Affinity Apheresis for Treatment of Bacteremia Caused by *Staphylococcus aureus* and/or Methicillin-Resistant *S. aureus* (MRSA)

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***Staphylococcus aureus* (SA) bacteremia is associated with high mortality, and often results in metastatic infections. The methicillin-resistant SA (MRSA) is an urgent health care issue, as nosocomial infections with these bacteria represent limited treatment alternatives. Samples of whole blood containing challenge inoculums of SA and MRSA strains were passed through columns packed with surface-heparinized polyethylene beads. The bound bacteria were eluted and quantitatively determined by culturing and by real-time PCR. Significant amounts of both SA and MRSA adhered to the heparinized beads (more than 65% of inoculated bacteria). After rinsing with buffer at high ionic strength, viable bacteria or bacterial DNA were eluted from the columns, indicating that the binding was specific. The conclusions that can be made from these experiments are that, as earlier reported in the literature, the high affinity of SA to heparin is retained in whole blood, and MRSA in whole blood binds to heparin with similar or higher affinity than SA. It should be possible to lower the amount of SA and/or MRSA from the blood of infected patients to levels that could be taken care of by the immune system. In previous studies, we have shown that passing blood from septic patients over beads coated with end-point-attached, biologically active heparin is a useful technique for regulating the levels of heparin-binding cytokine. These findings in combination with the present findings indicate the possibility of creating an apheresis technology for treatment of sepsis caused by SA and/or MRSA.**

Keywords: Immobilized heparin, bacteria, *Staphylococcus aureus*, MRSA, apheresis

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When microorganisms enter the bloodstream, a potentially life-threatening disease may occur. For instance, bacteremia that is often acquired in the hospital may result in severe sepsis. Premature and newborn babies as well as trauma and intensive care patients are vulnerable to such infections.

Staphylococcus aureus (SA) is the leading cause of bacteremia, and a steady increase in the incidence of severe infection such as sepsis caused by SA has been uniformly noted in the Western world [37]. SA bacteremia is associated with high mortality and often results in metastatic infections, such as infective endocarditis [10, 33].

Antibiotic resistance of bacteria is an increasing problem within medical treatment and there is a need for alternative therapies to address this. Thus, the methicillin-resistant SA (MRSA) is an urgent health care issue, as nosocomial infections with these bacteria present limited treatment alternatives. MRSA bacteremia is associated with a significant increase in mortality [6]. Inadequate antibiotic therapy is the main prognostic factor associated with mortality in patients with MRSA infections [12, 29]. Furthermore, MRSA infections are associated with high costs and extended hospital stay.

Humans serve as natural reservoirs for SA [19]. Thus, hospital-acquired MRSA strains are found not only in hospitals, but have also spread to long-term care facilities. In addition, community-acquired MRSA strains are rapidly emerging [5, 7].

Many microbes utilize glycosaminoglycans such as heparan sulfate (HS) on the surface of the mammalian cell as receptors. This mechanism is general and valid for many bacteria, viruses, and parasites. The HS molecules, structurally related to heparin, are highly negatively charged, partially sulfated, carbohydrate portions of proteoglycans that are present on the surface of almost all mammalian cells. HS chains are built up by alternating D-glucosamine and glycuronic acids (L-iduronic and D-glucuronic acids).

HS-binding microbes are exemplified by viruses like Herpes simplex virus type 1 (HSV-1), the causative agent of orolabial herpes; HSV-2, the causative agent of genital herpes; cytomegalovirus (CMV), the major complicating agent in immunosuppressed patients; dengue virus (causing recurrent fevers); and human immunodeficiency virus (HIV) [20]; and by bacteria including SA, *Helicobacter pylori*, *Streptococcus pyogenes*, *S. sanguis*, *S. mutans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*; and parasites including *Plasmodium falciparum* (causing malaria) and *Trypanosoma cruzi* (causing trypanosomiasis) [32].

Heparin is a glycosaminoglycan that is isolated from mammalian tissue and has been recognized for its blood anticoagulant properties. Heparin has for more than 70 years been used in the clinic as a blood anticoagulant and antithrombotic agent. Owing to the close structural resemblance between heparin and HS, they have almost identical biological activities *in vivo*, including binding to the microorganisms that have affinity for HS. As preparations of heparin are more homogenous than preparations of HS, the affinity of microbes to heparin is higher than that to HS, and as heparin is available in larger quantities we have used this molecule in the present attempt of microbial affinity apheresis.

When the immune system is fighting pathogens like bacteria, proinflammatory cytokines signal immune cells to travel to the site of infection [4]. In sepsis, the microbial invasion not only results in an excessive release of cytokines (*e.g.*, TNF-α, IL-1, IL-6, IFN-γ) and release of danger signals from the invaded tissues, the condition also induces an anti-inflammatory response (such as IL-10, TGF-β), and other signaling pathways such as the complement and coagulation cascades. As a result, multiple organ dysfunctions may occur.

Various extracorporeal blood purification systems, such as hemodialysis, hemofiltration, and hemodiafiltration, have been used for removal of inflammatory mediators by nonspecific binding to membranes, with so-far limited improved outcomes in septic patients [22, 31]. The effectiveness of extracorporeal immunadsorption techniques utilizing surface-bound antibodies for removal of specific bacterial endotoxin (lipopolysaccharide, LPS) or host-derived factors is still under investigation [28].

In a study on blood samples from septic patients, we have shown that the level of one proinflammatory cytokine, TNF-α, was restored to normal levels after passage over beads coated with biologically active heparin, thus indicating the possibility of breaking the "cytokine storm" [1]. As also has been observed by other researchers, in connection with cardiopulmonary bypass [11], no further activation of the inflammatory system in blood, due to surface activation, occurred during passage over the heparinized beads (*i.e.*, no increase of the cytokine Rantes was demonstrated) [30].

We hypothesize that when infected blood is passed through a device, consisting of a column packed with beads coated with biologically active heparin (*i.e.*, end-point attached), many bacteria including SA would be removed. Our aim was to investigate to what extent SA and MRSA are removed from whole blood after passage over surface-heparinized polyethylene beads.

MATERIALS AND METHODS

Bacterial Strains

One type strain of *S. aureus* (CCUG1800T, not methcillin resistant) was used as a control. Five MRSA strains were obtained from the Culture Collection, University of Gothenburg (CCUG). Three of those strains were PVL-positive and typed as USA300 (CCUG: 51485, 53251, and 54860), whereas the other two strains were typed as USA100 (CCUG: 46314, 55517). An additional MRSA strain, CCUG 35600, was used as a positive control in the real-time PCR.

The bacterial cultures were incubated in Brain Heart Infusion broth (BHI) overnight at 37°C. A 0.5 ml aliquot of each culture was then transferred to a test tube containing 4.5 ml of fresh BHI and incubated at 37°C for two additional hours. The culture tubes were centrifuged at 4,000 rpm for 10 min at 4°C, decanted, and washed once with distilled water (5 ml). After addition of a small volume of PBS to each pellet, followed by vortexing (approximately 15 sec), the concentration of bacteria was determined by adding additional PBS (ca. 5 ml) and measuring the optical density at 590 nm and comparing with a standard curve. A suspension of estimated 5×10^6 bacterial cells/ml was prepared by mixing one volume of bacterial suspension with 9 volumes of citrated blood.

In all experiments, the concentration of bacteria was also determined by viable counts. In short, samples of the bacterial suspensions were diluted in 10-fold serial dilutions starting from 1/100 to $1/10^5$ in PBS. Each 10-fold dilution (100 µl) was spread onto blood agar plates and incubated at least for 24 h at 37°C. The day after, the number of colony-forming units (CFU) was counted.

Preparation of Heparin Column

Polyethylene (PE) beads, with an average diameter of 0.3 mm (lot no. 180153), were supplied by the Polymer Technology Group (Berkeley, USA) and the columns (Polyprep, 0.8×4 cm) were obtained from BIORAD (USA). Heparin and polyethyleneimine (PEI) were purchased from Scientific Protein Laboratories (Waunakee, WI, USA) and BASF (Ludwigshafen, Germany), respectively. All chemicals used were of analytical grade or better.

The PE-beads were etched with an oxidizing agent (potassium permanganate in sulfuric acid). These hydrophilized beads, *inter alia* containing OH-groups and double bonds, were later used as controls. Immobilization of heparin onto the beads was performed as described by Larm *et al.* [14]. The resulting PE-beads, with covalently end-point-attached heparin, were sterilized with ethylenoxide (ETO) and stored dried. The amount of heparin was determined to be 2.0 mg heparin/g beads with the MBTH method [14, 26].

For the binding experiment, 0.43 g of each bead type, corresponding to approximately 1 ml of bed volume, was added to the columns. The column was rinsed with distilled water using vacuum to allow all the material to be packed at the bottom. A filter was put onto the

bed and the column rinsed with 250 ml of distilled water, preventing air to enter the bed. The columns were stored overnight in vacuum with water above the gel bed.

Binding of Bacteria to the Beads

The packed columns were washed twice with 5 ml of PBS, followed by 5 ml of PBS with 2 M NaCl added (PBS-2M), and finally with 5 ml of PBS three times. A volume of 0.5 ml of the mixture of each of the six bacterial cultures and blood (SA-B) (see Bacterial Strains) was added to the heparinized (Hep) and non-heparinized (Nonhep) columns. After absorption of the volume, 1 ml of PBS was added and the void was collected (void). The column was washed twice with 5 ml of PBS and all wash solutions were collected (wash). Finally, bound bacteria were eluted with 5 ml of PBS-2M, added twice to the columns (eluate). It took approximately 2–5 min for the added volumes of the various suspensions/solutions to pass through the columns.

Samples of SA-B, 0.5 ml of SA-B with added PBS (10 ml), and 0.5 ml of SA-B with PBS-2M (10 ml) were prepared and stored for the same time period as the samples passing through the columns. All samples were then stored on ice. The tubes with 10 ml of suspensions were all centrifuged at 4,000 $\times g$ for 25 min at 4°C. The supernatants were carefully removed and 1 ml of PBS was added to each tube.

Then 0.5 ml was saved for real-time PCR and stored frozen.

Quantification of MRSA by Real-Time PCR, SyberGreen PCR

A 500 μl volume of sample was used for preparation of DNA. The samples were washed twice in DNAse/RNAse-free ultrapure water (800 μl; Invitrogen) and centrifuged at 12,000 $\times g$ for 10 min, at 4°C. Finally, the pellets were suspended in 500 μl of the ultrapure water. A 300 μl volume of the suspension was transferred to new tubes and the DNA extraction performed with a Tecan MiniPrep 75 Automated Liquid Handler (Tecan Nordic AB, Mölndal, Sweden) using Bugs'n Beads (Nordiag AB, Hägersten Sweden).

Real-time PCR based on SyberGreen fluorescent dye, which selectively binds to double-stranded DNA templates, was performed with the Rotorgene method (Corbett Rotor-Gene 3000, QIAGEN AB, Sollentuna, Sweden). The analysis was performed according to the manufacturer´s manual.

Two primers for the *S. aureus*-specific *nuc*A gene (sequences 5'-GCGATTGATGGTGATACGGTI-3' and 5'-AGCCAAGCCTTGACGA ACTAAAGC-3') were used for DNA quantification [2].

Included in each real-time PCR run were a negative control (nuclease-free water) and positive SA controls (three concentrations of DNA, CCUG 35600), and a positive DNA extracted control (10^6 bacteria/ml of CCUG 35600).

Statistics

Students' t-test was used for comparison of binding of bacteria between heparinized and non-heparinized beads.

RESULTS

Generally, the numbers of recovered staphylococci in void, wash, and eluate samples gave similar results with either of the analyses methods (Fig. 1), except that the eluates
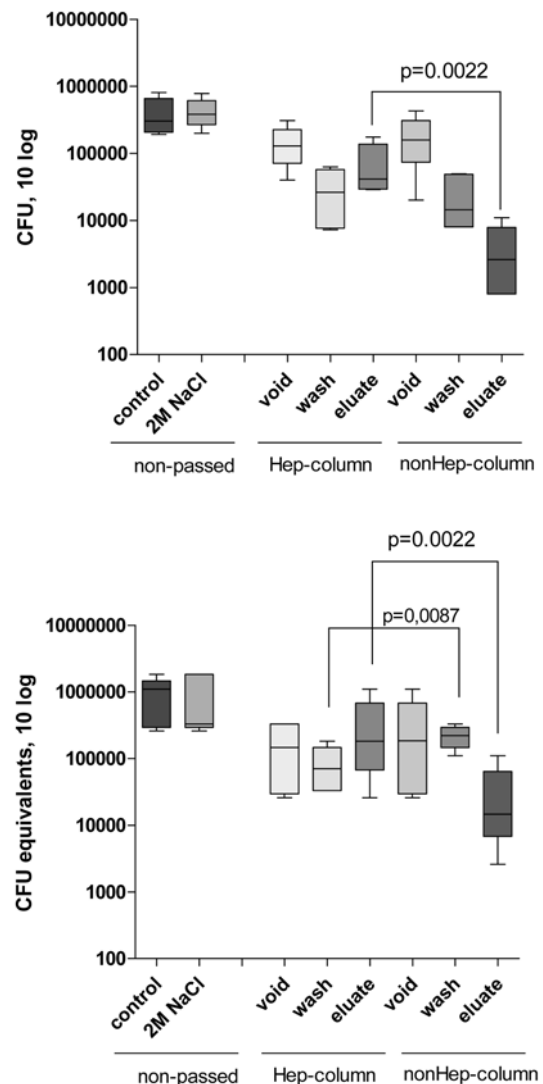

Fig. 1. Number of staphylococci in void, wash, and eluate, by passing blood containing approximately 4×10^5 CFU (upper graph) or 1×10^6 bDNA (CFU equivalents) (lower graph) through heparinized (Hep-column) and nonheparinized (nonHep-column) columns.

Median values of all six strains are shown, and the boxes represent the 25 percentiles with the lowest and highest values indicated.

contained more bacterial DNA (bDNA) copies in relation to void samples compared with CFU for Hep columns, and wash samples contained similar amounts of bDNA to void samples for nonHep columns.

A significant difference was found between the Hep and nonHep columns regarding leaking of bDNA during the washing procedure (Fig. 1), since the washing solutions of the nonHep columns contained higher amounts of bDNA, and reached the same range as the void samples. The numbers of bDNA, as well as CFU values, in the eluate of the Hep columns on the other hand were significantly

higher than those of the nonHep columns, indicating that bacteria bind more sufficiently to the Hep columns.

The reason for the reduced numbers of CFU in washing solutions and eluates compared with the numbers of the void samples for both types of columns would probably be partly explained by reduction of the viability of bacterial cells retained or bound to the columns for an extended time period.

Comparing the recoveries for each staphylococcal strain in void, wash, and eluate samples, based on percentages of the total numbers of CFU or bDNA in corresponding non-passed blood controls, the bacteria bound more strongly to the Hep columns compared with nonHep columns regarding the content in the void samples (Fig. 2). However, owing to the variability, no statistically significant difference was achieved. On average, based on paired comparisons, a 3-fold higher concentration of bDNA was found in the void samples of nonHep columns. Regarding wash samples, significantly more bDNA was washed out from the nonHep columns (a 4-fold difference, based on paired comparisons). Furthermore, significantly more CFU/bDNA was released

by the 2 M NaCl solution from the Hep columns. On average, 40 and 80 times more bacteria were recovered from the Hep columns, regarding viable counts and bDNA, respectively, thus indicating a significant binding of staphylococci to the Hep columns, but not to nonHep columns.

It should be mentioned that the percentages of mean total recoveries of the Hep columns were 60% for CFU, and 82% for bDNA. The corresponding figures for nonHep columns were 50% and 85%, respectively.

DISCUSSION

Significant amounts of SA and/or MRSA were removed from inoculated blood on passage through a column packed with heparinized beads. The various types of MRSA strains bound as effectively as did the SA strain. This finding implies that it should be possible to create an apheresis technology where the number of bacteria in the blood of patients with bacteremia caused by SA and/or MRSA is reduced to levels that could be taken care of by the immune system. This reduction may be carried out without deleterious inflammatory activation of the blood, which always occurs in connection with extracorporeal circulation in non-heparinized systems, sometimes leading to sepsis [11, 23].

In the present work, PE-beads that have been made hydrophilic by oxidation were used as references, and indeed bacteria adhered to these beads but with nonspecific binding, as demonstrated by the 40–80-fold higher recovery of bacteria in the eluate of the Hep columns as compared with the nonHep column. As discussed above, the use of these non-heparinized hydrophilic beads for removal of SA and/ or MRSA will also result in further activation of the inflammatory system, which will aggravate the condition of the patient. In another paper, using the same experimental set-up, we showed that no deleterious surface activation of blood from septic patients occurred in the surface-heparinized system. Instead, blood was activated by the non-heparinized control beads, as shown by an increase of the chemokine Rantes, whereas the concentration of the proinflammatory cytokine TNF-α was lowered to normal levels by the Hep columns [1].

Extracorporeal blood purification treatment in septic patients, by using conventional continuous renal replacement techniques, was shown to remove various inflammatory mediators from the circulation to a minor extent [22, 31, 36]. Increasing the hemofiltration rate improved the survival in intensive care unit patients with acute renal failure (13% sepsis), when compared with the conventional rate [27], although the mediator removal from blood was considered less satisfactory. The use of high permeable/high cut-off (HCO) membranes of different chemical compositions with various modalities of the blood purification (hemofiltration,
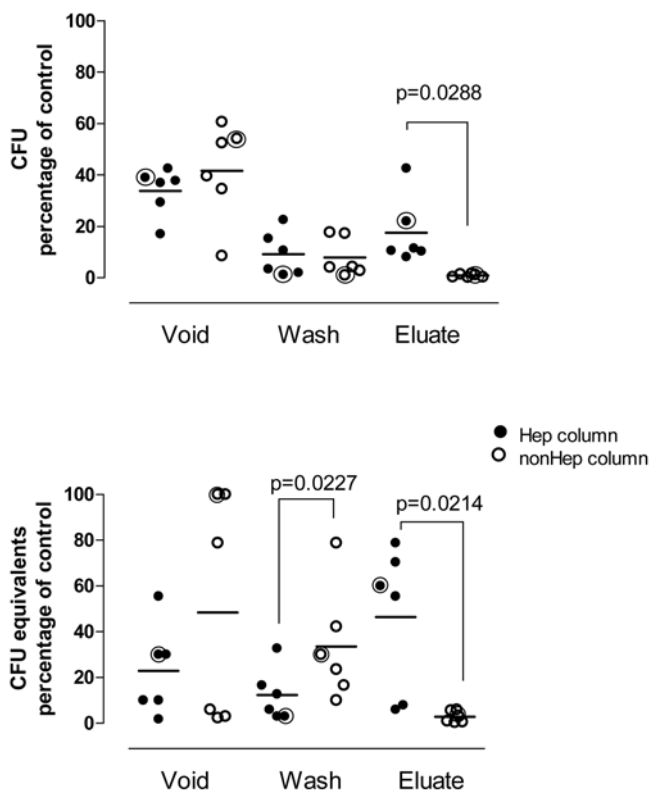

Fig. 2. Recoveries of the MRSA strains and *S. aureus* in void, wash, and eluate, by passing blood containing approximately 4×10^5 CFU (upper graph) or 1×10^6 bDNA (CFU equivalents) through heparinized (Hep column) and nonheparinized (nonHep column) columns.

Each point represents the percentage of recovery to the total CFU or bDNA in non-passed sham-treated controls. *S. aureus* is indicated by a circle. Means are indicated. Paired t-test was used for the statistics.

hemodialysis, hemodiafiltration) points towards a more efficient removal of mediators. However, an improved outcome in septic patients with HCO treatment has not yet been sufficiently documented.

Immunoadsorption or hemoperfusion techniques employing membrane- or surface-bound antibodies in the extracorporeal device, bind specific target mediators, thereby removing them from blood. Examples of such target mediators are bacterial lipopolysaccharide (LPS), IL-6, and complement factor C5a [28]. Although biocompatibility problems with the surfaces and matrices have been overcome, the clinical effectiveness of these devices needs further investigation.

During a long evolution, many pathogenic microorganisms have learned to utilize eukaryotic cell-surface glycoconjugates (*i.e.*, glycolipids, glycoproteins, and proteoglycans, like HS) as receptor molecules for cell attachment to facilitate tissue colonization and invasion processes. Specific proteins called adhesins on the surface of bacteria, viruses, fungi, and parasites interact with carbohydrate chains of glycoconjugates, which enable microbes to colonize mucosal surfaces and tissue lesions [32]. The capacity of bacteria to bind to HS proteoglycans could be regarded as a virulence factor [9], and two heparin-binding proteins have been isolated from SA [15]. The two MRSA strains that showed the lowest eluate values (bDNA and CFU) of the Hep column (Fig. 2), and which also corresponded to the lowest void values, indicated that their binding to heparin was particularly strong, and therefore less easily reverted by the salt solution. Different isolates of MRSA may vary in their level of expression of HS-binding proteins. It is known that the growth condition for bacteria affects their HS-binding capacity [16, 18].

Both bacterial virulence and load are important factors for the host response and the outcome of sepsis [34]. Indeed, it should be mentioned that not only live but also dead bacterial cells or fragments of their cell walls in the bloodstream may induce harmful host responses during severe sepsis. In several studies using quantitative PCR, a relation has been observed between the concentration of bacteria in the blood and the severity of sepsis. An association was observed between a high quantitative bacterial load of *Streptococcus pneumoniae* in blood samples and increased mortality or severity of disease (septic shock or the need for mechanical ventilation) [25]. In patients with bacteremia of *N. meningitidis*, the bacterial load also correlated with the mortality and severity of the disease [3, 8, 13]. In a case report, the SA DNA load of the blood was shown to correlate with the fever development in one studied patient [24]. A range from $3 \times 10^3 - 10^7$ DNA copies/ml blood have been observed in bacteremic patients [8, 17, 21, 24, 35]. In general, it appears the blood bacterial load is a prognostic parameter of the severity of the disease. Thus, the dual function of heparinized beads by reducing both the load of

SA/MRSA and TNF-α in blood without triggering the coagulation system should have beneficial effects on the outcome in septic patients.

When utilizing the properties of surface-immobilized heparin in the clinic, it is important that the biological activities of the heparin molecules are retained after coupling. This is best achieved when the molecules are coupled *via* the reducing terminal sugar residues, and consequently end point attached to the surface as in the current device. Similarly, in nature, almost all glycosaminoglycans including heparin and the HS molecules are linked *via* the terminal reducing end to a protein backbone (end point attached).

The present results indicate that a new technology for treatment of patients suffering from SA- and/or MRSA-induced sepsis could be developed.

When other medical treatments of bacteremia caused by MRSA have proven ineffective, affinity apheresis by utilizing the affinity of the bacterium for immobilized heparin could be an alternative by reducing the bacterial load as well as levels of proinflammatory cytokines.

Acknowledgments

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