and the acidic glycosaminoglycan heparan sulphate, a component of cell-surface or basement membrane-associated proteoglycans produced by both epithelial and mesenchymal cells.⁸ Our working hypothesis was that *H. pylori* could use heparan sulphate-specific adhesin(s), in addition to the previously reported sialic acid-specific haemagglutinin(s) or adhesin(s),^{5–7} to colonise gastric tissues.

Materials and methods

Strains and culture conditions

The 17 strains of *H. pylori* examined were from our laboratory collection.^{4,7,9} Generally, bacterial strains were grown under micro-aerophilic conditions at 37°C for 3–5 days on Blood Agar Base No. 1 (Lab M) with defibrinated horse blood 5% v/v. The bacteria were harvested with 0.01 M potassium phosphate buffer, pH 7.2, containing 0.15 M NaCl (1 ml/plate), washed

once and suspended in 0.1 M sodium acetate buffer, pH 4, containing 0.15 M NaCl (acetate buffer), to a final density of 10^{10} cfu/ml.

Chemicals

Highly purified preparations of glycosaminoglycans (hyaluronan, bovine lung heparan sulphate, bovine cartilage chondroitin sulphate, porcine skin dermatan sulphate, and porcine intestinal heparin) were obtained as described previously.^{10,11} Heparin oligosaccharides, isolated after partial de-aminative cleavage of heparin with nitrous acid and fractionated by gel chromatography, essentially as described previously,¹² were kindly provided by Dr U. Lindahl (Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden). Dextran (M_r 500 000) was obtained from Pharmacia and dextran sulphates (M_r 5000 and 500 000) were from Sigma. All other chemicals were of analytical grade and were purchased from various commercial sources.

Binding assay

Heparan sulphate was derivatised at the amino-terminal serine residue with *p*-hydroxyphenyl propionate and radio-iodinated.¹⁰ The binding of ^{125}I -heparan sulphate by *H. pylori* was quantified as described previously.¹³ Briefly, samples of an ^{125}I -labelled heparan sulphate solution (50 μl containing *c.* 20 ng, 25 000 cpm, of heparan sulphate) either in 0.1 M sodium phosphate buffer (PBS), pH 7.2, or in 0.1 M sodium acetate buffer, pH 4, containing 0.15 M NaCl, and supplemented with sodium azide 0.05% w/v and

bovine serum albumin 0.1% w/v, were mixed with 100 μ l of *H. pylori* cell suspension (10^9 cells) in polystyrene centrifuge tubes and kept at room temperature for 1 h. After adding 2 ml of ice-cold PBS or acetate buffer containing Tween 20 0.1% v/v, the mixtures were centrifuged (4500 g, 10 min) and the radioactivity of the pellets was measured in a gamma counter. *Staphylococcus aureus* served as a positive control.¹³ Other binding assays were performed in the presence of divalent cations (Ca^{++} , Mg^{++} , Mn^{++} , Fe^{++}) and EDTA (final concentrations 1 mM), and at various pH values and ionic strengths. Incubation mixtures without bacteria were used as background cpm controls. The bound heparan sulphate was expressed as a percentage of the total radiolabelled heparan sulphate incubated with the cells, and was corrected for the background cpm.

¹²⁵I-heparan sulphate binding-inhibition assay

H. pylori suspensions (100 μ l containing 10^9 cells) were incubated at 20°C for 1 h with 100 μ g (50 μ g/ml final concentration) of unlabelled heparan sulphate, chondroitin sulphate, dermatan sulphate, heparin, hyaluronan, heparin oligosaccharides, fetuin, hog gastric mucin, dextran sulphate (high and low mol. wt), and dextran. The treated cells were washed with 2 ml of PBS, suspended in 100 μ l of either PBS or acetate buffer, and used in the binding assay described above.

Enzymatic digestion of bacterial cells

H. pylori suspensions (100 μ l containing 10^9 cells) were treated with 100 μ g (50 μ g/ml final concentration) of pepsin (in 0.1 M sodium acetate buffer, pH 5.8), *Streptomyces griseus*-pronase E, *Tritirachium album*-proteinase K, trypsin, and chymotrypsin (in 50 mM Tris-HCl buffer, pH 7, containing 5 mM CaCl_2), or *Vibrio cholerae*-neuraminidase (in PBS) at 37°C for 1 h, as described in the Worthington Enzyme Manual.¹⁴ Cells were washed twice with PBS (4 ml), suspended in acetate buffer (pH 4) at the original cell concentration, and used in the binding assays described above.

Results

Preliminary screening of 17 *H. pylori* strains for binding of ¹²⁵I-labelled heparan sulphate showed that 90% of the strains studied bound this glycosaminoglycan. *H. pylori* strains 25 and 1139, which showed moderate binding and which have previously been examined for haemagglutinins,^{7,9} were selected for further characterisation of heparan sulphate-binding.

Increasing amounts of heparan sulphate were incubated with strain 25 (fig. 1), and saturation of the binding sites was obtained within the concentration

range used. Scatchard plot analysis of the binding data gave a straight line (fig. 1, inset), which indicated the presence of one class of heparan sulphate receptor. The dissociation constant (K_d) of 9×10^{-9} M, calculated from the slope of the line, indicated that heparan sulphate bound to *H. pylori* with a high affinity. Binding was time-dependent (fig. 2), and reversible by unlabelled heparan sulphate (fig. 3). Cell-bound ¹²⁵I-heparan sulphate was displaced by an excess of both unlabelled heparan sulphate (fig. 4a) and the analogous glycosaminoglycan heparin (fig. 4b). Binding was pH- and ionic strength-dependent (table I); binding was optimal at pH 4 and was

Fig. 1. Saturability of heparan sulphate-binding by *H. pylori* strain 25. Bacteria were mixed with the indicated amounts of ¹²⁵I-heparan sulphate and binding assays were performed. Binding results were analysed by Scatchard analysis (inset).

Fig. 2. Time course of heparan sulphate-binding by *H. pylori* strains 25 (—) and 1139 (---). Bacterial cells suspended in PBS (pH 7.2) were incubated with ¹²⁵I-heparan sulphate. At the times indicated, bacteria were washed and the amount of heparan sulphate bound was determined. The data points represent the means of triplicate measurements and the SD of each mean value was < 6%.

Fig. 3. Competition of ¹²⁵I-heparan sulphate binding to *H. pylori* strains 25 (—) and 1139 (---). Bacterial suspension (100 µl containing 10⁸ cells in PBS, pH 7·2) were incubated with 25000 cpm of ¹²⁵I-heparan sulphate. After incubation for 1 h at 22°C, 100 µg of unlabelled heparan sulphate was added and the mixtures were incubated at 22°C. At the times indicated, the bacteria were washed and the amount of ¹²⁵I-heparan sulphate remaining bound was determined. Data (the means of triplicate measurements) are expressed as the relative percentage of control (untreated cells) values. The SD of each mean value was < 10%.

inhibited in high ionic-strength solutions, e.g., 2 M NaCl virtually abolished binding. Divalent cations (Ca⁺⁺, Mg⁺⁺, Mn⁺⁺, Fe⁺⁺) and EDTA (all at 1 mM final concentration) did not affect heparan sulphate binding by the bacteria (data not shown).

Table I. Effect of pH and ionic strength on the binding of ¹²⁵I-labelled heparan sulphate by *H. pylori*

Factor	Binding of ¹²⁵ I-heparan sulphate to <i>H. pylori</i> (%)	
	Strain 25	Strain 1139
pH		
0·1 M Na-acetate (pH 2)	54·0	43·5
0·1 M Na-acetate (pH 4)	76·0	77·7
0·1 M Na-acetate (pH 6)	30·0	17·0
0·1 M Na-phosphate (pH 7)	20·1	12·7
0·1 M Na-phosphate (pH 8)	8·0	5·0
0·1 M Tris (pH 9)	6·3	4·5
Ionic strength* (M NaCl)		
0·075	20·0	8·4
0·15	15·2	8·5
0·30	13·4	9·6
0·60	10·3	8·0
1·00	11·6	6·3
2·00	4·2	4·5

*Sodium phosphate buffer (0·1 M, pH 7·2) was adjusted to various ionic strengths with NaCl. The data represent the means of triplicate measurements and the SD for each mean value was < 6%.

Pre-treatment of *H. pylori* with unlabelled heparan sulphate and heparin inhibited binding of ¹²⁵I-heparan sulphate (table II). However, other glycosaminoglycans of similar size (chondroitin sulphate and dermatan sulphate), highly glycosylated glycoproteins (hog gastric mucin and fetuin), and various carbohydrates (fucose, mannose, galactose, glucosamine and N-acetyl-D-glucosamine) did not inhibit binding. High and low-mol. wt dextran sulphates (but not high-mol. wt dextran) were also found to inhibit heparan sulphate-binding (table II). Heparin oligosaccharides were also tested for their ability to inhibit heparan sulphate-binding (fig. 5). The degree of inhibition

Fig. 4. Displacement of cell-bound ¹²⁵I-heparan sulphate by unlabelled heparan sulphate and heparin. The unlabelled heparan sulphate (a) or heparin (b) was added to bacterial suspensions of *H. pylori* strains 25 (—) and 1139 (---) in PBS, pH 7·2, containing bound ¹²⁵I-heparan sulphate and the suspensions were incubated at 22°C for 1 h. Bacteria were washed and the amount of ¹²⁵I-heparan sulphate remaining bound was determined. Data (the means of triplicate measurements) are expressed as the relative percentage of control (untreated cells) values. The SD of each mean values was < 8%.

Table II. Inhibition of ^{125}I -heparan sulphate-binding to *H. pylori* by pre-treatment of the bacteria with glycosaminoglycans and dextrans

Inhibitor	Binding* of ^{125}I -heparan sulphate to <i>H. pylori</i> (%)	
	Strain 25	Strain 1139
Control (without inhibitor)	20.0	13.0
Heparan sulphate	1.5	1.5
Chondroitin sulphate	18.0	13.2
Dermatan sulphate	19.6	12.4
Heparin	1.3	2.5
Hyaluronan	19.8	13.0
Fetuin	25.3	8.5
Hog gastric mucin	21.7	19.0
Dextran sulphate (high-mol. wt)	1.8	1.7
Dextran sulphate (low-mol. wt)	2.0	1.4
Dextran	20.7	13.0

*Binding assays were performed with bacterial cells suspended in phosphate buffer (pH 7.2). The data represent the means of triplicate measurements and the SD for each mean value was < 6%.

Fig. 5. Competition between heparin oligosaccharides and ^{125}I -heparan sulphate for binding to *H. pylori*. Bacterial suspensions (100 μl containing 10^9 cells in sodium acetate buffer, pH 4) were incubated with 50 μg of unlabelled heparin oligosaccharides. After incubation for 1 h at 22°C, ^{125}I -heparan sulphate was added to the mixtures and incubated at 22°C for 1 h. The bacteria were washed and the amount of heparan sulphate bound was determined. Data (the means of triplicate measurements) are expressed as the relative percentage of control (untreated cells) values. The SD of each mean value was < 6%.

increased gradually with increasing chain length. Heparin oligosaccharides longer than 10 saccharide residues gave $\geq 50\%$ inhibition.

Heat-treatment (80°C, 10 min) of the bacteria reduced their heparan sulphate-binding activity by c. 90% (table III). Binding was unaffected after treatment of the bacteria with pepsin and neuraminidase, but was reduced by 50–70% by exposure to pronase E, proteinase K, trypsin and chymotrypsin (table III). Results of heat- and enzyme-inactivation studies suggested that the heparan sulphate-binding component(s) was proteinaceous.

Table III. Effect of heat and enzymatic treatment of *H. pylori* on heparan sulphate-binding

Treatment	Relative percentage* of heparan sulphate-binding by <i>H. pylori</i>	
	Strain 25	Strain 1139
Control (acetate buffer, pH 4)	100	100
Heat (80°C, 10 min)	10	8
Chymotrypsin	35	49
Pronase E	30	44
Pepsin	100	100
Proteinase K	30	44
Trypsin	28	45
Neuraminidase	100	100

*Data are expressed as relative percentage of control (untreated cells) values. The data represent the means of triplicate measurements and the SD for each mean value was < 6%.

Discussion

H. pylori strains were screened for binding to the extracellular matrix component heparan sulphate. Of the *H. pylori* strains studied, 90%, regardless of haemagglutination patterns,⁷ bound ^{125}I -heparan sulphate. Strains 25 and 1139, which have previously been studied with regard to other cell-surface properties,^{7,9} were selected for further characterisation.

Binding of ^{125}I -heparan sulphate by *H. pylori* was consistent with the concept that the interaction involves protein receptor-ligand binding. Binding was (i) saturable, (ii) of high affinity and reversible by unlabelled heparan sulphate or heparin added in excess, (iii) inhibited by unlabelled heparan sulphate, heparin and heparin oligosaccharides but not by other glycosaminoglycans or glycoproteins, and (iv) sensitive to proteinase and heat treatments. The low binding at high pH (pH 8) and the high binding at low pH (pH 4), as well as the inhibition of binding at high ionic-strength (2 M NaCl) suggest that charge interactions are important for optimal binding. However, the specificity of the interaction appears to be similar to that of heparan sulphate-binding by herpes simplex virus,¹² in which heparin oligosaccharides inhibit heparan sulphate-binding. The proteinaceous binding-site on *H. pylori* appears to have a strong positive charge at pH 4, as heparan sulphate has a negative charge at pH 4.⁵ Interestingly, other negatively-charged glycosaminoglycans, such as dermatan sulphate and chondroitin sulphate, did not inhibit binding (table II), thus suggesting that binding also has some specificity for polysaccharides with a particular chain geometry and distribution of negative charges. On the other hand, in view of the fact that we used bovine lung heparan sulphate and porcine intestinal heparin in our studies, further studies need to be

performed to characterise the interactions between *H. pylori* and heparan sulphate derived from human gastric tissues.

The observation that pepsin-treated *H. pylori* retained the ability to bind ^{125}I -heparan sulphate, and that binding had a low pH optimum (pH 4), may be significant in the pathogenesis of the gastric infection caused by this microbe. Thus, the presence of pepsin and the acidic pH of the gastric environment associated with *H. pylori* colonisation may not affect the attachment of bacteria to heparan sulphate. In any case, the ability of *H. pylori* to adhere to and penetrate the gastric mucosa could require a variety of additional bacterial surface proteins, toxins and enzymes. Further studies need to be done to determine whether antibodies to heparan sulphate-binding protein(s) are present in the various stages of the gastric disease process, and whether patients' sera block heparan sulphate binding by *H. pylori*. Studies on the isolation, purification and characterisation of the *H. pylori*-

heparan sulphate binding protein(s) (HSBPs) are now in progress.

A number of tissue-invading bacteria and parasites bind to various connective tissue matrix components, which probably enables them to target and penetrate certain tissues.^{15,16} Several such pathogens have been shown to interact with fibronectin,¹⁷ various collagens and laminin. However, to our knowledge no pathogenic bacterium or parasite, except *Streptococcus pyogenes*, *Str. mutans* and *Staphylococcus aureus*^{13,18,19} has been reported to bind heparan sulphate. However, heparan sulphate has been proposed as the cell receptor for members of the herpes virus group.^{12,20} A systematic study should now be performed to explore whether other tissue-invasive pathogens use heparan sulphate or other proteoglycans as tissue receptors.

This study was supported by grants from the Swedish Medical Research Council (16 × 04723 to T.W. and grant 567 to L.-Å.F.) and a grant from the Medical Faculty of Lund University to F.A. We thank A. Kreger for his valuable comments and discussions.

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