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Clinical isolates of *Enterococcus faecalis* aggregate human platelets

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ABSTRACT

Many endocarditis pathogens activate human platelets and this has been proposed to contribute to virulence. Here we report for the first time that many clinical isolates of *Enterococcus faecalis*, a common pathogen in infective endocarditis, aggregate human platelets. 84 isolates from human blood and urine were screened for their ability to aggregate platelets from four different donors. Platelet aggregation occurred for between 11 and 65 % of isolates depending on the donor. In one donor, a significantly larger proportion of isolates from blood than from urine caused platelet aggregation. Median time to aggregation was 11 minutes and had a tendency to be shorter for blood isolates as compared to urine isolates. Immunoglobulin G (IgG) was shown to be essential in mediating activation and aggregation. Platelet aggregation could be abolished by an IgG-specific proteinase (IdoS), by an antibody blocking FcRγIIa on platelets, or by preabsorption of plasma with an *E. faecalis* isolate. Fibrinogen binding to bacteria or platelets does not contribute to platelet activation or aggregation under our experimental conditions. These results indicate that platelet activation and aggregation by *E. faecalis* is dependent on both host and bacterial factors and that it may be involved in the pathogenesis of invasive disease with this organism.

Keywords: PLATELET ACTIVATION; INFECTIVE ENDOCARDITIS; ENTEROCOCCUS FAECALIS.
1. Introduction

*Enterococcus faecalis* is part of the normal colon flora but can cause a variety of diseases in humans. Such infections typically occur in a hospital setting and affect persons with a suboptimal immune status. Treatment of *E. faecalis* infections is complicated both by the pronounced natural and acquired antibiotic resistance of the bacterium and probably also by the propensity of the bacterium to form biofilms on foreign materials. One of the most severe conditions caused by *E. faecalis* is infectious endocarditis (IE) and *E. faecalis* is a common cause of IE [1]. IE is characterized by the formation of vegetations on heart valves that contain bacteria, platelets and fibrin. Parts of a vegetation can embolize to peripheral tissues, including the brain, and cause septic infarctions. The infection can also damage the heart valves resulting in leakage and heart failure. The acute form of IE is a life-threatening condition most often caused by *Staphylococcus aureus* whereas less fulminant infections are typically caused by enterococci and viridans streptococci. Several surface-located adhesive components, including a recently defined pilus structure, have been shown to be important for *E. faecalis* virulence in animal models of IE [2, 3]. In experimental rabbit IE, *E. faecalis* bacteria are trapped within the vegetations formed and the bacteria are thereby protected from opsonizing antibodies [4]. Many endocarditis pathogens activate human platelets and this has been suggested to be involved in the pathogenesis of the disease. The molecular mechanisms leading to platelet activation have been described in detail for many pathogens (recently reviewed in [5]). Most mechanisms involve a plasma protein, such as fibrinogen, which forms a bridge between the platelet and the bacterium. This in itself does not mediate activation, and plasma immunoglobulin G (IgG) specific for
the bacterium is also required to mediate platelet activation through the FcγRIIa receptors on the platelet surface. This mechanism has been described for *S. aureus* ClfA [6] and FNBPA [7], *Streptococcus pyogenes* [8], and *Streptococcus agalactiae* [9]. Direct interactions between bacteria and platelets can also occur. A surface protein from *Streptococcus sanguis* or *Streptococcus gordonii* can bind directly to the platelet surface, but to mediate aggregation bacteria specific IgG is also required [10, 11]. A slower form of aggregation occurs for *S. aureus* through interactions with specific IgG and complement which induce platelet aggregation after a longer lag-time [6]. Aggregation of rabbit platelets in response to *E. faecalis* and *Enterococcus faecium* has been previously described, but the molecular mechanisms involved have not been determined [12]. However, interactions between *E. faecalis* and human platelets have not been reported. In this study we investigate the ability of *E. faecalis* isolates from two different groups of clinical samples to aggregate human platelets.
2. Materials and methods

2.1. Bacteria and culture conditions

*E. faecalis* isolates from human blood (n=42) and urine (n=42) were collected from the accredited diagnostic laboratory for Clinical Microbiology, Lund University Hospital, Sweden. The laboratory provides service to a large geographical area, which includes four hospitals and numerous primary care facilities in the county of Skåne in southern Sweden. Blood isolates were collected retrospectively for the period 2006-2008 and were from 42 distinct patients. Twenty three isolates were from 11 different wards of the Lund University hospital, 13 isolates were from 9 different wards of the Helsingborg hospital, 3 isolates were from 2 different wards of the Ångelholm hospital, and 3 isolates were from 3 different wards of the Landskrona hospital. The urine isolates were collected prospectively during a three-week period in 2008 from 42 distinct patients. Fifteen urine isolates were from the Lund University hospital, 6, 2, and 3 isolates were from the hospitals in Helsingborg, Ångelholm, and Landskrona respectively. Eighteen isolates were from primary care units. *E. faecalis* was identified based on the ability to convert tellurite to tellurium, a typical antibiotic susceptibility-pattern and growth of grey colonies on Cephalexin-Aztreonam-Arabinose agar [13]. The isolates were confirmed to be Gram positive cocci, catalase negative, vancomycin sensitive, able to utilise pyruvate, cleave L-pyrrolidonyl-beta-naphthylamide (PYR), and produce leucine aminopeptidase (LAP) [14]. *Streptococcus pyogenes* strain AP1 was grown as described previously [15]. *E. faecalis* bacteria were cultivated in tryptic soy broth (Difco) with the addition of 0.5% glucose (TSBG) at 37 °C with 5 % CO₂ for 16-18 hours (stationary growth phase) or to OD₆₂₀ of 0.5 (exponential growth phase). Bacteria were quantified by serial dilutions onto TSBG agar.
2.2. Platelet preparation

Blood samples were collected from four healthy donors who had not taken antiplatelet medication in the previous ten days. Five ml of blood was collected into citrated vacuum tubes. Centrifugation at 150 g for 10 minutes produced an upper platelet rich plasma (PRP), which was removed. In experiments presented in figure 1, platelets were manually counted using light microscopy. Subsequent centrifugation at 1500 g for 10 minutes produced an upper platelet poor plasma (PPP). In the experiment presented in figure 3B, 400 µl of PRP was centrifuged for 5 minutes at 1000 g and the soft platelet pellet was resuspended in the same volume of PPP. The PPP had been preabsorbed by incubation for 15 minutes with 5x10^8 bacteria followed by centrifugation at 10000 g for one minute.

2.3. Aggregometry

A dual channel platelet aggregometer (ChronoLog model 490) was used to assess platelet aggregation by turbidometry. PPP was used in the reference cell to establish a baseline of 100% transmission. PRP was used in the test cell and the change in transmission over time was an indication of the platelet aggregation. Bacteria from 10 ml of an overnight culture were harvested by centrifugation at 2500g for 10 minutes, washed two times in phosphate buffered saline (PBS), and resuspended in 500 µl of the same buffer. Twenty µl of bacterial suspension (corresponding to approximately 1x10^8 bacteria) was added to 400 µl of PRP in the test well and the platelet response was monitored for a maximum of 25 minutes. As a positive control, platelet aggregation was determined in response to soluble collagen I
(Triolab, Sweden). Results were analyzed using the Aggrolink, version 5.2.1 software.

2.4. Determination of platelet activation by E. faecalis

Twenty µl of PRP was incubated for 25 minutes at room temperature with 25 µl of HEPES buffer pH 7.4, either in the presence or absence of washed bacteria (approximately 2.5x10⁶ bacteria). After 25 minutes, 5 µl fluorochrome conjugated antibody (CD42PerCP, CD62PE, and PAC-1FITC) (all from BD Biosciences) was added and after 10 minutes the incubation was stopped by addition of 500 µl of 0.5 % formaldehyde in ice cold PBS. Samples were analyzed using a FACSCalibur flow cytometer in logarithmic mode with a gate setting for the CD42 positive platelet population. 50,000 cells were acquired and analyzed using Cell Quest software (Becton Dickinson).

To assess the role of plasma factors in platelet activation, PRP was treated with specific blockers, 20 µg/ml ReoPro (Eli Lily) or 50 µg/ml AT10 (Serotec), for 30 minutes at room temperature.

2.5. Electron microscopy

For scanning electron microscopy (SEM), 400 µl of PRP was incubated with bacteria (approximately 1x10⁸) or collagen (2.5 µg/ml). At various time points 100 µl of reaction mixture was added to 900 µl of fixation solution (4 % formaldehyde, 2.5 % glutaraldehyde in PBS) and incubated for one hour. The samples were centrifuged for 10 minutes at 1000g and the pellet was resuspended in 50 µl of fixation solution and absorbed onto poly-L-lysine-coated cover slips and incubated for one hour. The sample was further fixed overnight. Fixed specimens were dehydrated for 10 min at each step of an ascending
ethanol series and critical point dried in a Balzers critical point dryer in liquid carbon dioxide using absolute ethanol as an intermediate solvent. Samples were examined in a Jeol J-330 scanning electron microscope at an acceleration voltage of 5 kV and a working distance of 10 mm.

2.6. Other methods

For plasma absorption experiments $1 \times 10^9$ bacteria in 200 µl of PBS was incubated with 20 µl of heparinized human plasma for 45 minutes at 37 ºC followed by centrifugation at 6000 g for 5 minutes. The pellet was resuspended in 1 ml of PBS and subjected to centrifugation. This washing step was repeated three times. Elution of bound material was achieved by incubation with 50 µl 0.1 M glycine pH 2.0 for 5 minutes. Eluted material was subjected to SDS-PAGE and staining with commassie brilliant blue according to standard protocols. Human fibrinogen (Sigma) was radiolabelled and tested for binding to bacteria as previously described [15]. The cysteine proteinase IdeS was purified as a fusion to GST as previously described [16]. 400 µl of PRP was treated with 80 µg of GST-IdeS for 15 minutes at 37 ºC, conditions known to cleave the entire IgG pool of human plasma [17].

2.7. Statistics

Statistical analysis was performed using GraphPad Prism 4 for Macintosh, version 4.0C. Differences in distribution of isolates were tested using Fisher’s exact test, differences in time to aggregation was evaluated with Wilcoxon’s rank number test, and differences in levels of CD62P and PAC-1 presentation was tested using Students T-test.
3. Results

3.1 Aggregation of human platelets by *E. faecalis*

In a pilot experiment, aggregation of platelets from one donor (donor 1) was tested using eight different isolates of *E. faecalis* from human blood (denoted Bef1-Bef8). Six of these isolates induced platelet aggregation in the aggregometric assay after a lag-time of 10-14 minutes. One positive isolate (Bef5) and one negative isolate (Bef6) were chosen for further analyses. The importance of the bacteria-platelet ratio for aggregation was determined by adding different amounts of bacteria of isolate Bef5 and Bef6 to a fixed number of platelets in PRP. Bacterial number was determined by plating and platelets were manually counted. The result of a representative experiment is shown in Fig. 1, illustrating that as few as 0.3 Bef5 bacteria per platelet could induce aggregation. Bef6 failed to induce aggregation at any of the ratios tested in incubations for up to 25 minutes (data not shown). Bef5 bacteria from both exponential and stationary growth phase induced aggregation (data not shown) and for practical reasons all subsequent experiments were carried out using bacteria from the stationary growth phase.

3.2. Platelet aggregation by *E. faecalis* isolates from different clinical sources

Forty-two isolates from human blood cultures and 42 isolates from human urine were tested for their ability to induce platelet aggregation in PRP from four different donors. Twenty μl of bacterial solution (approximately 1x10^8 bacteria) was added to 400 μl of PRP and the reaction was followed for 25 minutes. There was significant variation in the incidence of aggregation among donors (Fig. 2A, lower panel). In donor 1, aggregation was induced by 48 % (40/84) of all isolates, in donor 2 by 11 % (9/84) of all isolates, in donor 3 by 21 % (18/84) of all isolates, and in donor
4 by 65 % (55/84) of all isolates. In donor 2, a significantly higher proportion of isolates from blood than from urine induced aggregation (p<0.01) whereas no significant difference was detected in the other donors. Median time to aggregation among all aggregating isolates was 11 minutes and in donors 1, 3 and 4, isolates from blood that induced aggregation had a shorter lag time than isolates from urine though this difference was not statistically significant. Platelets from donor 2 aggregated rapidly in response to seven isolates (Fig 2A). These seven isolates were from three of the four different hospitals and the bacteria were isolated at different time points. The isolates had a MIC for gentamycin ranging from 2 µg/ml to 16 µg/ml and the response to these isolates was not conserved among the other donors. This suggests that these isolates are not clonally related.

Figure 2B illustrates the number of donors that respond with aggregation to the different isolates. The ability to induce aggregation was widely distributed among isolates and was not associated with particular isolates. Only 3/84 isolates were capable of inducing aggregation in all donors, and only 20/84 isolates failed to induce aggregation in any donor. Thus the majority of isolates tested induced aggregation of platelets from one to three donors.

3.3. Donor and IgG dependency of platelet aggregation by E. faecalis

Since specific IgG has been demonstrated to be essential for platelet aggregation by other bacterial species we tested the role of IgG for E. faecalis. PRP from all four donors was treated with the highly IgG specific proteinase, IdeS [16]. In samples pretreated with buffer both collagen and Bef5 bacteria induced platelet aggregation. As expected, collagen was able to induce platelet aggregation of IdeS-
treated PRP, whereas the *E. faecalis* isolate failed to induce aggregation of plasma pretreated with IdeS. Identical results were obtained in all four donors and in figure 3A the results from donor 1 are shown.

To further assess the role of IgG, platelets from donor 1 were pelleted and resuspended in plasma that had been preabsorbed with bacteria of either the aggregating isolate Bef5 or the non-aggregating isolate Bef6. Isolate Bef5 induced aggregation of platelets resuspended in normal plasma or plasma pretreated with the non-aggregating isolate Bef6, but aggregation was not achieved when platelets were resuspended in plasma preabsorbed with Bef5. Data from a representative experiment (n=3) are shown in Fig. 3B.

Stimulation of platelets by various agonists, including ADP, results in phenotypic changes that reflect activation. We monitored these changes in PRP using three-color flow cytometry following stimulation with *E. faecalis* bacteria or ADP as a positive control. Platelets were identified using a CD42 specific antibody, the activation status of the fibrinogen receptor (GPIIb/IIIa) was determined using an antibody specific for the active conformation (PAC-1), and release of the alpha granules, indicating that platelet activation has occurred, was determined using a CD62 specific antibody. In the absence of activation, 4 ± 3 % (SD) presented CD62P and 5 ± 2 % of platelets presented PAC-1 on their surface (data not shown). As expected, ADP-activated platelets presented CD62P and PAC-1 on their surface (Fig. 4A and 4B, black bars). *E. faecalis* Bef5 also increased CD62P and PAC-1 presentation on platelets (Fig. 4A and 4B), indicating that platelet activation occurred. To determine the role of plasma IgG for platelet activation by bacteria, we carried out assays in the presence of AT10 which blocks IgG-binding to platelets [18]. Activation of platelets by Bef5 was significantly diminished when the IgG receptor was blocked
(Fig. 4A and 4B, grey bars, p < 0.001), indicating that IgG binding to the platelets is involved in platelet activation by the bacteria. Platelet activation in response to ADP does not involve IgG binding to the Fc receptor on platelets and was therefore not affected when the IgG receptor was blocked (Fig. 4A and 4B, grey bars). To determine the role of plasma fibrinogen for platelet activation by the bacteria, we carried out assays in the presence of ReoPro which blocks fibrinogen-binding [19]. We confirmed that ReoPro blocked the fibrinogen receptor (GPIIb/IIIa) on platelets since PAC-1 presentation was abolished by activated platelets in the presence of this blocker (Fig. 4B, white bars, p < 0.001). Importantly, ReoPro did not significantly influence platelet activation and CD62P presentation in response to Bef5 (Fig. 4A, white bar), indicating that fibrinogen binding to the platelets is not involved in platelet activation by the bacteria. Platelet activation in response to ADP does not involve fibrinogen binding to platelets and CD62P presentation was therefore not affected by ReoPro (Fig. 4A). The results shown in Fig 4 are for Donor 1, and the same profile of results was seen for two other donors tested. Platelet activation did not occur in the presence of Bef6 in any of the three donors (data not shown), and this is in concordance with the inability of this isolate to mediate platelet aggregation in the same donors.

3.4. Lack of plasma protein binding by E. faecalis

Sixteen isolates of E. faecalis, Bef1-Bef16 from human blood, were tested for binding of plasma proteins and radiolabelled fibrinogen. The positive control, the AP1 strain of S. pyogenes, absorbed several proteins from human plasma. None of the E. faecalis isolates had any protein bound to the surface after plasma exposure as judged by SDS-PAGE analysis of the eluted material. In addition, S. pyogenes bound over 90
% of added radiolabelled fibrinogen whereas the *E. faecalis* isolates bound less than 5%
(data not shown).

3.5. Visualization of the bacteria-platelet aggregates by SEM

PRP from donors 1 and 2 was incubated with the aggregating isolate Bef5, the
non-aggregating isolates Bef6, or collagen. At different time points, aliquots were
withdrawn into a fixative solution and prepared for SEM. Resting platelets were seen
as single discoid cells (Fig. 5A and 5B). Platelet activation was induced by collagen
and the morphological signs of activation occurred in both donors (Fig. 5C and 5D).
Activated platelets formed pseudopodia, adhered to one another, and aggregated.
Platelet activation also occurred in response to Bef5 in both donors. The platelets
from donor 2 aggregate more rapidly and formed larger aggregates than platelets from
donor 1 (Fig 5E-5H). The Bef6 isolate, which did not aggregate or activate the
platelets from either donor in aggregometry and flow cytometry, was used as a
negative control. As expected, platelets in the presence of Bef6 showed no signs of
aggregation (Fig. 5I and 5J) despite the fact that the bacteria were seen in close
proximity to the platelets on many occasions.
4. Discussion

Despite the importance of *E. faecalis* as a pathogen in IE and the potential role of platelet activation in the pathogenesis of this disease, the interaction between human platelets and *E. faecalis* has previously not been investigated. Our study demonstrates that many isolates of *E. faecalis* can induce platelet aggregation and that host factors contribute to this. There was a considerable variation in the incidence of aggregation and the lag-time to aggregation between donors. Platelet activation was proven to be dependent on plasma IgG interacting with platelet Fc receptors. Since *E. faecalis* is part of the normal flora, IgG specific for the bacterium should be present in most donors and this was the case for the four donors used in our study (data not shown). It therefore appears as if the specificity rather than the quantity of the IgG antibodies determines if a specific donor will respond with platelet aggregation to *E. faecalis*. Clearly, we cannot exclude that other components than IgG are important for platelet activation. A role for bridging plasma proteins, such as fibrinogen, described for other bacterial species, seems unlikely in our system since the bacteria did not bind fibrinogen or large quantities of other proteins in plasma. In addition, blockade of the fibrinogen receptor on platelets did not affect *E. faecalis* induced activation. A role for fibrinogen under other conditions than those used in this study can however not be ruled out since it has recently been demonstrated that *E. faecalis* grown in serum adhere to immobilized fibrinogen [20, 21].

Bacterial factors also contribute to the ability to aggregate platelets and some isolates induced aggregation of platelets from all donors indicating that these isolates are more efficient platelet activators than others. Moreover, isolates from blood had a tendency to cause aggregation more often and more rapidly than isolates from urine. This suggests that the bacterial factors that contribute to platelet activation may be
involved in virulence. In IE caused by *E. faecalis*, the ability to aggregate human platelets may contribute to the formation of vegetations on heart valves. Further studies are needed to establish a potential correlation between the source of isolation and platelet aggregation for IE pathogens.

The ability of a bacterium to activate human platelets has previously often been seen as a virulence trait. For example, several surface proteins of Gram-positive bacteria, mediating platelet aggregation, have been demonstrated to be important for virulence in animal models of IE [22-25]. However, platelet activation by Gram-positive pathogens most often requires specific host IgG, perhaps indicating that platelet activation should be regarded as a host immune response. Activated platelets can contribute to the immune response by capturing invading bacteria in a thrombus and release of pro-inflammatory factors including cytokines and also antibacterial peptides upon activation [26]. The environment in a platelet thrombus is therefore rich in antibacterial substances. The potential importance of this is illustrated by the finding that thrombocytopenic animals had significantly more bacteria in their vegetations in a rabbit model of streptococcal IE [27]. In addition, *S. aureus* isolates from IE episodes are more likely to be resistant to the action of thrombin induced platelet-microbicidal proteins (tPMPs), than isolates from sepsis associated with soft tissue *S. aureus* infections [28]. tPMP sensitive *S. aureus* strains are also less virulent in a rabbit endocarditis model [29]. Resistance to tPMPs correlates with the source of isolation for *S. aureus* but not for *E. faecalis*, which is generally not very susceptible to tPMPs [30]. Importantly, no study has compared the frequency of the ability to aggregate platelets for bacterial isolates from different infections and from the normal flora.
Clearly, an important interaction occurs between bacteria and platelets during infection and this area requires further elucidation. Platelet activation may represent a double-edged sword, on one hand contributing to the innate defence against bacteria, while at the same time contributing to the pathogenesis of specific infections. It is particularly important to clarify these interactions since they may represent potential targets for drug or vaccine development. This is especially relevant for *E. faecalis*, which is becoming increasingly resistant to antibiotics. A better understanding of the pathogenesis of *E. faecalis* infections will also facilitate the development of alternative therapeutic approaches.
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6.5. References


6.6 Legends to figures

FIG 1. *E. faecalis* mediates platelet aggregation. Platelet aggregation in response to isolate Bef5 of *E. faecalis* was determined using a Chrono Log aggregometer. Bef5 bacteria (6.7x10^6-4.3x10^8) were added to 400 µl of PRP from donor 1 (1.2x10^8 platelets/ml) and samples were analyzed by aggregometry. The scale of the Y-axis represents the relative light absorption of the sample where 100 represents the PRP after addition of bacteria and 0 the absorption of PPP. When platelets aggregate the opacity and light absorbance of the sample decreases. The number of bacteria added per platelet is given at the right side of the figure.

FIG 2. Platelet aggregation by *E. faecalis* varies between donors and between isolates. Platelet aggregation was monitored for *E. faecalis* isolated from human blood (B) or urine (U) in PRP from four different donors (donors 1-4). A. The number of isolates that induced aggregation in each donor is shown in the lower panel of A. In donor 2, a significantly higher proportion of isolates from blood caused aggregation as compared to isolates from urine (p<0.01). In the upper panel the time to aggregation for positive isolates is shown on the Y-axis for blood isolates (filled circles) and urine isolates (open circles).

B. The number of isolates from blood (black bars) or urine (white bars) that induce aggregation in 0, 1, 2, 3, or 4 donors are shown.

FIG 3. Platelet aggregation by *E. faecalis* is dependent on plasma IgG. (A) PRP from donor 1 was pretreated with the IgG-specific proteinase IdeS or with buffer alone and aggregation was monitored after the addition of 1x10^8 *E. faecalis* bacteria
(ef) or collagen (coll). (B) PRP from donor 1 was centrifuged and platelets were resuspended in normal plasma (washed platelets (WP)+plasma), in plasma preabsorbed (pa) with the aggregating isolate Bef5 (WP+plasma pa Bef5), or in plasma preabsorbed with the non-aggregating isolate Bef6 (WP+plasma pa Bef6). 1x10^8 bacteria of isolate Bef5 was added and platelet aggregation was monitored.

**FIG 4.** *E. faecalis* mediates platelet aggregation which is dependent on plasma IgG and independent of fibrinogen. ADP or Bef5 was added to PRP from donor 1 and after 25 minutes platelet activation was determined using flow cytometry. The percentage of the platelet population positive for CD62P (A) and PAC-1 (B) was determined in the presence of buffer (black bars), an antibody to block platelet Ig-binding (AT10, grey bars), or an antibody to block platelet fibrinogen-binding (ReoPro, white bars). Data represents mean ± standard error of the mean, n=5. P values were determined using the Students t test, p < 0.001 ***.

**FIG 5.** *E. faecalis* forms large platelet-bacteria thrombi in plasma. Platelets from donor 1 (left column of the figure) or donor 2 (right column of the figure) were subjected to SEM after fixation without prior stimulation (A and B), after 2 minutes incubation with collagen (C and D), after 15 seconds incubation with the aggregating isolate Bef5 (E and F), after 15 minutes incubation with isolate Bef5 (G and H), or after 15 minutes incubation with the non-aggregating isolate Bef6 (I and J). The white bar represents 10 µm.
Figure 3

B. Relative opacity

A. Relative opacity