Collagen Binding of *Staphylococcus aureus* Is a Virulence Factor in Experimental Endocarditis

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The role of *Staphylococcus aureus* collagen binding in the development of experimental endocarditis was studied. Two isogenic strains of *S. aureus*, 1 carrying an insertional inactivation of the gene encoding collagen-binding protein, were compared in a rat model of catheter-induced infective endocarditis (IE). Separate groups of rats with traumatized aortic valves were intravenously challenged with 1 of the strains. In rats sacrificed 24 h after inoculation, the collagen-binding strain significantly outnumbered the mutant strain (P < .001); however, 1 h after challenge, there was no difference in numbers of the 2 strains. The results were substantiated, using a 1:1 mixture of the parent strain and the mutant as an inoculate. Our findings suggest that collagen binding of *S. aureus* is important in the sustenance of experimental IE and plays a limited role during the initial attachment of the microorganism to traumatized aortic valves.

Infecive endocarditis (IE) can be related to cardiac valve defects resulting from clinical conditions, including rheumatic fever, stenosis, prosthetic valve implants, congenital heart disease, and degenerative changes. Such defects are thought to produce an abnormal, high-velocity jet stream of blood that can damage the cardiac endothelium [1]. Platelets and fibrin adhere to the exposed subendothelial matrix and form sterile vegetations on the cardiac valves, a condition called nonbacterial thrombotic endocarditis (NBTE). Following bacteremia, microorganisms can colonize NBTE and cause IE [2–5]. Additional platelets and fibrin are deposited over the microorganisms, leading to enlargement of the vegetation, and markedly impede the penetration of phagocytic cells [6].

IE is commonly caused by viridans streptococci, which occasionally enter the bloodstream from the oral microflora [7]. *Staphylococcus aureus* and coagulase-negative staphylococci (e.g., *S. epidermidis* and *S. hemolyticus*) are also often isolated as the infectious agents [8–11]. In intravenous drug users, IE is mainly caused by *S. aureus* infection of the tricuspid valve, even when the patient has no known history of valvular dysfunction [12]. In prosthetic valve IE, coagulase-negative staphylococci (e.g., *S. epidermidis*) are more commonly isolated [13].

An early crucial event in the infectious process is the adherence of microorganisms to tissue and matrix proteins [14]. *S. aureus* has been shown to specifically bind to collagen [15–22], fibronectin [23–26], laminin [27], vitronectin [28, 29], and fibrinogen [30–32].

Sterile vegetations consist partly of fibrin and fibronectin. It has been suggested that fibronectin facilitates adherence of *S. aureus* to vegetations and is of importance in the development of IE [33–35]. Recently, Moreillon et al. [36] provided evidence that *S. aureus* clumping factor is effective in promoting experimental IE in rats. However, the role of other binding functions in IE has not been elucidated. Collagen, for example, is present in valvular and aortic tissues and has been reported to be found in sterile vegetations [2]. It is also possible that collagen, being part of the basal membrane, is exposed on the surface of damaged heart valves. Thus, collagen is a possible candidate for host-protein bacterial interaction.

To develop alternative therapeutic measures against IE, such as passive immunization, vaccination, or application of antiadhesive compounds, it is essential to fully understand the mechanisms behind infection and the interrelation between host tissue and various adhesive functions of the microorganism. To that end, we sought to assess the role of *S. aureus* binding to collagen in a rat model of catheter-induced IE. We used 2 isogenic strains of *S. aureus*, 1 carrying an insertional inactivation of the gene encoding collagen-binding protein [37].

Materials and Methods

**Bacterial strains.** The gene encoding the collagen-binding protein in *S. aureus* has been cloned, and the nucleotide sequence has been determined [38]. The gene for collagen-binding protein in an *S. aureus* clinical strain, Phillips, was insertional inactivated using a gene encoding gentamicin resistance [37]. The mutant, which is unable to bind to collagen and is resistant to gentamicin, is called PH100. Phillips and PH100 strains were supplied by J. Patti (Texas A&M University, Houston).

Bacteria were grown in brain-heart infusion (BHI) broth, diluted in PBS (pH 7.3) to 10^5, 10^6, 10^7, and 10^8 cfu/mL, and stored at −70°C. This ensured a standardized concentration and quality of bacteria for use in each experiment. Aggregation of bacteria, as
seen by microscopic examination, was negligible both after growth in BHI and when recovered from heart tissues.

Adherence to immobilized collagen, fibronectin, or fibrinogen in vitro. Microtiter wells (Costar, Cambridge, MA) were coated overnight at 20°C with collagen type II or III (Southern Biotechnology, Birmingham AL), fibronectin (Sigma, St. Louis), or fibrinogen (Kabi-Pharmacia, Stockholm) at 20–0.2 μg/mL in PBS. (Data shown in figure 1 are for 4 μg/mL.) Plates were coated again with 2% bovine serum albumin fraction V (ICN Biomedicals, High Wycombe, UK) in PBS for 1 h at 37°C. Radioactive labeling was done by growing bacteria to late logarithmic phase in BHI containing 50 μCi of [3H]thymidine/mL (specific activity, 88 Ci/mmol). Specific incorporation was ~700 cfu/cpm. Washed [3H]thymidine-labeled S. aureus Phillips and PH100 cells were added to the wells in a serial dilution in PBS plus 0.05% Tween 20 (PBST) (2 × 10⁸ to 1.6 × 10⁶ bacteria/100 μL), and the wells were incubated at 37°C for 2 h.

The microtiter plates were gently rinsed, using a semiautomatic siphon microplate washer (Nunc, Roskilde, Denmark), five times with PBST to remove nonadherent bacteria. Plates were washed twice (30 min each) with 2 × 100 μL of 3% SDS to remove adherent bacteria. Radioactivity was determined by use of scintillation fluid (Ready Safe; Beckman Instruments, Fullerton CA) in a Beckman scintillation counter. Adherence to collagen of 1 strain in the presence of the other strain was tested. Increasing dilutions of Phillips were added to a constant concentration of radiolabeled PH100. This mixture was then allowed to bind to immobilized collagen in microtiter wells. The amount of radiolabeled bacteria was determined as above. The same type of experiment was also done with radiolabeled Phillips added to an increasing amount of nonlabeled Phillips.

Adherence to endothelial cells. Endothelial cells were isolated from human umbilical cords and cultivated as previously described [39]. The cells were transferred to flat-bottom microtiter wells and grown to confluence, usually for 2–3 days. Radiolabeled bacteria in PBS were added to achieve 2 × 10⁴ to 10⁷ cfu/100 μL. After 1 h of incubation at 37°C, nonadherent bacteria were removed by five gentle rinses with PBS. Attached bacteria were counted as above. Although negligible, bacterial background adherence (i.e., binding to microtiter wells treated only with endothelial cell growth medium) was subtracted. Wells were not precoated to enhance endothelial cell adherence, so bacterial background binding was restricted to sites not covered by endothelial cells.

Induction of NBTE. Wistar female rats (BK Universal, Sollentuna, Sweden), weighing 185 g, were anesthetized with pentobarbital. For each rat, a polyethylene catheter (outer diameter, 0.7 mm) was introduced via the right carotid artery into the left ventricle, passing the aortic valve, according to the method described by Heraief et al. [40]. The catheter was gently removed after 24 h, and the rat was then intravenously challenged via the tail vein.
with 1 mL of bacterial suspension at the concentrations indicated below.

In vivo adherence to heart valves. Animals were catheterized and inoculated as above. However, the amount of bacteria, $1 \times 10^6$ cfu/mL of either strain, was selected such that $~10^3$ bacteria could be recovered from the heart valves. A linear dose-response was found in a pilot experiment (data not shown). Rats were sacrificed 1 h after inoculation, and aortic valves (with vegetations as a result of the catheterization) and pulmonary valves (undamaged) were removed. The valves were washed thoroughly with sterile saline and homogenized in 1 mL of PBS by use of a Teflon homogenizer (KEBO, Stockholm) attached to an electric drill. Serial dilutions were plated on blood agar plates to count viable bacteria: The lowest detection level was 2 cfu/valve.

Another group of animals was challenged with a 1:1 mixture of both strains, similar to the approach of Winberg et al. [41]. Aortic valves were recovered as above, and homogenized valves were plated at various dilutions on plates without gentamicin. Single colonies were randomly picked and tested for their susceptibility to gentamicin, and the ratio of Phillips to PH100 was determined.

Quantification of bacteria recovered from heart valves 24, 48, or 72 h after infection. One group of animals was anesthetized, and their catheters were removed. The animals were then infected via the tail vein with $1.4 \times 10^5$ cfu of either Phillips or PH100. This inoculum size was determined in a pilot experiment, and for Phillips, it resulted in infection in almost 100% of the animals (data not shown). Twenty-four hours after inoculation, animals were sacrificed and the number of bacteria associated with the heart valves were determined as described above.

In addition, another group of animals was challenged with a 1:1 mixture of both strains and sacrificed after 24, 48, or 72 h. Aortic valves were recovered, and the ratio of Phillips to PH100 was estimated as above. In this experiment, an inoculum of $3 \times 10^5$ cfu was used to produce infection in all inoculated animals.

Results

Bacterial adherence to immobilized collagen, fibronectin, and fibrinogen in vitro. The ability for Phillips and PH100 to bind to immobilized collagen, fibronectin, and fibrinogen was compared. Figure 1A shows that Phillips bound better than PH100 to collagen type II. Binding was also measured to collagen type III and to osteoblasts grown in vitro, which produce mainly collagen type I. The results were the same as those with collagen type II (data not shown). However, binding to immobilized fibronectin (figure 1B) or immobilized fibrinogen (figure 1C) showed that Phillips and PH100 had equal binding capacity to these proteins.

In a similar adhesion experiment, the amounts of collagen, fibronectin, and fibrinogen immobilized on the plates was varied (0.2–20 µg/mL) while a constant concentration of bacteria ($3 \times 10^5$ cfu/mL) was maintained. Binding was dose dependent in every case except for PH100 on collagen (data not shown).

Adherence to collagen of 1 strain in the presence of the other strain was also tested. No interstrain interference on adherence was detected (data not shown).

In vivo adherence of bacteria to heart valves 1 h after infection. Primary adherence of bloodstream bacteria on valvar tissues was measured. Twenty-four hours after they were catheterized, animals were infected intravenously with Phillips (18 rats) or PH100 (17 rats). One hour later, the animals were sacrificed. The numbers of colony-forming units (cfu) that adhered to aortic heart valves with vegetations and to undamaged pulmonary heart valves were determined. For pulmonary heart valves, the means ± SEs for the logarithmic values of bacterial counts were 2.53 ± 0.12 for Phillips and 1.81 ± 0.11 for PH100, and for the aortic heart valves with attached vegetations, they were 3.60 ± 0.07 for Phillips and 3.54 ± 0.16 for PH100 (figure 2). There was no difference in adherence to aortic heart valves with vegetations by the 2 strains. However, Phillips adhered better than PH100 to the undamaged pulmonary valve ($P < .05$, two-tailed Student’s $t$ test). As expected, adherence to aortic valves with vegetations was better than to pulmonary valves for both strains.

Quantification of bacteria recovered from heart valves 24 h after infection. Eight rats each were infected with Phillips or PH100. Catheters were removed 24 h after insertion to avoid a possible foreign-body infection or an influence of the catheter on the infection. Animals were sacrificed 24 h after inoculation, and the numbers of cfu on aortic and pulmonary heart valves were determined. Figure 3 shows the logarithmic values for the number of bacteria on heart valves: There was a significant difference in the mean (±SE) for Phillips (5.36 ± 0.96) and PH100 (0.72 ± 0.37) ($P < .001$). The corresponding values from the pulmonary valve were 1.88 ± 0.65 for Phillips and 0.16 ± 0.13 for PH100 ($P < .05$).

Ratio of Phillips and PH100 on aortic valves after inoculation of a 1:1 mixture. Phillips and PH100 were mixed 1:1 and $3 \times 10^5$ or $2 \times 10^6$ cfu were inoculated into animals. Animals were sacrificed and bacteria were recovered from the aortic valves with vegetations 1 h ($2 \times 10^5$ cfu) or 24, 48, and 72 h ($3 \times 10^5$ cfu) after inoculation. The ratio of Phillips to the total amount of recovered bacteria from each valve was...
Figure 3. No. of bacteria recovered from aortic (left) and pulmonary (right) valves after 24 h. Horizontal lines indicate mean values. Symbols on bottom line indicate <2 bacterial cells recovered from each valve (detection limit). ●, S. aureus Phillips; ○, S. aureus PH100.

Figure 4. % of S. aureus Phillips recovered from aortic valves with vegetations at various times after animals were infected with 1:1 mix of Phillips and PH100. Ratio of Phillips to total amount of recovered bacteria from each valve was estimated and compared with ratio in inoculate ($\chi^2$ test). * $P < .001$ and ** $P < .10$ vs. inoculum.

Figure 5. Bacterial adhesion to endothelial cells. Indicated amounts of radiolabeled bacteria were added to confluent human umbilical vein endothelial cells grown in microtiter wells. Adherence after 1 h at 37°C was measured by scintillation counting (cpm) after cells were washed. Duplicate experiments are shown. ●, S. aureus Phillips; ○, S. aureus PH100.

Adherence of strains to endothelial cells. The difference in adherence of the 2 strains in vivo to the undamaged pulmonary valve 1 h after infection prompted us to determine if there was a difference in adherence in vitro to endothelial cells: We found no difference between Phillips and PH100 (figure 3). To confirm the similar binding to endothelial cells, we used scanning electron microscopy. For each strain, the number of bacte-

rrial cells adhering to endothelial cells was counted and not found to be different. About 15–30 bacteria were found per cell when $10^8$ cfu/mL were added to a nearly confluent layer of endothelial cells (data not shown).

Discussion

We studied the adherence of S. aureus to rat heart valves with catheter-induced NBTE. Two isogenic strains of S. aureus were used, differing only in their ability to bind to collagen.

The strains were tested for their binding capacity to immobilized collagen, fibrinogen, and fibronectin. No difference in binding to fibronectin or fibrinogen was found, whereas binding to collagen type II and type III differed significantly between the 2 strains. The theoretical possibility of a polar effect affecting downstream genes, resulting from the introduction of the GmR gene, seems unlikely since genes encoding matrix-binding proteins are usually monocistronic. The genes for fibronectin-binding proteins [26] and clumping factor [32] of S. aureus, protein G from group G streptococci [42], and the fibronectin-binding protein from Streptococcus dysgalactiae [43] have been shown to carry transcription terminators. We therefore assume that the difference in virulence between the 2 strains used here is the result of the difference in collagen binding. The cna gene was also reintroduced into the collagen-binding protein–deficient mutant PH100 on an autonomously replicat-
ing plasmid using a polymerase chain reaction–amplified DNA fragment encompassing the *cna* gene. The resulting strain, PHC, regained a capability to adhere to immobilized collagen that was intermediate to that of Phillips and PH100; however, when PHC was used in a mixed-inoculation experiment (PH100 and PHC), the plasmid was lost at a high rate, possibly because no antibiotic selection pressure could be applied in vivo, and therefore results were inconclusive (unpublished data).

The amount of bacteria recovered from aortic (with vegetations) and pulmonary heart valves was determined after hematoxylin stains with either strain. The experiments done 1 h after these infections showed primary adhesion of the inocula; the experiments done 24 h after infection showed the virulence and the ability of the inocula to maintain infection.

Phillips adhered better than PH100 to the undamaged pulmonary heart valve (*P* < .05). This cannot be explained by the dissimilar ability to adhere to undamaged endothelial cells, since both strains bind equally well to endothelial cells in vitro. Instead, the explanation can be found in the in vivo situation in the pulmonary valve in the experimental situation. Scanning electron microscopy of the pulmonary valve of infected animals occasionally revealed areas with patchy loss of endothelial cells and exposure of the underlying subendothelial matrix. Considering that *S. aureus* is the prime causative agent in right-sided endocarditis (pulmonary and tricuspid valves) where prior cardiac dysfunctions are not necessarily required for adherence, collagen binding of *S. aureus* might be of clinical significance for this type of IE.

In agreement with the findings of others, we noted that the number of bacteria found on the aortic valve was higher than on the pulmonary valve as a result of the vegetations, which form a substratum for attachment [3, 4]. The adherence ratio (log number of bacteria recovered on the aortic heart valve after 1 h – the log number of the inoculum cfu) was −4.5, the vast majority of bacteria having been trapped in spleen and liver (data not shown). The adherence ratio for Phillips on the pulmonary valve was −5.5. In a similar 1-h adherence experiment with *Streptococcus mutans*, these adherence ratios were −4.5 (same as for *S. aureus*) for aortic valve and −7 for pulmonary valve (Schennings T and Flock JI, unpublished data). The higher incidence of *S. aureus* than *S. mutans* in right-sided endocarditis can be explained by the better adherence of *S. aureus* than *S. mutans* to valvular tissue without vegetations. The explanation is not necessarily found in a different prevalence of bacteremia with these bacteria.

Bacterial counts 24 h after infection clearly showed a difference between the 2 strains: Phillips was more abundant than PH100 (*P* < .001). Thus, we conclude that propagation, or survival, is enhanced as a result of adherence to collagen. This conclusion is also supported by the fact that collagen is present in valvular and aortic tissues and also has been found in sterile vegetations [2]. It should be noted that among 25 clinical IE *S. aureus* isolates, 10 were unable to bind to collagen and lacked the gene for collagen-binding protein (personal communication, U. Ryding, Department of Infectious Diseases, Lund University Hospital, Lund, Sweden). Bacterial adherence might, therefore, be multifactorial and require several adherence mechanisms, some of which might compensate for the lack of others.

A lower inoculum size (1.4 × 10^5 cfu) had to be used in the 24-h experiment than in the 1-h experiment to avoid overloading the system. To determine the correct inoculum size, as few as 10^5–10^6 cfu were first used in a 1-h adherence experiment. Most of the animals had <2 cfu/heart valve (detection limit); however, such small inoculum sizes resulted in endocarditis after 24 h. This suggests that either very few bacteria on the heart valve are sufficient to produce IE or that there is an infective source causing transient bacteremia that leads to colonization on the valves and vegetations.

Our findings were substantiated using a 1:1 mixture of the bacterial strains. Use of a mixed inoculate and calculation of the ratio between the strains recovered from the vegetations provides for a more sensitive virulence comparison than does the use of separate inocula. This is because the range of cfu recovered from heart valves tends to be high as a result of individual variations among the animals. The basis of such individual variations is differences in the inoculum size, nonspecific defense mechanisms, and clearance of bacteria from the bloodstream through the reticuloendothelial system. Furthermore, the success of the infection is dependent on surgical manipulations required to induce infection. In the rat endocarditis model used here, the implantation of a catheter via the carotid artery as a prerequisite for formation of a sterile vegetations on the aortic valve and serving as a substrate for bacterial adherence might vary from animal to animal. It is also worth noting that using a 1:1 mixture enabled us to recognize that Phillips tends to outnumber PH100 (*P* < .10) even as soon as 1 h after inoculation. This finding suggests that collagen binding might already exert a positive effect on the survival of Phillips at an early stage of infection. However, it is likely that such a positive effect is more pronounced at a later stage of infection, since we found a clear predominance of Phillips (*P* < .001) on valves 24 h after infection with mixed or separate inocula.

We conclude from these studies that the ability of *S. aureus* Phillips to adhere to collagen is of minor importance for initiation of endocarditis caused by this microorganism but clearly facilitates the maintenance of IE.

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**References**