An appraisal of the virulence factors associated with streptococcal endocarditis

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Summary. Platelet aggregation is believed to be a virulence factor in infective endocarditis. Other factors may be adhesion to components of thrombotic vegetations, particularly platelets, fibronectin and fibrinogen. Two strains from the *Streptococcus sanguis* group (SSG) were chosen for comparative study on the basis that one aggregated both human and rat platelets and the other lacked this capacity. Both strains caused endocarditis in the rat model but the aggregating strain was found in higher numbers in the excised vegetations. The non-aggregating strain was unable to bind to human or rat platelets but could bind insoluble fibronectin, insoluble fibrinogen and platelet-fibrin clots from both sources, albeit to a lesser extent than the aggregating strain. These results suggest that whereas adhesion to, and aggregation of, platelets are not essential events in the initiation of the pathogenesis of experimental endocarditis, they may be factors contributing to virulence.

Introduction

Viridans streptococci form part of the normal oral flora and their entry to the blood stream as a result of dental procedures, for example, may lead to infective endocarditis; this involves the colonisation of platelet-fibrin vegetations on the endothelial surface of the heart. One possible factor in the pathogenesis of infective endocarditis is direct aggregation of platelets by bacteria, but it is also possible that bacteria may colonise the damaged valvular surface by their adherence to platelets and extracellular matrix proteins such as fibronectin and fibrinogen. Such conclusions have been based generally on assays in vitro with human components and on to a limited number of assays in vivo in animals. There has been no comprehensive analysis hitherto of the relationship between binding and platelet-aggregating properties in vitro for the human and animal system and the capacity to cause endocarditis in the animal model.

Members of the *Streptococcus sanguis* group (SSG) are the oral streptococci most frequently isolated from infective endocarditis. Previous work (Manning et al., unpublished observations) showed that SSG strain FSS2 aggregates both human and rat platelets to the same final percentage but with different lag phases. In the same study, 25 SSG strains were compared for their ability to aggregate platelets. A lag phase of > 3 min was found for 92% of strains with human platelets whereas all strains had a lag phase of < 3 min with rat platelets. Strain FSS2, isolated from a patient with endocarditis, is, therefore, a typical SSG strain in its ability to aggregate both human and rat platelets. Strain L50, a dental plaque strain that does not aggregate human or rabbit platelets, has now been shown to be unable to aggregate rat platelets. Thus, the aim of this study was to compare the two SSG strains for other possible virulence factors in tests with both human and rat products, and to relate these properties to the capacity of the strains to cause endocarditis in the rat.

Materials and methods

Bacterial strains and culture conditions

The two strains studied were FSS2, isolated from a patient with endocarditis at Freeman Hospital, Newcastle upon Tyne, and L50, a dental plaque strain kindly provided by Dr M. Herzberg, University of Minnesota, Minneapolis, USA. Both strains were stored in glycerol 30% v/v at −70°C. Purity was checked by plating out on Columbia Agar (Oxoid) supplemented with horse blood (Amadeus) 5% v/v. Organisms were grown in Brain-Heart Infusion Broth (Oxoid) supplemented with horse blood (Amadeus) 5% v/v at 37°C for 18 h in an anaerobic jar. Where radiolabelling was necessary, 3H-thymidine (Dupont) was added to the medium at 74 kBq/ml. For all assays, the cultures were washed three times in phosphate-buffered saline (PBS), pH 7.3, (g/L—NaCl 8·0; KCl 0·2; Na₂HPO₄·12H₂O 2·9; KH₂PO₄ 0·2) and routinely resuspended to an OD₆₆₀ of 1·00 ± 0·02.
Interaction with platelets

Preparation of standardised platelet suspensions. Human blood, containing 0.1 M tri-sodium citrate (9:1 v:v) as an anticoagulant, was supplied by the Red Cross Blood Bank, Sydney, Australia, from normal donors who were known to be free of infection and had taken no medication for at least 2 weeks prior to donation. A supernate of platelet-rich plasma (PRP) was prepared by centrifugation of the blood from at least two donors at 100 g for 10 min at 20°C, and platelet-poor plasma (PPP) was prepared by further centrifugation of the remainder of the blood at 2350 g for 10 min. PRP for the aggregation experiments was adjusted to an OD$_{405}$ of 0.5 with PPP. Rat blood was collected, under anaesthesia, from the heart into a syringe containing 0.1 M tri-sodium citrate (9:1 v:v) and PRP and PPP were prepared by the same method as for human blood.

Platelet aggregation assay. PRP (250 µl) was equilibrated to 37°C in a recording aggregometer (model Chronolog Corp., Haverton, PA, USA) and adenosine diphosphate (ADP) was added to a final concentration of 5–20 µM, as a positive control for the method as for human blood.

Table I. Results of in-vitro adhesion assays with human components

<table>
<thead>
<tr>
<th>Adhesion assay with</th>
<th>Strain FSS2</th>
<th>Strain L50</th>
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<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
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<tr>
<td></td>
<td>1 x 10^8</td>
<td>1 x 10^8</td>
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<tr>
<td>Platelets</td>
<td></td>
<td></td>
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<tr>
<td>1 x 10^8 cells/ml</td>
<td>44.9 (5.7)</td>
<td>192.4 (10.0)</td>
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<tr>
<td>Platelet-fibrin clots</td>
<td>33 (0.4)</td>
<td>221 (6.2)</td>
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<tr>
<td>Insoluble fibronectin</td>
<td>76 (0.7)</td>
<td>504 (40)</td>
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<tr>
<td>Insoluble fibrinogen</td>
<td>79 (1.0)</td>
<td>414 (7.1)</td>
</tr>
<tr>
<td>Rat fibrinogen</td>
<td>50 (0.5)</td>
<td>1924 (10.0)</td>
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</table>

Platelet adhesion assay. All microtitration plates and centrifuge tubes were pre-coated with PBS containing bovine serum albumin 1% and Tween 20 0.05% (PBS-BSA). An iso-osmotic solution of Percoll (Pharmacia) 90% v:v in PBS was diluted with PBS to determine the concentration required for each strain to be deposited on the bottom of the tube after centrifugation at 5000 g for 20 min. The respective concentrations for strains FSS2 and L50 were 50% and 25%. PRP was washed three times in PBS containing 5 mM EDTA to produce platelet ghosts and resuspended to an OD$_{440}$ of 0.5. Platelet ghosts (100 µl) and radio-labelled bacterial suspension (100 µl; 1 x 10^8 cells/ml and 1 x 10^8 cells/ml, respectively) were added to wells of a microtitre plate (Nunc, Denmark) and shaken for 20 min at room temperature. The suspension from each well and a PBS wash (100 µl) were carefully layered on to the pre-determined concentration of Percoll and centrifuged at 5000 g for 20 min. Platelets with adherent bacteria remained at the top of the centrifuge tube whereas unbound bacteria were centrifuged to the bottom. Controls were bacteria incubated with PBS only. Samples (200 µl) were taken from the top and the bottom of the Percoll and counted in a scintillation counter to determine the numbers of bound bacteria. Assays were performed in triplicate with two separate batches of cells.

Adhesion to platelet-fibrin clots. PRP (250 µl) and 0.3 M CaCl$_2$ (100 µl) were added to wells of a tissue-culture plate (24-well plate; well size 16 mm x 16 mm; Nunc) and incubated at 37°C for 30 min to form a clot. A radiolabelled bacterial inoculum (1 x 10^7 cells/ml and 1 x 10^8 cells/ml) was added to each well (300 µl) and shaken at room temperature for 1 h. Control clots were shaken with PBS only. The supernate was removed and the clot surface was washed several times in PBS to remove non-adherent bacteria, dissolved with streptokinase (0.3 U/ml; Boehringer Mannheim, Germany) and transferred to scintillation vials. Bacterial suspension (300 µl) was added to the dissolved control clots to determine the amount of radioactivity associated with each cell; the number of bound bacteria in the samples could then be calculated. Assays were performed in duplicate with two different batches of cells.

Binding to insoluble fibronectin and fibrinogen

Rat fibronectin was prepared by the method of Vuento and Vaheri. Rat fibrinogen was then prepared from the fibronectin-deficient plasma by precipitation with ammonium sulphate 25% followed by chromatography on a Sepharose CL-6B column (2.5 x 70 cm) with PBS as the equilibration buffer. The proteins were judged to be pure by polyacrylamide gel electrophoresis. Human fibronectin and fibrinogen were purchased from Sigma. Each protein (200 µl; 5 µg/ml in PBS) was pipetted into wells of a micro-
titration plate (Nunc) and left overnight at 4°C. Control wells contained PBS only. Unbound protein was removed by washing in PBS-BSA before the addition of a radiolabelled bacterial suspension (100 μl; 1 x 10⁸ cells/ml and 1 x 10⁹ cells/ml). Microtitration plates were shaken for 30 min at room temperature and washed several times in PBS. Adherent bacteria were solubilised by the addition of sodium dodecyl sulphate (SDS) 2.5% in 0.2 M NaOH (100 μl) to the well for 30 min followed by neutralisation with 0.05 M acetic acid (400 μl). The contents of each well were then counted on a scintillation counter to determine the number of bound organisms. Assays were performed in triplicate on two different batches of cells.

In-vivo endocarditis model

Bacterial endocarditis was established in female Sprague-Dawley rats weighing c. 200 g by the technique of Santoro and Levison.11 A polyethylene catheter (0.5 mm internal diameter; Dural Plastics, NSW, Australia) was inserted across the aortic valve via the right carotid artery, sealed and left in place throughout the experiment. The infecting strain (5 x 10⁷ cfu in 0.5 ml PBS) was injected through the tail vein 48 h after catheterisation. Control rats were sham-operated with placement and withdrawal of the catheter and given strain FSS2 or catheterised and given PBS. At post-mortem examination the abdominal organs were inspected for evidence of metastatic disease and the hearts for correct placement of the catheter. Following removal, catheters were placed in brain heart infusion broth (10 ml) for qualitative culture. All aortic vegetations were excised aseptically, weighed and homogenised in 5 ml of sterile PBS 96 h after bacterial injection. Colony counts were performed on blood agar plates and incubated at 37°C for 18 h. Bacterial titres were recorded as total cfu, and cfu/mg of vegetation. All strains from resected vegetations were identified by colonial morphology, haemolysis on blood agar plates, Gram’s stain and capacity to aggregate platelets. Differences in bacterial titres within vegetations among groups were assessed by the non-parametric Mann–Whitney U test.

Results

In-vitro assays

Strain FSS2 aggregated both human and rat platelets to a final value of 85–90%; the lag phase was 8 min for human platelets and < 0.5 min for rat platelets. Strain L50 was unable to aggregate either human or rat platelets.

Strains FSS2 and L50 at concentrations of 1 x 10⁸ cells/ml and 1 x 10⁹ cells/ml were compared for their capacity to bind platelets, insoluble fibronectin, insoluble fibrinogen and platelet-fibrin clots. Table I gives the results for human components and table II for rat components.

The number of adherent bacteria at the 10-fold higher cell concentration was only increased by 10-fold in one case, the binding of insoluble human fibronectin by strain L50; binding may have been approaching saturation level at the higher cell concentration. The values at the higher concentration highlight the differences between the strains.

With human components strain FSS2 was more active than strain L50 in all assays. The most obvious difference, up to 200-fold, was in platelet adhesion but there were two-fold and three-fold differences, respectively, in binding to insoluble fibrinogen and platelet-fibrin clots; the difference for insoluble fibronectin was marginal. The same general trend was seen as with rat components; the difference for platelet adhesion was 12-fold and the respective differences for binding to insoluble fibronectin, insoluble fibrinogen and platelet-fibrin clots were three-fold, seven-fold and two-fold respectively.

Strain FSS2 adhered better to human platelets than to rat platelets by a factor of 16 but the results were similar in all other assays. Strain L50 was unable to adhere to either human or rat platelets but bound three times more effectively to insoluble human fibronectin and fibrinogen then to rat components. Adhesion to platelet-fibrin clots was the same for both strains.

Animal model of endocarditis

Twenty-six rats were used in the production of experimental endocarditis; eight were given strain FSS2 (two rats from this group died before harvest), seven strain L50, three PBS and eight were sham-operated.

The catheters from the PBS controls showed no visible growth of organisms on culture, whereas there was growth on the catheters of infected animals. All vegetations with organisms yielded pure cultures and the organisms were the same as those used to infect the animal. No bacteria were detectable in any of the animals given PBS or in five of eight sham-operated animals. The other three sham-operated animals had 750, 1 x 10³ and 3 x 10⁴ total cfu respectively.

The distribution of total log₁₀ cfu recovered from the vegetations of each rat inoculated with strains FSS2 and L50 is shown in the figure. The values for strain FSS2, with a range of 5.26–11.23 and a median of 9.26, were significantly higher (p = 0.035) than for strain L50, for which the range was 0–8.0 and with a median of 5.32. The distribution of log₁₀ cfu/mg of tissue for both strains showed a similar difference. In four of six animals inoculated with strain FSS2 and two of seven animals inoculated with strain L50, the total recovered cfu exceeded the number injected.

The pathological condition at necropsy of each animal related to the treatment of the animal. Sham-operated, non-cannulated rats were generally healthy and all organs were normal. All cannulated rats showed some degree of congestion in the lungs and
Table II. Results of in-vitro adhesion assays using rat components

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<tr>
<th>Adhesion assay with</th>
<th>Mean (SD)</th>
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<tr>
<td></td>
<td>Strain FSS2</td>
<td>Strain L50</td>
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<tr>
<td></td>
<td>1 x 10^9 cells/ml</td>
<td>1 x 10^9 cells/ml</td>
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<tr>
<td>Platelets</td>
<td>1.7 (2.2)</td>
<td>12.1 (9.0)</td>
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<tr>
<td>Insoluble fibronectin</td>
<td>6.5 (0.7)</td>
<td>44.9 (5.2)</td>
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<tr>
<td>Insoluble fibrinogen</td>
<td>10.6 (1.2)</td>
<td>54.2 (7.1)</td>
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</tr>
<tr>
<td>Platelet-fibrin clots</td>
<td>5.7 (0.9)</td>
<td>13.2 (1.6)</td>
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Figure. Distribution of the total log_{10}cfu of bacteria cultured from each vegetation of catheterised rats infected with strains FSS2 and L50. The bars represent the median value for each strain.

Discussion

The ability to aggregate platelets has been associated with the capacity to cause endocarditis. Strain FSS2, an endocarditis isolate, aggregates human and rat platelets (Manning et al., unpublished observations). The present studies in conjunction with previous work show that strain L50 is unable to aggregate platelets from either source within the defined period.

The adhesion of bacteria to platelets in endocardial vegetations may lead to further deposition of platelets and the development of platelet-fibrin matrices, followed by enlargement of the vegetations. The adhesion of bacteria to platelets has been suggested as a prerequisite for platelet aggregation. Strain FSS2 adheres to both human and rat platelets but in much higher numbers to human platelets. Results obtained for Staphylococcus aureus showed that the ability of staphylococcal cells to adhere to platelets depended upon the availability of platelet-receptor sites for the organism rather than on the affinity of the organism for the platelet surface. If SSG strains behave similarly, these results would suggest that the lesser binding of strain FSS2 to rat platelets may be due to fewer platelet receptors, although the number must still be sufficient to trigger aggregation. Strain L50 adheres very poorly to human or rat platelets and, apparently, in insufficient numbers to cause aggregation.

Both strains bind to insoluble human and rat fibronectin; strain FSS2 is marginally superior in regard to human fibronectin and three times better for rat fibronectin. There is now an awareness of the role of fibronectin in the adherence of circulating organisms to damaged heart valves. Fibronectin is present in increased amounts at the sites of endothelial injury and repair, on the surface of thrombotic lesions, on the surface of platelets and within platelet α-granules. Fibronectin exists in soluble form in plasma and can change conformation when bound in an insoluble form to other molecules and substrates such as gelatin, collagen and plastic. S. sanguis has also been shown to bind to insoluble fibronectin even in the presence of large amounts of soluble fibronectin suggesting that adherence to immobilised fibronectin may be more important in streptococcal endocarditis. Studies in vitro have shown that organisms bind better to artificial thrombi formed in the presence of fibronectin than thrombi formed from fibronectin-deficient plasma and studies with animal models have shown that the adhesion of S. sanguis to insoluble fibronectin in vitro correlated with the ability to produce endocarditis in rats. This evidence indicates that binding to insoluble fibronectin plays a role in endocarditis.

Little information is available on the role of fibrinogen in infective endocarditis. Fibrinogen is present in a soluble and an insoluble form and may act in a similar way to fibronectin. Fibrinogen, as fibrin, is also one of the major components of vegetations. Both strains have been shown in this study to bind insoluble fibrinogen, strain L50 less effectively.

The assay of adhesion to platelet-fibrin clots simulates the adherence of bacteria to vegetations and may involve the same interactions as adhesion to soluble fibronectin and fibrinogen, since platelet-fibrin clots have been shown to contain fibronectin and fibrinogen.

It is not known whether strain L50 can cause endocarditis in man, but the comparison in vitro of the characteristics of the two strains and their potential to
cause endocarditis in the rat leads to some conclusions. Infection with strain FSS2 produced significantly larger vegetations and also produced embolic spread manifested principally as multifocal renal abscess. However, the finding that strain L50 could cause endocarditis suggests that platelet aggregation is not an initiating factor in the pathogenesis of endocarditis, although it may still be a virulence factor. These results are in agreement with recent results obtained by Herzberg et al.9 with the rabbit model. The difference in the capacity to aggregate platelets is reflected in similar differences in adhesion to platelets. Both strains bind insoluble fibronectin and insoluble fibrinogen and adhere to platelet-fibrin clots; the results reflect the differences in this potential to cause endocarditis. The extent of the reaction of a strain with these mammalian tissue components may be important factors in the pathogenesis of endocarditis.

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References