Thrombocidins, microbicidal proteins of human blood platelets

Krijgveld, J.

Citation for published version (APA):
Chapter 2

Neutralization of Platelet Microbicidal Activity in Rabbits Enhances Susceptibility for Viridans Streptococcal Experimental Endocarditis

Jacob Dankert\textsuperscript{a}, Jeroen Krijgsveld\textsuperscript{a}, Janneke van der Werff\textsuperscript{b}, Willem Joldersma\textsuperscript{b} and Sebastian A.J. Zaat\textsuperscript{a}

\textsuperscript{a}Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands,
\textsuperscript{b}Department of Hospital Epidemiology, University Hospital Groningen, Groningen, The Netherlands
Chapter 2

ABSTRACT

Oral viridans streptococci (VS) entering the bloodstream can adhere to and colonize cardiac platelet-fibrin vegetations, causing infective endocarditis (IE). Adherent bacteria may however be removed due to the action of thrombocidins (TCs), bactericidal proteins from blood platelets. To investigate this we vaccinated rabbits with crude TCs. The sera from vaccinated rabbits, but not from non-vaccinated controls, contained antibodies recognizing purified human TCs, and neutralized both human and rabbit platelet-released bactericidal activity. In two experimental IE models, vaccinated rabbits had a higher incidence of IE due to TC-susceptible VS than non-vaccinated rabbits. The incidence of IE due to a TC-resistant VS strain was not different in vaccinated and non-vaccinated rabbits. Apparently antibodies raised by vaccination with human TC neutralized the rabbit platelet bactericidal activity in vivo, thereby increasing the incidence of IE due to TC-susceptible VS. VS isolated from blood cultures of IE patients were proportionally less susceptible to crude TC preparations than gingival sulcus isolates and blood isolates from non-IE patients. Collectively, our data show that bactericidal platelet proteins like TCs play a significant role in preventing development of IE due to TC-susceptible circulating VS.
INTRODUCTION

Infective endocarditis (IE) denotes infection of the endocardial surface of the heart (31). Major determinants in the pathogenesis of IE are the presence of vegetations (VGs) at the damaged surface of the endocardium (1) and the ability of circulating VS to adhere to (5, 32) and to colonize these VGs (5, 10, 11). VGs are produced by blood turbulence due to various heart diseases or local and systemic stress conditions (1). Although many microorganisms have been implicated in IE, viridans streptococci (VS) are the most common isolates (21, 29, 31). VS abundantly cover the oropharyngeal mucosa (18) and may gain access to the bloodstream, most commonly after traumatization of the mucosa (9) or because of gingival diseases (9, 36).

Platelets are the earliest and numerically the predominant cells in VGs (2, 8, 32), and adherence of VS onto VGs is followed by a rapid accumulation of additional platelets and fibrin at the VG surface (8, 12, 16, 24, 31). Recently, we have shown that persistence of VS adherent on VGs in the rabbits was related to the susceptibility of VS test strains to bactericidal factors released from platelets by thrombin stimulation at the site of VGs (5). Human as well as rabbit platelets release such bactericidal factors (4, 5, 38, 39), which share a low molecular weight, a cationic nature, and a broad antimicrobial spectrum. Based on the reported amino acid compositions of rabbit platelet microbicidal proteins (39) and on the amino acid sequence of thrombicidins (22), these microbicidal proteins from rabbit and human platelets are similar, but not identical. In order to study the role of thrombicidins in the development of IE we vaccinated rabbits with crude human thrombicidins to induce antibodies neutralizing the microbicidal factors from rabbit platelets. The vaccinated rabbits showed an enhanced susceptibility for IE due to thrombicidin-susceptible VS.

MATERIALS AND METHODS

Patients and viridans streptococcal isolates

We collected a total of 178 viridans streptococcal (VS) isolates. Of these isolates 73 were grown from blood cultures of 67 patients with evidence of IE, diagnosed using the Duke criteria (14). Twenty-two isolates were cultured from blood specimens of 21 non-IE bacteremic patients. The remaining 83 isolates were cultured from gingival sulcus samples collected from 8 IE patients and 28 persons with a non-IE cardiac disease. Gingival sulcus swabs from patients were collected prior to antibiotic treatment. Culturing was done as previously described (5). Isolates were identified using standard methods (15, 28). The ability of the isolates to produce dextran was assessed after growth on mitis-salivarius agar (Oxoid, Unipath Ltd, Basingstoke, United Kingdom)

Isolates were stored in skim milk (Oxoid) at −20°C, cultured on sheep blood (5% v/v) agar (Oxoid) plates at 37°C in 5% CO2 for 48 h, and maintained at 4°C for one week. Before each test, bacteria were freshly grown in Mueller-Hinton (MH) broth (pH 7.4) (Difco Laboratories, Detroit, Mich) on a rotary shaker at 90 rpm for 24 hours without
aeration. Numbers of cfu were routinely determined by plating appropriate dilutions on sheep blood agar plates and counting after incubation in 10% CO\textsubscript{2} at 37°C for 48 h.

**Test organisms**

For experimental IE studies in rabbits 3 VS strains were selected from a collection used in previous experimental IE studies, on the basis of their differential susceptibility to releasates containing thrombocidins from thrombin stimulated platelets (4, 5). *Streptococcus oralis* strain J30 (formerly *S. sanguis* II (5)) had been isolated from the oral cavity of a patient with a non-IE cardiac disease. Both *Streptococcus mitis* strain S224 (non-dextran producing, formerly *S. mitior* dx-, (5)) and *S.sanguis* strain U108 (dextran producing, formerly *S. sanguis* I, (5)) had been isolated from blood cultures of IE patients. After 30 min of exposure to standard releasates of thrombin-activated platelets (see below) *S.oralis* J30 and *S.mitis* S224 showed 50% and 30% survival, respectively. *S.sanguis* U108 was resistant, showing 90% survival. The VS test strains were stored, grown and maintained as mentioned for the patient isolates. *Bacillus subtilis* ATCC 6633 was used to assay the bactericidal activity in platelet releasates and supernatants from sonicated platelets (see below). *B.subtilis* was stored at -20°C in skim milk, maintained on sheep blood agar at 4°C for 2 to 4 weeks, and was grown in brain heart infusion (BHI) broth (BBL, Microbiology Systems, Cockeysville, Md) for 14 -16 h before use.

**Preparation of bacterial suspensions**

After centrifugation (4,000 x g; 4°C; 10 min) bacteria were washed three times with PBS (8.1 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 140mM NaCl, 3mM KCl; pH 7.2) and resuspended in 0.9% (w/v) NaCl. Suspensions were sonicated for 30 s (50 kHz) (Bransonic 32; Bransonic Power Co, Danbury, Conn.) and adjusted to an optical density at 540 nm of 1.0 (model 24 spectrophotometer; Beckman Instruments Inc. Palo Alto, Calif.) with 0.9% NaCl. These standardized suspensions contained approximately 10\textsuperscript{8} cfu/ml, and were diluted in 0.9% NaCl to obtain the appropriate inocula for the individual experiments. Prior to each experiment inocula were checked by serial plating.

**Preparation of fresh human and rabbit platelet suspensions**

Fresh platelet suspensions and releasates were prepared as previously described (4, 5), using siliconized glassware (Vacutainer Systems, Becton Dickinson, Meylan, France) throughout collection of blood and all platelet handling procedures. Briefly, blood from healthy subjects and from healthy New Zealand White rabbits was freshly collected in siliconized tubes containing 0.1 ml of 0.11 M sodium citrate (pH5.5). After centrifugation of blood (225 x g, 37°C, 20 min), the platelet rich plasma (PRP) was centrifuged (2,000 x g, 37°C, 10 min), the pelleted platelets were washed twice in Tyrode's salt solution (Sigma Chemical Co, St. Louis, MO) and resuspended to a final concentration of 2-4 x 10\textsuperscript{8} platelets per ml in Dulbecco Modified Eagle Medium (Gibco Laboratories, Grand Island, NY) containing 10 mM sodium citrate (DMEM-C; pH 7.2).

**Preparation of platelet releasates**

Washed human and rabbit platelets were stimulated with 1 NIH unit/ml of human thrombin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service,
Amsterdam, the Netherlands) at 37°C for 15 min. The releasates from the stimulated platelets were collected after centrifugation (200 x g, 37°C, 20 min), stored at -20°C and used within 1 month.

**Preparation of platelet sonicate supernatants**

Human and rabbit platelet suspensions (2-4 x 10^8 platelets per ml) in DMEM-C were sonicated (50 kHz) for 3 min. The cell debris and unbroken cells were removed by centrifugation (2,000 x g, 37°C, 20 min). This supernatant is referred to as sonicate-sup. Sonicate-sups from platelet suspensions from 5 human subjects were pooled and stored at -20°C in portions of 0.5 ml. These pooled human platelet sonicate-sups were used to vaccinate rabbits.

**Bactericidal activity of platelet releasates and sonicate-sups**

Bactericidal activity of platelet releasates and sonicate-sups was tested using *B. subtilis* ATCC 6633. Inocula of 1.5 x 10^3 cfu/ml were incubated in 0.5 ml of undiluted releasates or sonicate-sups, or in 2-fold serial dilutions (1:2 to 1:1024 diluted with DMEM-C) of either preparation at 37°C in siliconized glass tubes (Vacutainer Systems). After 30 min of exposure, aliquots of 0.3 ml were transferred to tubes with 0.7 ml PBS containing 0.01% (w/v) sodium polyanetholesulfonate (SPS; "liquoid"), to neutralize the platelet bactericidal activity (4). The samples were sonicated (50 kHz) (Bransonic) for 30 s to disperse clumped bacteria, and quantitatively cultured on sheep blood agar. Based on the number of cfu before incubation, the mean percentual survival of *B. subtilis* in each dilution was calculated.

**Susceptibility of clinical VS isolates to human platelet releasates**

Susceptibility of VS isolates was tested as described above for *B. subtilis*, using standard human platelet releasates. Standard releasates were releasates killing more than 99.9% of *B. subtilis* when diluted 1:64 and 50% when diluted 1:256. Based on earlier studies (4, 5), VS isolates with more than 50% survival after 30 min of exposure to standard releasates were defined as resistant.

**Vaccination of rabbits with human platelet sonicate-sups**

Healthy female and male New Zealand White rabbits weighing 2.1 -3.1. kg, were vaccinated intramuscularly in the hind leg with 1 ml of pooled human platelet sonicate-sups. Sonicate-sups rather than releasates from thrombin activated platelets were used, in order to avoid the induction of antibodies against thrombin (33). Portions of human platelet sonicate-sups to be used for the first vaccination were emulsified with equal volumes of complete Freund adjuvant. Repeat vaccinations were done with 1 ml of sonicate-sups mixed with 1 ml of incomplete Freund adjuvant 3, 5 and 7 weeks after the first vaccination. Blood (5 ml) of the rabbits was collected prior to vaccination and subsequently at 2 week intervals. Sera, obtained by centrifugation (100 x g, 5 min) of the clotted blood were stored at -80°C in 1.5 ml portions.
Detection of antibodies against human platelet sonicate-sups and purified human platelet thrombocidins in sera from rabbits immunized with human platelet sonicate-sups

Sera from rabbits immunized with human platelet sonicate-sups were tested for the presence of antibodies recognizing proteins in human and rabbit sonicate-sups by immunodiffusion according to Ouchterlony (25). Rabbit sonicate-sups were prepared essentially as described for the human platelet sonicate-sups. To analyse whether rabbits immunized with human platelet sonicate-sups had produced antibodies recognizing the major cationic antibacterial proteins from human platelets, thrombocidin (TC)-1 and TC-2, a Western blot was performed. Human platelet sonicate-sup, purified TC-1 and TC-2 (22), and purified Human Neutrophil Protein 1-3 (HNP) were electrophoresed in triplicate in a tricine SDS-PAGE system (30). After electrophoresis the gel was divided into 3 equal parts, each containing an identical set of samples. One part was stained with Coomassie Brilliant Blue. The other two parts were analysed by Western blotting as described previously (35). One blot was incubated with rabbit serum collected after the fourth vaccination, and the second blot with pre-immune serum. Rabbit antibodies were visualized with a mouse-anti-rabbit monoclonal antibody conjugated to alkaline phosphatase (Promega, Madison, WI), and nitroblue tetrazolium (NBT, Promega) and 3-bromo-1-chloro-3-indolyl phosphate (BCIP, Promega) as substrate.

Bactericidal activity in human and rabbit platelet releasates supplemented with serum from rabbits vaccinated with human platelet sonicate-sups

Portions of 0.3 ml of serum from rabbits vaccinated with human platelet sonicate-sups were added to 0.3 ml of undiluted human and rabbit platelet releasates. After incubation at 37°C for 30 min, 10^3 cfu of B. subtilis ATCC 6633 were added, and incubation was continued for 30 min in order to assay bactericidal activity. Then aliquots of 0.3 ml were collected, mixed with 0.7 ml PBS containing 0.01% SPS, and quantitatively cultured as described above. Similar experiments were performed with the 3 VS test strains used in the experimental IE studies. Releasates supplemented with rabbit serum collected prior to vaccination served as controls in the assays.

Rabbit model of non-bacterial thrombotic VGs

Left-sided non-bacterial VGs were induced in New Zealand White rabbits weighing 2.1 to 3.1 kg. A polyethylene catheter (external diameter 0.8 mm; internal diameter 0.4 mm) was inserted into the left carotid artery and placed in the left ventricle as previously described (20). Of these rabbits, 95 had received 4 vaccinations with human platelet sonicate-sups. The last vaccination had been performed at least 3 weeks prior to catheterization. The catheter either remained in place during bacterial challenge of the rabbits, or was removed 24 h prior to bacterial challenge. In the latter case, rabbits are less susceptible to IE (12, 13, 17, 40).

Production and evaluation of bacterial adherence and IE

Rabbits with the catheter left in place were injected with 1 ml of 0.9% NaCl containing either 10^4 or 10^5 cfu of the test organism in the marginal ear vein, 24 h after
placement of the catheter. Rabbits from which the catheter was removed were challenged with $10^5$ cfu of the test organism, 24 h after removal of catheter. Rabbits were sacrificed by intravenous injection of pentobarbitone at 5 min or 48 h after challenge with the test organisms. The heart was removed under aseptic conditions and the VG was excised immediately and rinsed 3 times with 5 ml of 0.9% NaCl. VGs were weighed, homogenized and quantitatively cultured as previously described (5). Bacterial adherence was defined as the presence of bacteria on VGs at 5 min after challenge. IE was defined as culture-positivity of VGs at 48 h.

**Rabbit blood cultures after challenge**

Blood from rabbits was collected by puncturing the central artery of the ear at 5 and 30 min after challenge. Immediately after killing of the rabbits 5 ml of blood was collected from the right ventricle. Blood was quantitatively cultured as previously described (5). Bacteremia was expressed as the number of cfu per ml of blood.

**Statistics.** The incidence of culture positive VGs at 5 min and the incidence of IE, and the incidence of IE in vaccinated and non-vaccinated rabbits was compared with Fisher’s exact test or by the chi-square test with Yate’s correction for sample sizes larger than 50.

**RESULTS**

**Bactericidal activity in releasates from thrombin-activated human and rabbit platelets and in human platelet sonicate-sup**

In releasates from thrombin-activated rabbit and human platelets, as well as in human platelet sonicate-sup at least 99.9% of the inoculum of $10^3$ cfu of *B. subtilis* ATCC6633 was killed within 2 to 3 min of incubation. Human platelet releasates of which a 64-fold dilution killed 99.9% and a 256-fold dilution killed 50% of the *B. subtilis* inoculum in 30 min, were designated as standard releasates.

**Antibodies to human and rabbit platelet antigens and to purified thrombicidins in sera of rabbits vaccinated with human platelet sonicate-sup**

Sera from rabbits collected after the third and fourth vaccination with the pooled human platelet sonicate-sup reacted with human as well as rabbit platelet sonicate-sup used as antigens in the Ouchterlony immunodiffusion test. The precipitation lines indicated close antigenic relatedness between human and rabbit platelet antigens (not shown). Serum collected after the fourth vaccination was used in a Western blot of tricine SDS PA gels containing human sonicate-sup, purified human TC-1 and TC-2, and purified HNP (Fig. 1A). Most proteins in the platelet sonicate-sup, and the purified human TC-1 and TC-2 were stained in the blot, indicating that antibodies against platelet sonicate sup antigens including TC-1 and TC-2 were present in serum collected after the 4th vaccination of the rabbits (Fig. 1B). No reaction of this immune serum was observed with HNP. The pre-immune rabbit serum did not recognize sonicate-sup antigens, nor TC-1 or TC-2 (not shown).
Bactericidal activity in human and rabbit platelet releasates is neutralized by serum from rabbits vaccinated with human platelet sonicate-sups

Releasates from thrombin-activated rabbit or human platelets were incubated with serum from rabbits which had been vaccinated with pooled sonicate-sups of human platelets. Subsequently, bactericidal activity was tested using $10^3$ cfu of *B. subtilis* ATCC 6633 and $10^3$ cfu of *S. oralis* J30. The releasates incubated with rabbit sera collected prior to vaccination, or after the first vaccination killed >99% of the *B. subtilis* and 50% of the *S. oralis* J30 inoculum (Table 1). This reduction of viable numbers was similar to that observed in releasates with added DMEM-C instead of serum. Rabbit sera collected after the third vaccination markedly reduced the bactericidal activity in the releasates. Sera collected after the fourth vaccination neutralized the bactericidal activity in the releasates almost completely (Table 1). Sera of rabbits vaccinated at least three times with sonicate-sups of human platelets also neutralized the bactericidal activity released from thrombin activated rabbit platelets against *the S. mitis* S224 test strain (not shown). The platelet releasate-resistant *S. sanguis* U108 was not killed in releasates, or in releasates supplemented with sera from the vaccinated rabbits (not shown). The results of the killing assays were not influenced by agglutinating antibodies, since no bacterial agglutination was observed in microscopic examinations of *B. subtilis* and the 3 VS test strains exposed to sera from either non-vaccinated or vaccinated rabbits.

![Figure 1. Tricine gel electrophoresis of human platelet sonicate-sup, purified TC-1, TC-2, and HNP. A, Coomassie Brilliant Blue-stained gel. B, Western blotting with serum from rabbits vaccinated 4 times with human platelet sonicate-sups.](image)
Table 1. Bactericidal activity against *B. subtilis* ATCC6633 and *S. oralis* J30 of releasates from thrombin activated human and rabbit platelets supplemented with sera from rabbits vaccinated with sonicate-sups of human platelets.

<table>
<thead>
<tr>
<th>Releasates supplemented with&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Human platelet releasate</th>
<th>Rabbit platelet releasate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean reduction&lt;sup&gt;b&lt;/sup&gt; of</td>
<td>Mean reduction&lt;sup&gt;b&lt;/sup&gt; of</td>
</tr>
<tr>
<td></td>
<td><em>B. subtilis</em></td>
<td><em>S. oralis</em> J30</td>
</tr>
<tr>
<td>DMEM-C</td>
<td>&gt;99.9</td>
<td>50±3</td>
</tr>
<tr>
<td>Pre-immune serum</td>
<td>&gt;99.9</td>
<td>52±1</td>
</tr>
<tr>
<td>Serum after 1st vaccination</td>
<td>&gt;99.9</td>
<td>53±4</td>
</tr>
<tr>
<td>Serum after 2nd vaccination</td>
<td>&gt;99.9</td>
<td>48±5</td>
</tr>
<tr>
<td>Serum after 3d vaccination</td>
<td>28±4</td>
<td>34±5</td>
</tr>
<tr>
<td>Serum after 4th vaccination</td>
<td>14±3</td>
<td>11±4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Releasates were supplemented with 0.3 ml of DMEM-C or the indicated sera and incubated at 37°C for 30 min prior to assessing the bactericidal activity against *B. subtilis* and *S. oralis* J30.

<sup>b</sup> Percentage ± S.D. reduction after exposure for 30 min at 37°C.
Table 2. Number of culture-positive VGs at 5 min after intravenous challenge with $10^4$ cfu of three viridans streptococcal strains J30, S224 and U108 and the incidence of IE at 48 h post challenge in non-vaccinated rabbits and in rabbits vaccinated intramuscularly four times with 1 ml of pooled human platelet sonicate-sup.

<table>
<thead>
<tr>
<th>Test strain</th>
<th>Percentage survival of test strains in rabbit platelet releasate</th>
<th>No. colonized VGs / no. VGs evaluated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-vaccinated rabbits</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>S. oralis J30</td>
<td>50</td>
<td>19/25 (76%)</td>
</tr>
<tr>
<td>S. mitis S224 (dx)$^b$</td>
<td>30</td>
<td>8/9 (89%)</td>
</tr>
<tr>
<td>S. sanguis U108</td>
<td>90</td>
<td>4/18 (22%)</td>
</tr>
</tbody>
</table>

$^a$, p-values at 48 h in non-vaccinated rabbits versus vaccinated rabbits.
$^b$, dx-, dextran negative
Microbicidal proteins in rabbit endocarditis

Initial adherence and development of experimental IE in non-vaccinated and in vaccinated rabbits with the catheter left in place during challenge

To test the effect of neutralization of platelet-released bactericidal activity on the development of IE, rabbits vaccinated with human platelet sonicate-sups were challenged with $10^4$ cfu of VS test strain J30, S224 or U108. The incidence of IE in non-vaccinated rabbits 48 h after challenge with either of the platelet releasate susceptible strains J30 and S224 was low (Table 2). Only 4 of 30 non-vaccinated rabbits challenged with these 2 strains developed IE. Conversely, in total 17 of 22 vaccinated rabbits developed IE due to these 2 VS strains ($p<0.001$). The difference of IE frequency between vaccinated and non-vaccinated rabbits due to S. oralis J30 was significant ($p<0.001$), whilst no significance was reached for S. mitis S224 ($p=0.07$). The incidence of IE in non-vaccinated and vaccinated rabbits due to the releasate resistant strain U108 was not significantly different. No difference existed between the weights of VGs harvested from vaccinated and non-vaccinated rabbits at 48 h after challenge, nor between the bacterial densities in the VGs (data not shown).

At 5 min after challenge with either of the strains, the percentage of VGs with adherent bacteria was similar in non-vaccinated and in vaccinated rabbits (Table 2). In the non-vaccinated rabbits the 2 platelet releasate susceptible strains J30 and S224 had colonized significantly ($p<0.001$, and $p<0.01$, respectively) more VGs at 5 min than at 48 h post challenge, whilst in the vaccinated rabbits the number of culture positive VGs at 5 min and 48 h was similar.

The number of circulating bacteria at 5 or 30 min after challenge with either of the 3 VS strains was not different in the non-vaccinated and the vaccinated rabbits. Quantitative cultures of blood samples at 5 min after challenge yielded $6 \pm 3$ cfu and $5 \pm 3$ cfu of strain J30 per ml blood, and $4 \pm 3$ cfu and $6 \pm 2$ cfu per ml blood of strain S224, in non-vaccinated and vaccinated rabbits, respectively. Blood samples collected at 30 min after challenge were sterile.

Initial adherence and development of experimental IE in non-vaccinated and in vaccinated rabbits challenged after removal of the catheter

To further explore the effect of neutralization of platelet-released bactericidal activity on the development of experimental IE in rabbits, we used a low-susceptibility model for experimental IE (40). In this model the catheters were removed from rabbits with catheter-induced VGs 24 h prior to challenge with $10^5$ cfu of strains J30 or U108.

The initial colonization of VGs by both strains at 5 min post challenge was similar, irrespective of the presence of the catheter, or of the vaccination status of the rabbits (Table 3). Numbers of adherent bacteria 5 min after challenge in all three groups of rabbits (Table 3) were also similar (not shown).

At least 90% of the non-vaccinated rabbits which had the catheter in place during challenge with strains J30 or U108 developed IE. Removal of the catheter 24 h prior to challenge significantly reduced the frequencies of IE for both test strains. None of the rabbits challenged with J30, and only 30% of the rabbits challenged with U108 had IE (Table 3). However, strain J30 produced IE in 4 of 6 (66%) of the vaccinated rabbits from which the catheter had been removed 24 h prior to challenge ($p=0.015$ vs non-vaccinated rabbits from which catheters had been removed). Thus, even in this low susceptibility
Table 3. Number of culture-positive VGs at 5 min after intravenous challenge with $10^5$ cfu of two viridans streptococcal strains J30 or U108 and the incidence of IE at 48 h post challenge in non-vaccinated rabbits and in rabbits vaccinated intramuscularly four times with 1 ml of pooled human platelet sonicate sups. Non-vaccinated rabbits were either challenged while the catheter remained in place, or 24 h after removal of the catheter. The vaccinated rabbits were challenged 24 h after removal of the catheter.

<table>
<thead>
<tr>
<th>Test strain</th>
<th>Non-vaccinated rabbits catheter in place</th>
<th>Non-vaccinated rabbits catheter removed</th>
<th>Vaccinated rabbits catheter removed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min/48 h</td>
<td>5 min/48 h</td>
<td>5 min/48 h</td>
</tr>
<tr>
<td>S. oralis J30</td>
<td>30/35</td>
<td>15/16</td>
<td>6/6</td>
</tr>
<tr>
<td>S. sanguis U108</td>
<td>10/12</td>
<td>12/13</td>
<td>7/8</td>
</tr>
</tbody>
</table>

ND, not determined

*a, P<0.001 versus value at 48 h in non-vaccinated rabbits with catheters left in place

*b, P=0.015 versus value at 48 h in non-vaccinated rabbits with catheters removed

*c, P=0.003 versus value at 48 h in non-vaccinated rabbits with catheters left in place.
model of IE, neutralization of platelet bactericidal activity by vaccination with human
sonicate-sups rendered the rabbits susceptible for IE due to strain J30. Vaccination did not
significantly influence development of IE due to the platelet releasate resistant strain U108
(Table 3). The weights of the VGs as well as the VS densities in infected VGs at 48 h after
challenge were not different in non-vaccinated and vaccinated rabbits (not shown).

Susceptibility of viridans streptococci isolated from blood and gingival sulcus to
bactericidal activity in human platelet releasates

Susceptibility of the clinical VS isolates to standard human platelet releasates was
assessed. The proportion of resistant VS among IE isolates (63/73, 86%) was significantly
higher than among gingival sulcus isolates (22/83, 27%; p<0.005), and among VS isolates
from the blood of non-IE bacteremic patients (4/22, 18%; p<0.005).

*S.mitis* was the most common species cultured from blood of IE patients, as well
as from the gingival sulcus of these patients and persons with non-IE cardiac disease (Fig.
2). The proportion of *S.mitis* strains resistant to human platelet releasates was significantly
higher among the strains isolated from IE patients (35 of 45) than among gingival sulcus
isolates (10 of 44; P<0.005). Dextran-positive *S. mitis* isolates were distributed equally
among susceptible and resistant isolates (data not shown). All 32 *S.sanguis* isolates from
blood of IE patients and from the gingival sulcus cultures were resistant. The summarized
proportion of resistant *S.mutans, S.intermedius* and *S.salivarius* was significantly higher
among the IE isolates (7 of 7) than among gingival sulcus isolates (2 of 30; p<0.005)
(Fig.2).

![Figure 2. Species distribution and susceptibility of viridans streptococcal isolates to platelet releasate bactericidal activity. IE, isolates from blood specimens of IE patients. Sulc, isolates from the sulcus gingivalis of IE patients and patients with non-IE cardiac diseases.](image-url)
Bactericidal proteins released by platelets upon thrombin stimulation have been recognized as an important innate host defense mechanism against experimental IE in rabbits (4). These platelet proteins most likely are responsible for the clearance of adherent VS from VGs within 2 h after these bacteria had adhered (5). In the same experimental IE model, Sullam et al (34) observed that VGs of thrombocytopenic rabbits were colonized with higher numbers of S. aureus than non-thrombocytopenic controls. In addition, an S. aureus mutant strain with reduced susceptibility to rabbit platelet microbicidal proteins proliferated to higher densities in VGs, and, in contrast to the susceptible parent strain, were also cultured from kidneys and spleens after hematogenous dissemination (6).

In this study we present 2 lines of evidence further supporting the role of the bactericidal proteins released from platelets in the host defense against IE. Firstly, VS causing IE appeared to be selected for low susceptibility to human platelet bactericidal activity. Only 14% of the 73 VS isolates from the blood of IE patients were susceptible to the standard platelet releasates. In contrast, the proportion of susceptible VS among isolates from their sulcus gingival was high, and was similar that among sulcus isolates from persons with non-IE cardiac disease, and from the blood of non-IE bacteremic patients. Of note, the species distribution of the IE-causing VS isolates also highlighted this selectivity for platelet releasate resistance. Only 12% of the randomly collected 83 gingival sulcus isolates were of the intrinsically resistant S. sanguis, whilst this species represented 29% of all isolates causing IE. This frequency of S. sanguis among IE-causing VS is similar to frequencies reported earlier (7, 26, 27). S. mitis was the predominant species cultured from blood and the gingival sulcus. More than 90% of the gingival sulcus isolates and the non-IE bacteremia isolates were susceptible, whereas 78% of the isolates from blood cultures of IE patients were resistant to platelet bactericidal activity (Fig. 2). Finally, strains of S. mutans, S. intermedius and S. salivarius isolated from IE patients were resistant, while almost all gingival sulcus isolates of these species were susceptible. A similar selectivity for reduced susceptibility among IE isolates has been reported for staphylococci (37).

Since platelet bactericidal activity was assumed to contribute significantly to the defense against the onset of experimental IE due to VS (5), neutralization of this activity would enhance the susceptibility of rabbits for IE. We aimed to neutralize the platelet bactericidal activity by vaccination of rabbits to induce antibodies against platelet bactericidal proteins, and subsequently to test the susceptibility of these rabbits to IE due to VS. Human platelets contain at least 10 antibacterial proteins, designated thrombicidins, with different activity against various bacterial species (23). Similarly, rabbit platelets contain at least 7 different platelet microbicidal proteins (PMPs) (39). Because of these high numbers of microbicidal proteins in humans as well as in rabbits, and because the individual proteins have not been purified or are not available in sufficient quantities, rabbits were vaccinated with sonicate-sups prepared from human platelets. Sonicate-sups similar bactericidal activity as releasates from thrombin-stimulated platelets were used, to avoid induction of anti-thrombin antibodies (33), which might interfere with coagulation and formation of VGs.

The rabbit immune sera collected after 4 vaccinations contained antibodies that precipitated with human platelet sonicate-sup antigens, and recognized purified human
thrombocidins in a Western blot (Fig.4). The immune sera neutralized bactericidal activity of human as well as of rabbit platelets. The most likely explanation for this neutralization is that human and rabbit bactericidal proteins are antigenically related. To verify this assumption, purified rabbit platelet microbicidal proteins would have to be tested for their reactivity with the rabbit immune sera.

In the traditional experimental IE model, with the catheter left in place during challenge, vaccination with pooled human platelet sonicate-sups rendered the rabbits more susceptible to IE due to the releasate susceptible strains J30 and S224. Enhancement of susceptibility for IE due to vaccination was also found in rabbits with catheter-induced VGs and challenged with $10^5$ cfu of strain J30 after the catheter had been removed (Table 3). Removal of the catheters 24 h prior to challenge strongly reduced the susceptibility of non-vaccinated rabbits for IE. None of them developed IE, whilst more than 90% of non-vaccinated rabbits with the catheter left in place developed IE after challenge with $10^5$ cfu. Our data showed that neutralization of platelet bactericidal activity strongly enhanced the susceptibility of rabbits for IE due to the platelet releasate-susceptible strain, even in the absence of the catheter during challenge. The relevance of platelet released bactericidal proteins in the pathogenesis of experimental IE can also be deduced from the data obtained with strain U108, which is hardly killed during exposure to platelet releasates in vitro. The incidence of IE due to this strain was independent on the vaccination status of the rabbits.

The enhanced susceptibility to IE of the vaccinated rabbits was not due to an increased adherence of circulating VS to the VGs. In non-vaccinated rabbits the number of adherent VS per VG at 5 min after challenge with $10^4$ cfu and with $10^5$ cfu ranged from 1 to 38 (mean 14) cfu, and from 2 to 58 (mean 18) cfu, respectively. In vaccinated rabbits the number of adherent VS 5 min after challenge was not different (not shown). The magnitude and duration of bacteremia were not different either in the vaccinated and non-vaccinated rabbits. Blood samples taken at 30 min after challenge with $10^4$ cfu were culture-negative, and after challenge with $10^5$ cfu were either sterile or had low numbers of cfu, in both vaccinated and non-vaccinated rabbits. Thus, the increased incidence of IE in the vaccinated rabbits most likely was due to the neutralization of the platelet released bactericidal activity at the VG, preventing the clearance of susceptible VS early after their adherence to the VGs.

In the general population the presence of VGs is not rare. VGs were found in 2.4% of over 3,000 autopsies of non-IE hospitalized patients (3). The incidence of transient bacteremia after daily activities such as chewing candy (17 - 51%, (31)) or tooth brushing (7 - 50%, (9)) is very high. The incidence of IE is however rather low, being 1.7-3.8 cases per 100,000 person years (31). In persons having cardiac diseases involving IE, the incidence is approximately 100-fold higher, being 0.2 - 1.8 per 1,000 person years (19). However, even in this patient group, and certainly in the general population, much higher incidences of IE would be expected based on the incidence of VGs and transient bacteremias. Our experimental data and the observed high frequency of resistance to thrombocidin-containing human platelets releasates among VS blood isolates from IE patients indicate that thrombocidins may play a protective role against circulating VS adhering to VGs, thereby preventing IE due to low grade bacteremia in everyday life.
REFERENCES


Microbicidal proteins in rabbit endocarditis


