Adaptive gene expression of endocarditis-causing viridans group streptococci and Staphilococcus aureus
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Rapid multiplication of endocarditis-causing viridans group streptococci in platelet-fibrin clots is dependent on plasma components and streptococcal protease activity

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ABSTRACT

Endocarditis-causing viridans group streptococci (VS) colonizing platelet-fibrin vegetations on heart valves are capable of rapid growth. In infected vegetations bacterial densities are extremely high. In vitro, 3 VS strains grew rapidly and to high densities in platelet rich plasma (PRP) clots, but also in platelet poor plasma (PPP) clots. To determine which host components are required for this rapid growth, Streptococcus gordonii CH1 was grown in platelet clots, fibrin clots, serum, and plasma. Since PRP, PPP, platelets, fibrin, or serum were not supportive for growth, the rapid multiplication required as yet unknown plasma components entrapped within the clot during coagulation. S. gordonii CH1 also grew well in fibrinolysed PPP clots. Therefore, growth promoting components apparently were not affected by proteases of the fibrinolytic cascade. Addition of protease inhibitors to fibrinolysed PPP clots largely blocked bacterial growth, indicating that bacterial protease activity is essential for growth of VS in vegetations.
INTRODUCTION

Infective endocarditis (IE) denotes infection of the endocardial lining and of the heart valves. Viridans group streptococci (VS) originating from the oral cavity and upper pharyngeal tract microflora are the most common bacteria causing native IE. During transient bacteremia due to traumatization or infection of the oral or pharyngeal mucosa, VS are able to bind to vegetations (VGs), platelet-fibrin thrombi present on previously damaged endocardial or valvular lesions. Subsequently, adherent VS multiply and colonize these VGs (1,2). Since clearance of bacteria within the VG by professional phagocytes is hampered (2, 3), multiplication of VS continues until nutrient limitation occurs. Growth predominantly occurs just beneath the surface of the VG, while deep inside VGs clusters of bacteria, embedded in layers of fibrin, are metabolically inactive (2).

Several VS determinants are known to be involved in the initiation of IE. These include adhesins, providing VS the ability to adhere to different matrix molecules of the host (4,5), and resistance to antibacterial proteins from activated blood platelets, contributing to persistence of adherent VS (6,7,8). In contrast, characteristics of VS involved in the further progression of IE are scarcely studied. Such a characteristic may be the rapid multiplication of VS in the VG, as has been observed in experimental models of IE (1,6,7). It is conceivable that this rapid multiplication of VS, resulting in a high number of bacteria within 4 to 6 h after bacterial adherence to VGs (6), will contribute to their property to cause IE. By studying growth of VS in various human blood fractions in vitro, we aimed to identify factors either from the host or from VS that are supportive for the rapid bacterial growth.

MATERIALS AND METHODS

Bacteria and growth conditions
Streptococcus sanguis U108 (7), Streptococcus oralis J30 (formerly classified as Streptococcus sanguis II) (7), and Streptococcus gordonii CH1 (9) were cultured in Todd Hewitt (TH) broth and on TH-agar (Difco Laboratories, Detroit, MI) at 37°C. To prepare inocula for growth experiments, overnight bacterial cultures were centrifuged, the pellets were washed twice with phosphate-buffered saline (PBS, pH 7.4), and the bacteria were diluted in PBS to a final concentration of 2-4 x 10⁶ cfu/ml. For each growth experiment, 25 μl of this suspension (0.5 - 1 x 10⁵ cfu) was used as inoculum.

Reagents
Purified human fibrinogen was dissolved in 0.9% NaCl at a final concentration of 2 mg/ml. Human thrombin (CLB, Amsterdam, The Netherlands) was used at 10 U/ml, and tissue plasminogen activator (tPA; Boehringer Ingelheim B.V., Alkmaar, The Netherlands) at 0.1 mg/ml. One tablet containing a mixture of protease inhibitors (Complete™; Boehringer Mannheim GmbH, Mannheim, Germany) was dissolved in 5 ml of milliQ water, to obtain a 10x concentrated stock solution.
Preparation of human plasma, serum, and clots

After informed consent, blood from healthy volunteers was collected in polypropylene tubes containing a 3.2% buffered sodium citrate solution (blood-to-anticoagulant ratio, 9:1). Citrated blood was centrifuged for 10 min at 200xg, and three-fourth of the upper phase volume was collected to obtain platelet rich plasma (PRP). Platelet poor plasma (PPP) was collected after subsequent centrifugation of the remaining volume of blood for 15 min at 2,000xg. PRP and PPP clots were prepared by adding human thrombin and 1/10 volume of 0.5 M CaCl₂ to PRP and PPP, respectively. PRP and PPP serum were obtained after centrifugation of coagulated PRP and PPP for 10 min at 2,000xg. Fibrin clots were prepared by adding human thrombin and CaCl₂ to human fibrinogen suspensions. Fibrinolysis of PPP clots was initiated by addition of tPA. After incubation at 37°C for one hour the clots were completely lysed. The resulting suspension was designated as fibrinolate.

Bacterial growth experiments

Bacterial growth experiments were performed in 6 ml Falcon polypropylene tubes (Becton Dickinson Labware, Franklin Lakes, NJ) in duplicate. Twenty-five µl of bacterial inoculum was added to 250 µl of either of the different blood fractions. For reasons of comparison, identical VS inocula were added to 250 µl of TH broth. When required, 25 µl of the protease inhibitors stock solution was added. The final volume of all test samples, adjusted with sterile water if necessary, was 300 µl. After addition of the inoculum, coagulation of PRP, PPP, or fibrin to prepare clots was initiated, and continued for 90 min at room temperature. Then the clots were centrifuged briefly at 2,000xg and washed twice with PBS. PRP and PPP clots were subsequently submersed in 200 µl of fresh PPP, and fibrin clots in 200 µl of PBS. The test tubes were incubated at 37°C under continuous rotation (160 rpm). At various intervals, tubes were placed on ice, and ice-cold PBS was added to obtain a sample volume of 1.25 ml. The samples were ground using a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). The resulting suspensions were sonicated for 30 s in a sonicator bath (47 kHz; Branson Europa B.V., Soest, The Netherlands), 10-fold serially diluted in PBS, and plated on blood agar. The grinding and sonication procedures did not influence survival of VS strains (6).

RESULTS

We first determined the growth of S. sanguis U108, S. oralis J30, and S. gordonii CH1 in PRP clots. The growth rates of U108 and J30 in PRP clots were similar to those in TH. S. gordonii CH1 grew even more rapidly in PRP clots than in TH-broth (Fig. 1). After overnight incubation, the numbers of cfu of all three isolates in the PRP thrombi were at least 10-fold higher than those in TH-broth (Fig. 1).

To identify the components responsible for the rapid multiplication of VS, we studied the growth of S. gordonii CH1 in clots prepared from PRP, PPP, and fibrinogen, and in plasma, serum, and fibrinolate. S. gordonii CH1 was used in these experiments since this strain had a higher growth rate in the PRP thrombi than in TH-broth. Since logarithmic bacterial growth started approximately 0.5 h after incubation at 37°C (Fig. 1), the increase in bacterial numbers between 0.5 h and 5 h was used as a measure for the bacterial growth rate.
Rapid growth of VS in platelet-fibrin clots

Growth rates in PPP clots and PRP clots were essentially identical (Fig. 2A), and thus the number of platelets in the clots apparently had no influence on the bacterial growth. Soluble components released from thrombin activated platelets did not promote bacterial growth, since no increase in bacterial numbers was noted in PRP serum (Fig. 2A). Other soluble plasma or serum factors were not responsible for the growth-enhancing effect in clots either, as CH1 multiplied poorly in PPP (Fig. 2A), and the bacteria did not grow in PPP serum (data not shown) and in PRP serum. Furthermore, no growth was observed in clots prepared from human fibrinogen (Fig. 2A), implying that fibrin was not used as a nutrient source by S. gordonii CH1. Taken together, these data indicate that specifically PRP and PPP clots contain as yet unknown components, which are required for the rapid growth of VS. The availability of these clot components for the bacteria was only slightly affected by active fibrinolysis, as bacteria grew almost as well in a solution of degraded PPP clots (fibrinolate) as in intact PPP clots (Fig. 2A and 2B).

Finally, we determined whether the growth-enhancing components within the clots were directly available for bacterial growth or whether bacterial protease activity was required for further processing. To evaluate the role of bacterial proteases, the effect of host proteases was excluded by using PPP clot fibrinolates, which already contained all components resulting from the host fibrinolytic protease activity. Protease inhibitors were added to the fibrinolate before assessing growth of the bacteria. These protease inhibitors almost completely blocked the bacterial multiplication in PPP fibrinolate, whereas they only marginally influenced bacterial growth in TH-broth (Fig. 2B). Apparently, bacterial proteases are required for degradation of components present in the clot, that are not degraded by proteases from the host fibrinolytic pathway, providing nutrients required for the rapid bacterial growth within PRP or PPP clots.
DISCUSSION

Viridans group streptococci (VS) are commensals of the human oral cavity, and are in general not recognized as important pathogens. Nevertheless, VS are the most prevalent etiologic agents in native infective endocarditis (IE). Although several bacterial constituents are known to be involved in initiation of IE (4,5), little is known about the bacterial characteristics resulting in further progression of the disease. In the experimental rabbit model of IE, development of disease by VS is associated with rapid multiplication of the bacteria within the vegetation. Numbers of cfu of *S. oralis* J30 and *S. sanguis* U108 started to increase within VGs only 2 h after adherence (7). In the present study, rapid multiplication of these isolates was also observed in PRP plasma clots prepared in vitro, with similar growth rates as in the nutrient-rich TH broth. The more rapid growth of *S. oralis* J30 compared to *S. sanguis* U108, which was also observed in the experimental rabbit IE model (7), appeared to be a characteristic of this specific isolate. *S. gordonii* CH1, another VS strain often used in experimental IE studies (9), showed a similar rapid multiplication in PRP clots, and growth of
Rapid growth of VS in platelet-fibrin clots

this strain in the clots was even faster than in TH broth. Additionally, bacterial densities in the platelet clots were substantially higher than in TH broth after overnight incubation. Growth of VS in the in vitro PRP and PPP clot model closely resembled the in vivo bacterial growth in experimental IE models (1,6,7).

Neither platelets nor fibrin, the major constituents of VGs in vivo, were accountable for the observed enhanced growth of *S. gordonii* CH1. Although both plasma and serum are rich in soluble constituents, including glycoproteins that might function as nutrients for VS, rapid bacterial growth was not supported by these blood fractions either. Therefore, the rapid bacterial growth must depend on plasma components entrapped in the clots. The nature of these specific components remains to be determined. Since a VG is not only a site of local coagulation but also of fibrinolysis (10), we argued that fibrinolysis might influence growth of VS by altering the growth supportive clot components. This appeared not to be the case, as bacterial growth was almost as fast in total fibrinolates as in intact PPP clots. Alternatively, fibrinolysis might be required to release the growth-supportive components from the clot. Any of the growth-stimulating clot components present in the fibrinolate required further processing before they could be used by the bacteria, since rapid growth in fibrinolate was almost completely blocked by the addition of protease inhibitors. As the proteases of the fibrinolytic pathway had already actively degraded the clot to completion before addition of the protease inhibitors, the proteases involved in degradation of the clot components apparently were of bacterial origin. VS are known to produce many different protease and glycosidase activities (11,12,13). Proteases of VS have also been implicated in formation (thrombin-, Hageman factor-, activated factor X-like activities) and dissolution (plasmin-, kallikrein-, activated protein C-like activities) of clots (11). Straus identified four excreted proteases from an *S. sanguis* endocarditis isolate, of which two were able to degrade human serum albumin (14). Recently, an extracellular serine-type protease of *S. gordoni* CH1 was purified that hydrolyzes type IV collagen and collagen analogues (15). This protease is secreted under conditions considered to represent nutritional conditions at the endocardial thrombus. We now have shown the importance of VS protease activity for processing of clot components required for their growth, whereas such protease activity is not required for bacterial growth in TH broth. The involvement of specific bacterial proteases in intravegetational growth implies that these enzymes may indeed contribute to the virulence of VS in the pathogenesis of IE.

We conclude that specific components of plasma clots, other than platelets and fibrin, serve as nutrients for bacteria within a VG, and are required for rapid bacterial multiplication. VS proteases convert these components within the VG to nutrients supporting the rapid bacterial proliferation. These proteases might therefore serve as targets in the development of new strategies for the treatment of IE.
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