BACTERIAL PATHOGENICITY

A particle agglutination assay for rapid identification of heparin binding to coagulase-negative staphylococci

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The heparin-binding properties of six different species of coagulase-negative staphylococci were examined by a particle agglutination assay. Heparin (mol. wt 4000–6000), mildly treated with sodium periodate, was covalently coupled to amino-modified latex beads (0.72 µm diameter). The particle agglutination assay was validated by comparing results with the adhesion (percentage binding of adherent cells) of coagulase-negative staphylococcal strains to heparinised microtitration plates. Of 38 different coagulase-negative staphylococcal strains tested, 30 showed agglutination reactivity with heparin-coated latex beads. Strains of different coagulase-negative staphylococcal species agglutinated heparin-coated latex beads to various extents (e.g., cells of *Staphylococcus haemolyticus* strains reacted more strongly than cells of *S. epidermidis* strains). The agglutination reaction was significantly inhibited by fucoidan, suramin, λ-carrageenan and other sulphated compounds, but not by non-sulphated carbohydrate polymers such as hyaluronic acid. Agglutination of staphylococcal cells with heparin-coated latex beads was completely blocked by a cell-surface extract. These results suggest that structures responsible for heparin binding are exposed on the cell surface.

Introduction

Heparan sulphate glycosaminoglycans (GAGs) form a complex heterogeneous family of macromolecules composed of linear sulphated polysaccharide chains, which are associated with epithelial and mesenchymal cell surfaces and extracellular matrix proteins (ECM) [1].

A number of biological effects have been attributed to heparin since its discovery almost 80 years ago. Many of these are independent of its anticoagulant activity. Heparin and heparan sulphate are believed to possess various biological activities that include the ability to modulate tissue homeostasis, wound healing, cell differentiation, cell proliferation and inflammation [2].

Several studies have shown that heparin and other GAGs are involved in the adherence to eukaryotic cells of pathogenic microbes such as herpes simplex, *Chlamydia trachomatis*, *Bordetella pertussis*, *Streptococcus mutans*, *Trypanosoma cruzi*, *Helicobacter pylori* and *Leishmania donovani* [3–10].

A previous study showed that *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) interact

with heparan sulphate [11, 12] and other ECM molecules, i.e., fibronectin, vitronectin, thrombospondin and lactoferrin. All these proteins contain heparin-binding domains [13, 14]. CNS comprise >25 different species of staphylococci that belong to the skin commensals of man and animals. A few of these species can infect intravascular catheters and prosthetic devices, and one of them, *S. epidermidis*, is the dominant cause of infections associated with biomaterials [15–17].

Among new approaches to prevent the microbial colonisation of intravascular catheters, some reports have dealt with heparin and heparin-like substances coated on catheters [12, 15]. Heparinised devices are used clinically because of their thrombo-resistant properties. The risk of bacteraemia with heparinised catheters was reduced to half for many pathogens, but CNS were still the most frequent micro-organisms to be isolated in both bacteraemia and colonisation of heparinised catheters [12, 15].

Materials and methods

Chemicals

Heparin (mol. wt 4000–6000) was purchased from Fluka (Heidelberg, Germany). Amino-modified latex beads (AML, bead diameter 0.72 µm) were purchased from Seradyn Inc. Particle Technol Division (Indianapolis,

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IN, USA) and latex beads (control L-05031-02, bead diameter 0.8 μm), were from Difco. Azure A, bovine serum albumin, dextran sulphate (mol. wt 8000), fucoidan, λ -carrageenan from *Gigantia aciculata* and *G. pistillata*, colominic acid, Evans blue, hyaluronic acid from bovine trachea, pentosan polysulphate, sodium acetate, sodium periodate, pepsin, pronase E, proteinase K, trypsin, Trypan blue and tunicamycin (T-7765) from *Streptomyces* species were obtained from Sigma. Fragmin[®] (low molecular heparin; mol. wt 5000) and merthiolate were purchased from Kabi AB, Stockholm, Sweden). Suramin, commercially available as Germanin[®], was from Bayer AG (Leverkusen, Germany) and Tween 20 from Merck AG (Darmstadt, Germany). Blood agar (BA) base was purchased from Lab M (Salford), and supplemented with horse blood 4%. Brain Heart Infusion (BHI), Tryptic Soy (TS) and Todd-Hewitt (TH) broths were from Difco. All buffers and chemicals were of analytical grade.

Bacterial strains and growth conditions

S. aureus strains Cowan 1 and ISP 546, strains of six different CNS species isolated from patients with osteomyelitis and biomaterial-associated infections, and *Micrococcus* spp. isolated from blood cultures were studied. The strains were cultured on BA and in TH, BHI and TS broth at 37°C for 24 h on an orbital shaker. Bacterial cells were harvested in the late stationary phase by centrifugation (3000 rpm for 20 min), washed twice with 0.07 M phosphate-buffered saline (PBS), pH 7.2, and finally suspended in the same buffer at a final concentration of 10^9 cfu/ml, and used promptly in the particle agglutination assay (PAA). *Helicobacter pylori* strain 25 was included as a positive control [8, 9].

Cell surface extract

For cell surface extraction, cells of a strain of *S. haemolyticus*, SM 131, were washed twice, resuspended in 2.5 ml of 1.0 M LiCl, pH 5.0, and incubated for 2 h at 45°C with agitation. After centrifugation (3000 g, 30 min) supernate was dialysed overnight at 4°C against PBS. The protein content was determined by a BioRad microassay (BioRad, Richmond, CA, USA). The extract was stored at -20°C.

Tunicamycin treatment of staphylococci

S. epidermidis H9-E and *S. haemolyticus* SM131 and E 2498 cells were grown at 37°C for 24 h with continuous shaking in TH broth supplemented with tunicamycin 12.5 $\mu\text{g}/\text{ml}$ [18]. The cells were washed, suspended in PBS and tested in the PAA as described below.

Preparation of latex reagents

Heparin PAA reagents were prepared by both covalent coupling and electrostatic adsorption to latex beads.

Covalent coupling. Heparin was bound covalently to AML beads after mild periodate oxidation [19]. Heparin dissolved in 0.1 M sodium acetate buffer (NaAc, pH 5.5) at a concentration of 2 mg/ml was treated with 100 μl of 0.1 M NaIO₄, which was added in 20- μl volumes over 10 min. The reaction mixture was incubated for a further 20 min in the dark and oxidised heparin was separated from NaIO₄ by gel filtration with a Sephadex G-25 column equilibrated with 0.1 M NaAc buffer (pH 5.5). One hundred μl of AML particles (Seradyn, IN, USA; solids 10%, diameter = 0.722 μm) were incubated with 1 ml of oxidised heparin solution overnight at room temperature. Finally, the heparin-coated AML particles were washed twice in PBS and resuspended in the same buffer at a final concentration of 1% total solids and stored at 4°C until used.

Adsorption. Heparin was electrostatically adsorbed to the latex beads (Difco; diameter = 0.81 μm). Briefly, a 1.0-ml sample of latex particle suspensions was mixed with 3.0 ml of 0.17 M glycine-NaOH buffer (pH 8.2). Heparin (500 μg) was added and the mixture was kept at 30°C overnight on a horizontal shaker at 50 rpm. The mixture was centrifuged (9200 g, 15 min, 10°C), and the pellet was suspended in 2 ml of 0.17 M glycine-NaOH buffer containing BSA 0.05% and merthiolate 0.01% and kept at 4°C for 12 h.

The presence of heparin on the latex particles was detected by a chromatic method with Azure A stain [20]. Control latex beads were prepared with BSA 0.05% in the same manner as the heparin.

Particle agglutination assay (PAA)

Latex reagent (10 μl) was placed on glass slides, and an equal volume of a bacterial cell suspension (10^9 cfu/ml) was added. The two drops were mixed gently, and the agglutination reaction was read after 2 min [21]. Each strain was tested in duplicate and assays were repeated on two different days with separately prepared bacterial suspensions. Strains were tested for auto-aggregation by mixing one drop of a bacterial suspension with one drop of PBS on a glass slide.

Particle agglutination inhibition assay

The bacterial suspension (10^8 cells in 100 μl) was pre-incubated for 1 h at 20°C with 100 μl of inhibitor at a final concentration of 1 mg/ml and then mixed with PAA reagents. PBS was used as a control.

In another series of experiments, 100 μl of bacterial cell-surface extract (protein concentration 400 $\mu\text{g}/\text{ml}$) was incubated with an equal volume of coated latex beads for 30 min at 20°C. After that, 10 μl of bacterial cell suspension were mixed with 10 μl of mixture (cell surface extract + heparin-coated latex beads) and

checked for agglutination abilities. Agglutination was recorded after 2 min and scored as described above.

Heat and enzyme treatment

Washed cells of *S. haemolyticus* strains SM 131 and E 2498 were heated at 90°C for 30 min, cooled rapidly, and then used in the PAA. Bacterial cells were also treated with different enzymes (Table 3) at 37°C for 1 h. The cells were washed carefully twice with PBS to remove enzyme residues, resuspended in the same buffer to 10⁹ cfu/ml and tested in the PAA. The incubation conditions for each enzyme were according to the Worthington Enzyme Manual (Worthington Biochemical Corp., Freehold, NJ, USA); the ratio of enzyme to substrate was 100 µg/100 µl.

Periodate treatment

The bacterial cell suspensions (500 µl, 10⁹ cfu/ml) were pre-incubated overnight with an equal volume of 100 mM sodium periodate, at 4°C in the dark. After washing three times with PBS, bacterial cells were incubated further with 100 mM NaBH₄ for 1 h at 4°C, washed twice in PBS and finally resuspended in PBS at the original concentration [22].

Adherence assay

Bacterial adherence was quantified by a bioluminescence assay [15]. Heparin binding of staphylococcal cells was tested on microtitration plates (96 wells; Costar, Cambridge, MA, USA) with end-point-attached heparin (4 µg/cm²) [23]. One hundred µl (5 × 10⁸ cfu/ml) of bacteria were incubated on the heparin-coated surface for 1 h at 20°C. The wells were washed three times with PBS-Tween 20 0.05% and the number of adherent bacteria was quantified by measuring the ATP content of the bound cells, as previously described [15]. Briefly, ATP was extracted from adherent bacteria in 100 µl of trichloroacetic acid (TCA) 2.5%, 50 µl of the extraction fluid was transferred to 200 µl ATP Monitoring Reagent (AMR) with 750 µl 0.1 M Tris-acetate, 2 mM EDTA, pH 7.75 (TAE) and vortex mixed for 1 min. A blank containing 200 µl of AMR, 750 µl of TAE and 50 µl of TCA 2.5% was prepared simultaneously. The amount of ATP was recorded in a luminometer (LKB Wallac 1250 Luminometer, Turku, Finland). Results (percentage binding) were expressed as the mean value of triplicate samples.

Results

Evaluation of the agglutination assays-agglutination strength

The agglutination scored in the PAA showed different intensities with cells of different staphylococcal strains. According to this and for the purpose of semi-quantitative analysis, the agglutination was classified

in four numerically assigned categories: no macroscopically visible agglutination (0 = -), weak (1 = +), moderate (2 = ++), and strong (3 = +++) agglutination [14, 21].

PAA reactivity of CNS strains with heparin-coated latex beads

Of a total of 38 strains from six different CNS species tested, 30 agglutinated with heparin-coated latex beads. However, there was great strain variability, and different CNS species agglutinated heparin-coated latex beads to varying extents (e.g., cells of *S. haemolyticus* reacted more strongly than cells of *S. epidermidis* strains) (Fig. 1).

Influence of culture media

Reference type culture collection strains *S. aureus* Cowan1, *S. haemolyticus* SM31 and E 2498, *S. epidermidis* H9-E, 3380 and RP 12 and *S. saprophyticus* TW 111 were grown on BA, in BHI, TH broth and TS broth and tested for PAA reactivity with heparin-coated latex beads. TH broth and BA were optimal for CNS strains because only a few strains autoaggregated. Heparin binding to staphylococcal cells, harvested in the late stationary phase, was influenced by the culture medium and growth conditions. TH broth enhanced the expression of heparin-binding surface components (stronger agglutination reaction) of *S. aureus* and CNS strains compared to cells of the same strains grown on BA (Table 1).

Inhibition of PAA

Pre-incubation of *S. haemolyticus* SM131 cells with various glycosaminoglycans and polysulphated com-

Table 1. Effect of culture media on agglutination patterns of some *S. aureus* and CNS cells with heparin-coated beads

Strain	PAA score* in			
	TH	BA	BHI	TS
<i>S. aureus</i>				
Cowan 1	3	2	3	0
<i>S. haemolyticus</i>				
SM 131	3	3	AA	1-2
E 2498	2	2	2	2
<i>S. epidermidis</i>				
3380	2	1	1	NT
H9-E	3	2	NT	1
RP 12	AA	2	AA	1
<i>S. saprophyticus</i>				
TW 111	2	0	2	0

AA, autoaggregating; NT, not tested; BA, blood agar; TH, Todd-Hewitt broth; BHI, brain heart infusion broth; TS, tryptic soy broth. *PAA reactions were scored from strongly positive 3 or 2 to weakly positive 1, or negative 0.

Fig. 1. Agglutination patterns of staphylococcal cells with heparin-coated latex beads. **A**, *S. aureus* (2 strains); **B**, *S. epidermidis* (17); **C**, *S. haemolyticus* (5); **D**, *S. hominis* (4); **E**, *S. capitis* (3); **F**, *S. saprophyticus* (1); **G**, *Micrococcus luteus* (1); **H**, other CNS strains (7). Each dot represents one bacterial strain. Experimental procedures are described in *Materials and methods*.

pounds blocked the PAA reactivity with heparin-coated latex beads to different extents. Fragmin[®], fucoidan, suramin, λ -carrageenan, Trypan blue and Evans blue (at final concentrations of 1 mg/ml) almost abolished the agglutination of *S. haemolyticus* SM131 and E 2498 cells. Heparin blocked the agglutination to a lesser extent and hyaluronic and colominic acid did not affect the agglutination ability of *S. haemolyticus* SM131 cells with heparin-coated beads (Table 2).

The cell surface extract of *S. haemolyticus* SM131 cells completely inhibited the agglutination of heparin-coated latex beads (Table 2).

Protease and heat treatment

Cells of *S. haemolyticus* strains SM131 and E 2498 were sensitive to protease treatment (Table 3). Cell-surface structures of *S. haemolyticus* E 2498 were sensitive to all proteases used, whereas those of *S.*

Table 2. Influence of potential inhibitors on the agglutination of *S. haemolyticus* SM131 and E 2498 cells with heparin-coated latex beads

Inhibitor (1 mg/ml final concentration)	PAA score* with strain	
	SM 131	E 2498
PBS (control)	3	2
Cell-surface extract	0	0
Heparins and heparin-like molecules		
Fragmin [®] (mol. wt 5000)	1	1
Heparin (mol. wt 4000–6000)	2	1
Suramin	1	1
Fucoidan	1	1
λ -Carrageenan	1	1
Pentosan polysulphated	1	1
Sulphated dyes		
Evans blue	1	1
Trypan blue	1	1
Congo Red	1	1
Non-sulphated polymers		
Hyaluronic acid†	3	2
Colominic acid	3	2

*See footnote to Table 1.

† α -2-8-Polysialic acid.

haemolyticus SM131 were more protease-resistant (Table 3). Only pronase E and proteinase K treatment of cells of *S. haemolyticus* SM 131 abolished the agglutination reaction with heparin-coated latex beads. Heat treatment (90°C, 30 min) of cells of both *S. haemolyticus* SM 131 and E 2498 decreased agglutination intensity with heparin-coated latex beads (Table 3).

Tunicamycin treatment of staphylococci

Although tunicamycin, an antibiotic that was found to block the protein glycosylation of bacterial surfaces [18], at 12.5 µg/ml subinhibitory concentration, affects the growth of *S. haemolyticus* SM 131 and E 2498 and *S. epidermidis* H9-E, the agglutination reaction was partially abolished for cells of *S. haemolyticus* SM 131 and *S. epidermidis* H9-E, whereas no effect was observed in the agglutination by *S. haemolyticus* E 2498 cells, compared with heparin agglutination caused by non-antibiotic-treated bacterial cells (Table 4).

Periodate treatment

Further experiments were made to study the importance of carbohydrates in the binding of heparin to staphylococcal cells. The heparin agglutination was abolished by periodate treatment of cells of *S. haemolyticus* SM 131 and E 2498 (Table 3).

Bioluminescence assay

Agglutination results (PAA score) were compared with the heparin binding results of staphylococcal cells

Table 3. PAA reactivity of *S. haemolyticus* SM131 and E 2498 cells with heparin-coated latex beads after heat, enzymic and periodate treatments

Treatment	PAA score* with <i>S. haemolyticus</i>	
	SM 131	E 2498
Buffer (PBS, pH 7.2)	3	2
Pronase E	0	0
Trypsin	2	0
Proteinase K	1	0
Pepsin	1	0
Heat treatment (90°C 30 min)	1	1
Periodate treatment	0	0

*See footnote to Table 1.

Table 4. Effect of a subinhibitory concentration of tunicamycin, in Todd-Hewitt broth, on the ability to agglutinate heparin-coated latex beads of *S. haemolyticus* SM 131 and E 2498 and *S. epidermidis* H9-E

Tunicamycin (µg/ml)	Heparin-PAA reaction*		
	<i>S. haemolyticus</i>		<i>S. epidermidis</i>
	SM 131	E 2498	H9-E
0 (Control)	3	2	3
12.5	2	2	1

*See footnote to Table 1.

(Fig. 2). The three reaction groups positive for heparin-PAA with scores of 3, 2 and 1, corresponded to median values of 7%, 2.6% and 1% binding to heparinised microtitration plates. No visible agglutination reaction (0) corresponded to no detectable adherence of staphylococci to the heparinised microtitration plates, indicating a high sensitivity of the PAA test described in this study (Fig. 2).

Discussion

The attachment of bacteria to host tissues is the initial event in colonisation and the development of an infection. Staphylococci become associated with host tissue components in open wounds and biomaterial-associated infections by lectin-like bacterial components (often termed 'adhesins'), with specific structures on the host cells and ECM and by hydrophobic and electrostatic interactions [16, 17, 24].

Glycosaminoglycans such as heparin interact with and modulate the structure, function and metabolism of a wide variety of host proteins and enzymes including ECM, growth factors, pro-inflammatory chemokines and leucocyte proteases, such as cathepsin [2]. Heparin interacts with these proteins through multiple and specific electrostatic forces between the polyanionic groups of the glycosaminoglycan chain and the positively charged amino acids of those proteins [2].

Heparin-coated biomaterials have clinical application today [15–17] and it is important to study heparin-CNS interactions in order to understand the behaviour of heparinised biomaterials and also to develop methods to block the CNS adhesion on these surfaces. Therefore, this study focused on this topic and developed a rapid assay (PAA) for detecting heparin binding to CNS strains. Such an assay was shown to give reliable data on the binding properties of serum and ECM proteins to staphylococci [14, 21] and other bacteria [25–27]. By performing the tests in duplicate and repeating them on separate days, highly reproducible results were obtained. Amino-modified latex beads from Seradyn and non-activated latex beads from Difco were the most suitable for coating with heparin. Uncoated latex particles caused non-specific agglutination because of the strong negative charge and high surface hydrophobic properties [14, 25]. Coating of the particles with ovalbumin was inadequate as a negative control as staphylococci have often been found to bind to ovalbumin (unpublished data). BSA reduced the non-specific binding of the particles and was used as a negative control. PAA reagents were stable during storage for 3 months at 4°C. The reagents do not withstand freezing.

The fact that proteases, heat, periodate and tunicamycin treatments of staphylococcal cells decreased the

Fig. 2. Comparison between PAA score (□) and percentage heparin binding (▨) of cells of 10 CNS strains (*S. epidermidis* BD 7743, a; BD 1897, b; BD 16301, c; BD 6694, d; BD 13807, e; BD 9229, f; *S. haemolyticus* E 2498, i; MD-7, k; *S. hominis* BD 8127, j; BD 8008, l) and two *S. aureus* strains (ISP 546, g; Cowan 1, h) determined by bioluminescence method on heparinised microtitration plates. Bacterial preparations and binding assays are described in *Materials and methods*.

heparin-PAA reactivity suggests that both protein and carbohydrate moieties are responsible for heparin binding (Tables 3, 4). The agglutination of CNS with heparin was blocked by various polyanions but not by non-sulphated macromolecules, indicating the importance of the sulphation of these inhibitors. It is interesting that polysulphated oligosaccharides such as pentosan polysulphate, fucoidan and dextran sulphate were potent inhibitors (Table 2). These compounds all have O-sulphated groups and a saccharide backbone which are distinct from those of heparin and heparin sulphate. These results suggest that the agglutination of staphylococcal cells with heparin most probably involved hydrophobic surface proteins with charged sulphur-containing groups, as the binding was efficiently inhibited by polysulphated but not by non-sulphated polymers. The complete inhibition of the heparin-PAA reaction by a staphylococcal cell-surface extract indicates that the structures responsible for heparin binding are exposed on the cell surface.

This finding is particularly interesting as staphylococcal cells specifically interact with various ECM molecules, such as fibronectin, vitronectin, collagen, thrombospondin, lactoferrin and transferrin, all of which contain heparin-binding domains [13].

In conclusion, these data support the earlier observation that the attachment of CNS cells to heparinised biomaterials is mediated by surface-located proteinaceous structures [12, 15]. Nevertheless, hydrophobic and electrostatic interactions may be important in the pathogenesis of biomaterial-associated infections caused by CNS. Further studies on the hydrophobic surface components, or hydrophobins [24] of CNS should be carried out.

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