Effect of heparin binding on *Helicobacter pylori* resistance to serum

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The objective of this study was to evaluate the effect of heparin binding to *Helicobacter pylori* cells on their survival in the presence of fresh rabbit serum with or without active complement components. Three *H. pylori* strains were compared and the amounts of heparin added reflected the physiological concentrations that can be found in animal tissues. No growth of *H. pylori* was noted in the presence of serum. Serum with or without active complement produced a reduction in c.f.u. for strains SPM 326, CCUG 17874T and SS1. However, addition of heparin resulted in increased survival of bacterial cells in serum with or without active complement. It appears that heparin binding to *H. pylori* can prevent bacterial cell death due to the alternative complement system. Heparin binding could also protect from heated serum (complement-inactivated), indicating protection from other serum components besides complement. In vivo, the process of heparin binding could possibly result in facilitated colonization due to a higher survival rate.

Introduction

*Helicobacter pylori* is a Gram-negative, microaerophilic, spiral-shaped bacterium that chronically infects the stomach. This pathogen is associated with chronic gastritis and gastric ulcer and may lead to the development of gastric cancer (Uemura et al., 2001). Despite the observed production of specific antibodies to *H. pylori* and recruitment of various cell populations to inflamed gastric areas, the infection remains life-long. Infection with *H. pylori* is one of the most common bacterial infections worldwide. To date, numerous virulence factors responsible for gastric colonization, survival and tissue damage have been described and characterized (Dubreuil et al., 2002). However, the critical feature of this infection may reside in its ability to persist within the host for such a long period of time and not in its ability to damage host tissues. Many of the pathogenic events seen during *H. pylori* infection may be due to alteration of the host response by the bacterium. Interactions with various host serum and extracellular matrix proteins have been related to the infectious process (Dubreuil et al., 2002). Some of these interactions, as with heparin and heparan sulfate, are of high affinity (Kd = 9 × 10⁻⁷ M) (Ascensio et al., 1995) and could therefore play a crucial role in the fate of the pathogenic process. The interplay of host proteins binding to *H. pylori* could represent an effective way to evade the host immunological response and to adapt to an invading infection.

Heparin is produced by mast cells and is stored in intracellular granules. It is found particularly in liver, lungs and gut of various animals. A structural relative, heparan sulfate, is found in the extracellular cell matrix at the surface of most animal cells. This acidic glycosaminoglycan is produced by epithelial and mesenchymal cells. It is less sulfated than heparin. Recent studies indicate that *H. pylori* may favour the interaction with heparin, as the availability of this component may be enhanced in epithelial and subepithelial environment. Neutrophil-activating protein produced by this bacterium is able to cross the epithelial layer and subsequently activates mast cells, followed by degranulation and release of the granule content (Montemurro et al., 2002). Recently, vacuolating cytotoxin A (VacA) was also shown to recruit and activate mast cells (Sapajatura et al., 2002). The C-terminal domain of the 58 kDa subunit of *H. pylori* VacA binds heparin and heparan sulfate (Utt et al., 2001), indicating that heparan sulfate could represent a receptor/coreceptor for VacA cytotoxin, supporting the results of an earlier study (Reyrat et al., 1999). Experiments with cells in culture showed inhibition of cell vacuolation following pretreatment of *H. pylori* with heparin (Sommi et al., 1997). A heparin-binding protein was located at the cell surface by biotinylation and indirect immunofluorescence assays. This protein corresponded to a 55–60 kDa molecule (Utt & Wadstrom, 1997).

Duensing et al. (1999) described a novel, extragenomic mechanism of bacterial surface modulation that can amplify the adaptive and pathogenic potential of numerous bacterial species including glycosaminoglycans and related sulfated polysaccharides. The new mechanism involves specific bac-
terial recruitment of molecules, such as heparin, in the immediate environment during the infectious process. These molecules can then serve as universal binding sites for various mammalian heparin-binding proteins. Ascensio et al. (1995) have shown clearly that pre-incubation of *H. pylori* with heparan sulfate and other sulfated polysaccharides resulted in enhanced fibroblast growth factor binding, supporting this novel microbial pathogenesis strategy.

We performed a study to determine whether the binding of heparin, a glycosaminoglycan found in many animal tissues and known to bind to *H. pylori* with high affinity, could alter the survival of the micro-organism in the presence of serum.

### Methods

**Bacterial strains and culture media.** *H. pylori* strains SPM 326, CCUG 17874T and Sidney strain 1 (SS1) (Lee et al., 1997) were used in this study. Strain SPM 326 was obtained from a human isolate that was adapted to the mouse as described previously (Marchetti et al., 1995). All *H. pylori* strains tested are cytotoxin-associated antigen A (CagA)-positive (Xiang et al., 1995). Bacteria were grown for 24 h on Columbia agar plates under microaerophilic conditions as described previously (Xiang et al., 1995).

**Normal rabbit serum.** Fresh rabbit blood was collected from naive rabbits (a pool of three animals). The blood was centrifuged to sediment the cells and then filtered on 0.22 μm filters. When tested, the sera did not react with *H. pylori* whole-cell extract, as determined by immunoblot. Sera were kept for 7 days at most. Complement components were inactivated by heating the serum at 56 °C for 30 min.

**Serum bactericidal assay.** A modification of the bactericidal assays reported by Ngeleka et al. (1992) and Trust et al. (1981) was employed. Briefly, *H. pylori* was grown overnight on Petri plates and the growth scraped from the solid medium. Cells were washed twice in Veronal solution (4·1 g NaCl, 0·5 g gelatin, 0·5 g sodium 5,5-barbiturate, in 500 ml water, after adjusting the pH to 7.35 with HCl and adding 0·5 ml each of 2 M MgCl₂ and 0·3 M CaCl₂). The cell suspension was adjusted to an OD₅₃₅ of 1 (approx. 10⁹ c.f.u. ml⁻¹). Various quantities of heparin (sodium salt from porcine intestinal mucosa; Sigma) ranging from 0 to 10 mg ml⁻¹ were added to 200 μl of 1:100-diluted bacterial cell suspension and left for 30 min at 37 °C. Unbound heparin was then removed by washing with 1·5 ml Veronal buffer. This step was repeated twice. Next, 200 μl cell suspension was mixed with 200 μl of either fresh rabbit serum, heated serum or Veronal buffer, as a negative control. The tubes were incubated for 6 h in a microaerophilic atmosphere (CampyGen, Oxoid). Next, diluted aliquots (50 μl) were spread on Columbia agar plates and incubated at 37 °C for 72 h under microaerophilic conditions. Various concentrations of heparin were added in order to assess the effect of binding on bacterial survival in the presence of serum. Colony-forming units (c.f.u.) were determined for the different treatments.

**Results and Discussion**

In this study, we analysed the effect of heparin binding to *H. pylori* on survival *in vitro* in the presence of fresh serum. As *H. pylori* could be exposed to heparin during a gastric infection *in vivo* and since it is already known that it can bind to this bacterium, it was hypothesized that the process could be responsible, in part, for the augmented survival necessary for long-term establishment in an animal host.

For strain SPM 326, we observed that, in the presence of normal rabbit serum (without antibodies directed against *H. pylori*), the number of c.f.u. was reduced by about one logarithm in 6 h (Fig. 1a). No reduction or increase in c.f.u. was noted when cells were incubated in Veronal buffer (data not shown). The viability of this strain was also affected, though to a lesser extent, by addition of heated serum (56 °C for 30 min). Survival of the same strain in the presence of heparin (0·2 mg ml⁻¹) was much greater in the presence of normal untreated or heated serum. There was a clear protective effect upon heparin addition. No change in c.f.u. was noted when heparin-treated bacterial cells were incubated for 6 h in the presence of heat-treated serum (complement-inactivated). No bacterial growth was noted for any of the treatments tested. The same general response was noted with strain SS1, with comparable numbers of c.f.u. (data not shown).

We observed similar results for *H. pylori* CCUG 17874T (Fig. 1b). In this case, survival was reduced by almost two logarithms in the absence of heparin when incubated in normal serum. In contrast, in the presence of complement-inactivated serum, a one log reduction was noted. Thus, it

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**Fig. 1.** Resistance of *H. pylori* strains SPM 326 (a) and CCUG 17874T (b) in fresh rabbit serum in the absence or presence of 0·2 mg heparin ml⁻¹ after 6 h. △, Serum; ▲, complement-inactivated serum; ■, serum plus heparin; ■, complement-inactivated serum plus heparin. C.f.u. were determined by plating bacterial cells on Columbia agar plates and incubating at 37 °C for 72 h under microaerophilic conditions.
seems that strain CCGU 17874T is more sensitive to fresh rabbit serum with or without complement. Nevertheless, the protection provided by the addition of heparin (0.2 mg ml\(^{-1}\)) was important and of the same order of magnitude as noted for strains SPM 326 and SS1.

Dose–response curves for heparin protection were prepared with strains SPM 326 and CCGU 17874T (Fig. 2). When 0-1 mg heparin ml\(^{-1}\) was added, no significant protection was seen compared with a control to which no heparin was added. Protection from the normal serum effect was seen at 0.2 mg ml\(^{-1}\) and above. Maximal protection was attained at around 0.5 mg ml\(^{-1}\).

The observations that *H. pylori* strains are susceptible to complement (Gonzales-Valencia *et al.*, 1996) even in the absence of specific antibodies and that heparin binds several complement proteins (Sahu & Pangburn, 1993) could explain, in part, the protective effect noted in our study. It could contribute to immobilization of complement components by heparin that are no longer capable of effectively killing bacterial cells. For example, C4b, in particular, has been shown to bind heparin effectively (Blom, 2002). This complement protein subunit of the classical pathway can be captured on the surface of several pathogens, including *Neisseria gonorrhoeae*, *Bordetella pertussis*, *Streptococcus pyogenes* and *Escherichia coli*. This process could contribute to their resistance to serum and increased pathogenicity. The increase in resistance to serum observed in the presence of heparin could indicate a role in resistance to the alternative complement pathway. It is unlikely that heparin mediates binding to C3b, as this complement component was shown not to bind to heparin using radiolabelled compounds and affinity chromatography (Sahu & Pangburn, 1993). It is possible that heparin masks sites for binding of C3b and blocks initiation of the alternative complement pathway. Thus, limited access for the terminal membrane attack complex to the bacterial cell surface, provided by heparin binding, could be responsible for the serum protection observed.

Nevertheless, as some protection was also observed for heated serum (complement-inactivated), a mechanism other than complement protection seems to be responsible for part of the protection observed. In this study, the amounts of heparin that effectively increased survival of *H. pylori* are in the range available in vivo, as reported by Lovich & Edelman (1999), who concluded that approximately 0.3 mg ml\(^{-1}\) is found in arteries. It should also be noted that the amount added to bacterial cells did not exclude the fact that only a portion of the heparin added could bind to *H. pylori* cells effectively in the 30 min period allowed for the reaction to take place. In vivo, longer contact, through *H. pylori* heparin-binding proteins, could allow concentration of this compound on the surface of the micro-organism to concentrations much above that found in tissues. As heparin could allow *H. pylori* to avoid phagocytosis (Chmiela *et al.*, 1997), *in vivo* studies could reflect this process, which was not evaluated in the present study.

Studies should now be conducted in an animal model for *H. pylori* to confirm the results generated in this study. This approach could be troublesome, since the differential survival rate of bacteria *in vivo* could result in more rapid and efficient colonization, but not necessarily in the presence or absence of infection.

**Fig. 2.** Response of *H. pylori* strains SPM 326 (a) and CCUG 17874T (b) towards fresh rabbit serum in the presence of varying quantities of heparin. Heparin was added to bacterial cells as described in Methods. C.f.u. were determined at time zero (open bars) and after 6 h incubation in the presence of rabbit serum (filled bars) by plating bacterial cells on Columbia agar plates and incubating at 37 °C for 72 h under microaerophilic conditions.

References


