Platelet Microbicidal Activity Is an Important Defense Factor against Viridans Streptococcal Endocarditis

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To study the role of platelet microbicidal activity in host defense against infective endocarditis (IE) due to viridans streptococci (VS), the susceptibility to platelet releasate of blood and oral VS isolates from patients with and without IE was compared. The influence of neutralization of platelet microbicidal activity was studied in 2 experimental IE models. Resistance to platelet releasate was more prevalent among VS from blood of patients with IE than from blood of bacteremic patients without IE and among oral VS isolates. Serum from rabbits vaccinated with human platelet sonicate supernatants neutralized human and rabbit platelet-released microbicidal activity and had antibodies recognizing microbicidal proteins thrombocidin-1 and -2 and other human platelet proteins. In the 2 rabbit IE models, vaccination increased the susceptibility to experimental IE due to platelet releasate-susceptible VS. Thus, platelet-released microbicidal activity is an important host defense factor against IE due to VS.

Infective endocarditis (IE) denotes infection of the endocardial surface of the heart [1]. Major determinants in the pathogenesis of IE are the presence of sterile vegetations at the damaged surface of the endocardium [2] and of microorganisms circulating in the bloodstream that are able to adhere to [3, 4] and colonize these vegetations [3, 5, 6]. Vegetations are initiated by excessive blood turbulence due to various endocardial disease states or to local and systemic stress conditions [2]. Although many microorganisms cause IE, viridans streptococci (VS) are the most common [1, 7, 8]. VS abundantly cover the oropharyngeal mucosa [9] and gain access to the bloodstream, most commonly after traumatization of the mucosa [10] or because of gingival diseases [10, 11].

Platelets are the earliest and the predominant cells in vegetations [4, 12, 13]. Moreover, adherence of VS onto vegetations is followed by rapid accumulation of additional platelets and fibrin at the vegetation surface [1, 13–16]. We showed that persistence of VS adherent on catheter-induced vegetations in rabbits is related to the susceptibility of VS test strains to microbicidal factors released from platelets by thrombin stimulation [3, 17]. In the present study, we investigated whether VS blood isolates from patients with IE would be less susceptible to the microbicidal activity of thrombin-stimulated human platelets than VS blood isolates from patients without IE and oral VS isolates.

Human and rabbit blood platelets contain a spectrum of different cationic microbicidal proteins [17–21]. The human thrombocidins (TCs) [18, 19] and rabbit platelet microbicidal proteins (PMPs) [20, 21] share a low molecular weight, a cationic nature, and a broad range of antimicrobial activity [3, 17–21]. On the basis of the amino-acid sequence of TCs [19] and the amino-acid composition of the PMPs [21], the microbicidal proteins from human and rabbit platelets are similar but not identical. To further study the role of such microbicidal proteins from platelets in the pathogenesis of IE, we vaccinated rabbits with human platelet preparations containing TCs in an attempt to induce antibodies with cross-species neutralizing activities against the homologous microbicidal proteins from rabbit platelets.

Materials and Methods

Test organisms. Bacillus subtilis ATCC 6633, a platelet peptide-susceptible marker strain [1, 3, 20] was used to assay the microbicidal activity in platelet releasates and in supernatants from sonicated platelets (see below). B. subtilis was stored at ~20°C in skim milk, was maintained on sheep blood agar at 4°C for 2–4 weeks, and was grown in brain-heart infusion broth (BBL Microbiology Systems) for 14–16 h before use.

For experimental IE studies in rabbits, we used 2 standard experimental IE test strains, Streptococcus oralis J30 and S. sanguis U108. S. oralis J30 (formerly S. sanguis II [5]) is a TC-susceptible
strain originally isolated from the oral cavity of a patient with a non-IE cardiac disease. *S. sanguis* U108 (dextran producing; formerly *S. sanguis* I [3]) is a TC-resistant strain originally isolated from blood cultures of a patient with IE. These strains have been used extensively by various groups in experimental IE studies [3–5, 17, 22–24]. Additional experiments were done with the TC-susceptible, nonextran- 

producing *S. mitis* strain S224 (formerly *S. mitior* dx ) isolated from blood cultures of a patient with IE and used in experimental IE studies [3, 17].

The strains were stored in skim milk (Oxoid; Unipath) at −20°C, were cultured on sheep blood (5% vol/vol) agar (Oxoid) plates at 37°C in 5% CO₂ for 48 h, and were maintained at 4°C for 1 week. Before each test, bacteria were freshly grown to stationary phase in Mueller-Hinton broth (pH 7.4; Difco Laboratories) on a rotary shaker at 90 rpm for 24 h without aeration. Numbers of colony- 
forming units were determined routinely by plating appropriate dilutions on sheep blood agar plates and by counting after incubation in 10% CO₂ at 37°C for 48 h.

**Bacterial suspension preparation.** After centrifugation (4000 g, 4°C, 10 min) bacteria were washed 3 times with PBS (8.1 mM Na₃HPO₄, 1.5 mM KH₂PO₄, 140 mM NaCl, and 3 mM KCl [pH 7.2]) and were resuspended in 0.9% (wt/vol) NaCl. Suspensions were sonicated for 30 s (50 kHz, Bransonic 32; Branson Power) and were adjusted to an OD at 540 nm of 1.0 (model 24 spectrophotometer; Beckman Instruments) with 0.9% NaCl. These standardized suspensions contained ~10⁸ cfu/mL and were diluted in 0.9% NaCl to obtain the appropriate inocula for the individual experiments. Before each experiment, inocula were checked by quantitative culturing.

**Preparation of fresh human and rabbit platelet suspensions.** Fresh platelet suspensions and releasates were prepared, as described elsewhere [3, 17], by using siliconized glassware (Becton Dickinson Vacutainer Systems) throughout blood collection and all platelet handling procedures. In brief, blood specimens from healthy subjects or from healthy New Zealand White rabbits were collected in siliconized tubes containing 0.1 mL of 0.11 M sodium citrate (pH 5.5). After centrifugation of blood (225 g, 37°C, 20 min), the platelet-rich plasma was centrifuged (2000 g, 37°C, 10 min), and the pelleted platelets were washed twice in Tyrode’s salt solution (Sigma Chemical) and were resuspended to a final concentration of 2 × 10¹⁰ to 4 × 10¹⁰ platelets/mL in Dulbecco’s modified Eagle medium (Gibco Laboratories) containing 10 mM sodium citrate (DMEM-C; pH 7.2).

**Preparation of platelet releasates.** Washed human or rabbit platelets were stimulated with 1 National Institutes of Health U/mL of human thrombin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) at 37°C for 15 min. This procedure induces release of microbicidal peptides from mammalian platelets [3, 17, 20]. The releasates from the thrombin-stimulated platelets were collected after centrifugation (200 g, 37°C, 20 min), were stored at −20°C, and were used within 1 month. These releasates were used to test the in vitro susceptibility of VS to and to assess the neutralizing capacities of sera from vaccinated rabbits (see below).

**Preparation of platelet sonicate supernatants.** Human and rabbit platelet suspensions (2 × 10⁹ to 4 × 10¹⁰ platelets/mL) in DMEM-C were sonicated (50 kHz) for 3 min. The cell debris and unbroken cells were removed by centrifugation (2000 g, 37°C, 20 min). Sonicate supernatants from platelet suspensions from 5 human subjects were pooled and stored at −20°C in 0.5-mL portions. These pooled human platelet sonicate supernatants were used to vaccinate rabbits.

**Microbicidal activity of platelet releasates and sonicate supernatants.** Microbicidal activities of platelet releasates and sonicate supernatants were tested by using *B. subtilis* ATCC 6633. Inocula (1.5 × 10⁴ cfu/mL) were incubated in 0.5 mL of releasates or sonicate supernatants and in 2-fold serial dilutions (1:1 to 1:1024 diluted with DMEM-C) of either preparation at 37°C in siliconized glass tubes (Vacutainer Systems). After exposure for 30 min, 0.3- 

mL aliquots were transferred to tubes with 0.7 mL of PBS containing 0.01% (wt/vol) sodium polyanetholesulfonate (SPS; “liq-

uid”) to neutralize the platelet microbicidal activity [3, 17]. The samples were sonicated (50 kHz; Bransonic) for 30 s, to disperse clumped bacteria, and then were cultured quantitatively on sheep blood agar. On the basis of the number of colony-forming units before incubation, the mean (±SD) percentage of survival of *B. subtilis* in each dilution was calculated. Each assay result represents the mean of ≥4 independent runs on separate days.

**Susceptibility of clinical VS isolates to human platelet releasates.** We collected 198 VS isolates. Of these, 73 were grown from blood cultures of 67 patients with IE and 20 from sulcus swabs of 8 patients with evidence of IE, as diagnosed by the Duke criteria [25]. We cultured 22 isolates from blood specimens of 21 neutro- 

penicillin patients with bacteremia without IE (referred to throughout as bacteremic patients without IE) and 20 isolates from the sulcus of 18 of these patients. In total, 63 isolates were cultured from gingival sulcus samples from 28 patients without IE with a cardiac disease at risk for IE. All gingival sulcus swabs from patients were collected before antibiotic treatment.

Culturing was done as described elsewhere [3]. Isolates were identified by standard methods [26, 27]. The ability of the isolates to produce dextran was assessed after growth on mitis-salivarius agar (Oxoid). Isolates were cultured and stored, as described above for the test organisms.

**Susceptibility of clinical VS isolates for platelet microbicidal ac-

tivity was tested, as described above, for *B. subtilis*. The technicians performing the susceptibility assays were not informed about organ-

ism source (patient category, blood, or sulcus gingivalis isolate). The assays used stationary-phase bacteria and standard throbmin-

induced human platelet releasates with high-titered activity against *B. subtilis* (i.e., >99.9% killing of *B. subtilis* when diluted 1:64 and ≥50% when diluted 1:256). Susceptibility of these stationary-phase VS was similar to that of logarithmic-phase bacteria, as tested with a panel of 20 isolates. On the basis of results of an earlier study, we defined VS isolates with >50% survival after 30 min of exposure to undiluted standard releasates as resistant [3, 17, 28, 29].

**Vaccination of rabbits with human platelet sonicate supernatants.** 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 supernatants. We used sonicate supernatants, rather than releasates from thrombin-stimulated platelets to avoid the induction of antibodies against thrombin [30]. Portions of human platelet sonicate supernatants for the first vaccination were emulsified with equal volumes of complete Freund’s adjuvant. Repeat vaccinations were done with 1 mL of sonicate supernatants mixed with 1 mL of incomplete Freund’s adjuvant 3, 5, and 7 weeks after the first vaccination. Rabbit blood (5 mL) was collected before vaccination and subsequently at 2-week intervals. Serum, obtained
after centrifugation of clotted blood (100 g, 5 min), was stored at −80°C in 1.5-mL portions.

Detection of antibodies against human platelet sonicate supernatants and purified human platelet TCs in serum from vaccinated rabbits. To analyze whether rabbits vaccinated with human platelet sonicate supernatants had produced antibodies recognizing TC-1 and TC-2, the major cationic antibacterial proteins from human platelets [17], a Western blot was performed. Human platelet sonicate supernatant, purified TC-1 and TC-2, and a partially purified preparation of human neutrophil defensins 1–3 (HNP1–3) were electrophoresed in triplicate in a tricine SDS-PAGE system [31]. 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 2 parts were analyzed by Western blotting [32]. One blot was incubated with rabbit serum obtained after the fourth vaccination, and the second blot with pre-vaccination serum. Rabbit antibodies were visualized with a mouse anti–rabbit monoclonal antibody conjugated to alkaline phosphatase (Promega) with nitroblue tetrazolium (Promega) and 3-bromochloro-3-indolyl phosphate (Promega) as substrate.

Assessment of neutralization of microbicidal activity in human and rabbit platelet releasates by antibodies in serum from vaccinated rabbits. Serum samples (0.3 mL) from each rabbit vaccinated with human platelet sonicate supernatants were added to 0.3 mL of undiluted human and rabbit platelet releasates shown to have potent microbicidal activity in vitro against B. subtilis. 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 to assay microbicidal activity. We then collected 0.3-mL aliquots that were mixed with 0.7 mL of PBS containing 0.01% SPS and quantitatively cultured, as described above. Similar experiments were performed with the 3 VS test strains for use in the experimental IE studies. Releasates mixed with rabbit serum obtained before vaccination served as controls in the assays.

Rabbit model of IE. Sterile left-sided vegetations were induced in New Zealand White rabbits weighing 2.1–3.1 kg. A polyethylene catheter (external diameter, 0.8 mm; internal diameter, 0.4 mm) was inserted into the left carotid artery and was placed retrograde across the aortic valve into the left ventricle, as described elsewhere [22]. Of these rabbits, 95 had received 4 vaccinations with human platelet sonicate supernatants. The last vaccination was done 3 weeks before catheterization. Catheterization was done when the serum of vaccinated rabbits contained antibodies neutralizing the microbicidal activity of platelet releasates. The catheter either remained in place during bacterial challenge of the rabbits or was removed 24 h after placement. After another 24 h, these rabbits received the bacterial inoculum. In the latter model, rabbits are less susceptible to IE [14, 22, 24, 33].

Evaluation of bacterial adherence to vegetations and development of IE. Rabbits with the catheter left in place were injected in the marginal ear vein with 1 mL of 0.9% NaCl containing 10^6 cfu of the test organisms 24 h after catheter placement. Rabbits from which the catheter was removed were challenged with 10^5 cfu of the test organisms 24 h after catheter removal. Such inocula induce low frequencies (10%–20%) of IE in the respective models [3, 17, 24] and should allow for accurate measurement of differences in rabbit susceptibility to IE development. Rabbits were killed by intravenous injection of pentobarbital at 5 min or 48 h after challenge with the test organisms. The heart was removed under aseptic conditions, and the vegetation was excised immediately and was rinsed 3 times with 5 mL of 0.9% NaCl. The vegetation was weighed, homogenized, and quantitatively cultured, as described elsewhere [3]. Usually 1 vegetation was present in each heart. If ≥1 vegetation was present, the vegetations were pooled and analyzed as 1 vegetation. Bacterial adherence was defined as the presence of bacteria on vegetations 5 min after challenge. IE was defined as culture positivity of vegetations at 48 h. Only rabbits with correctly positioned catheters (catheter in-place group) and macroscopic vegetations (both groups) were included in this analysis.

Rabbit blood cultures after challenge. Blood samples from rabbits were collected by puncturing the central artery of the ear at 5 and 30 min after challenge. Immediately after rabbit sacrifice, 5 mL of blood was collected from the right ventricle. Blood was quantitatively cultured as described elsewhere [3]. The bacteremia level was expressed as colony-forming units per milliliter of blood.

Statistics. The incidence of culture-positive vegetations 5 min after challenge and the incidence of IE in vaccinated and nonvaccinated rabbits was compared by Fisher’s exact test or by χ^2 test with Yates correction for sample sizes >50.

Results

Microbicidal activity in releasates from thrombin-activated human and rabbit platelets and in human and rabbit platelet sonicate supernatants. In releasates from thrombin-activated rabbit and human platelets and in human and rabbit platelet sonicate supernatants, ≥99.9% of the inoculum of 10^3 cfu of B. subtilis ATCC 6633 was killed within 2–3 min of incubation. Human platelet releasates, in which a 64-fold dilution killed ≥99.9% and a 256-fold dilution killed 50% ± 5% of the B. subtilis inoculum in 30 min, were designated as standard releasates. After 30 min of exposure to such standard releasates, S. oralis J30 and S. mitis S224 showed 50% and 30% survival, respectively. S. sanguis U108 was resistant with 90% survival.

Susceptibility of VS from blood and gingival sulcus to microbicidal activity in human platelet releasates. Among VS isolates from blood of patients with IE, 63 (86%) of 73 were resistant to platelet microbicidal activity, compared with 5 (23%) of 22 isolates from blood of bacteremic patients without IE (P < .001). The proportion of resistant VS among isolates from blood of patients with IE (63 [86%] of 73) also was significantly higher than among VS isolates from the sulcus gingivalis of these patients (5 [25%] of 20; P < .001; figure 1). Conversely, the proportion of resistant VS among the VS blood isolates of bacteremic patients without IE (5 [23%] of 22) was low and not significantly different from the proportion of resistant VS among their gingival sulcus isolates (4 [20%] of 20). The proportion of resistant isolates among sulcus isolates from patients with IE (5 [25%] of 20), bacteremic patients without IE (4 [20%] of 20), and nonbacteremic cardiac patients without IE (16 [25%] of 63) was similar (figure 1). Thus, only blood from patients with IE had a high proportion of platelet releasate-resistant VS isolated.

All 34 S. sanguis isolates from blood of patients with IE and...
from the gingival sulcus cultures of the 3 patient groups were resistant to human platelet releasates, indicating that this species is intrinsically resistant (figure 1). S. mitis was the most common species cultured. The proportion of S. mitis resistant to human platelet releasate was significantly higher among S. mitis isolates from the blood of patients with IE (35 [78%] of 45) than among S. mitis isolate from their gingival sulcus (2 [18%] of 11; P < .001). In contrast, the proportion of resistant S. mitis among blood isolates (4 [22%] of 18) and gingival sulcus isolates (1 [9%] of 11) from bacteremic patients without IE was similar and was as low as the proportion of resistant VS among gingival sulcus isolates from patients with IE (2 [18%] of 11) and patients with non-IE cardiac disease (8 [22%] of 36; figure 1). Dextran-positive S. mitis isolates were distributed equally among susceptible and resistant isolates (data not shown). The proportion of resistant S. mutans, S. intermedius, and S. salivarius was significantly higher in blood isolates (7 [100%] of 7) than among gingival sulcus isolates of patients with IE (0 of 6; P < .001) or among blood (0 of 3) and gingival sulcus isolates (2 [7%] of 30; P < .001) from the 2 other patient groups (figure 1). Thus, VS isolates of various species cultured from the blood of patients with IE often were more resistant to platelet microbicidal activity than VS from blood of bacteremic patients without IE or from the sulcus gingivalis of any of the patient groups.

Rabbit vaccination with human platelet sonicate supernatants. We vaccinated rabbits with human platelet sonicate supernatants to induce antibodies that would neutralize platelet microbicidal activity. Serum obtained after vaccination 4 was used in a Western blot of tricine SDS-PAGE gels containing HNP1–3, purified human TC-1 and TC-2, and human sonicate supernatant proteins (figure 2A). In the Western blot, purified human TC-1 and TC-2 and most proteins present in the platelet sonicate supernatant were stained, including proteins with similar migration as purified TC-1 and TC-2 (figure 2B), indicating that antibodies against platelet sonicate supernatant antigens, including TC-1 and TC-2, had been elicited. The absence of staining of HNP1–3 indicated specificity of the immune response of the vaccinated rabbits (figure 2B). The prevaccination rabbit serum did not recognize the sonicate supernatant antigens, purified TC-1, TC-2, and HNP1–3 (figure 2C).

Microbicidal activity in human and rabbit platelet releasates is neutralized by serum from rabbits vaccinated with human platelet sonicate supernatants. Releasates from thrombin-activated rabbit or human platelets were incubated with serum from rabbits vaccinated with pooled sonicate supernatants of human platelets. Subsequently, microbicidal activity was tested by using $10^3$ cfu of B. subtilis ATCC 6633 and $10^3$ cfu of S. oralis J30. The releasates incubated with rabbit serum obtained before vaccination and after the first vaccination killed >99% of the B. subtilis and 50% of the S. oralis J30 inoculum (table 1). In releasates with DMEM-C added instead of serum, we observed a similar reduction of viable numbers. After incubation of platelet releasates with rabbit serum obtained after vaccination 3, the microbicidal activity against both bacterial species was markedly decreased. The microbicidal activity of the releasates almost was completely neutralized after incubation with serum obtained from rabbits after vaccination 4 (table 1). Serum from rabbits vaccinated ≥3 times with sonicate supernatants of human platelets also neutralized the microbicidal activity released from thrombin-stimulated rabbit platelets against the S. mitis S224 test strain (data not shown). The platelet releasate-resistant S. sanguis U108 showed >90% survival after incubation in releasates and in releasates mixed with serum from the vaccinated rabbits (data not shown). The results of the killing assays were not influenced by agglutinating antibodies, since no bacterial agglutination was observed in microscopic ex-
aminations of *B. subtilis* and of the 3 VS test strains exposed to serum from nonvaccinated or vaccinated rabbits.

**Circulating VS and initial adherence to vegetations of nonvaccinated and vaccinated rabbits in 2 experimental IE models.** To test the effect of antibodies neutralizing the platelet microbicidal activity on the development of experimental IE, we used nonvaccinated rabbits and rabbits vaccinated with human platelet sonicate supernatants. The catheter to induce vegetations was either left in place during challenge or removed 24 h before challenge with VS test strains J30 (releasate susceptible) or U108 (releasate resistant). An additional experiment with a limited number of vaccinated rabbits with the catheter left in place used *S. mitis* S224 (releasate susceptible) as the challenge strain.

Rabbits with the catheter in place were challenged with 10⁴ cfu of a VS test strain. The number of circulating VS at 5 min after challenge with either of the 3 test strains did not differ in nonvaccinated and vaccinated rabbits (table 2). All blood samples collected 30 min after challenge were sterile. For each of the 3 VS test strains, the percentage of vegetations with adherent bacteria 5 min after challenge was similar in nonvaccinated and vaccinated rabbits, as was the number of adherent colony-forming units per vegetation (table 2).

Rabbits from which the catheter was removed were challenged with 10⁵ cfu of the VS test strains. At 5 min after challenge with strain J30, blood of nonvaccinated and vaccinated rabbits had similar numbers of colony-forming units per milliliter (table 2). At 30 min after challenge, the size of the bacteremia was not significantly different (2 ± 2 and 2 ± 1 for nonvaccinated and vaccinated rabbits, respectively). The number of culture-positive vegetations and the number of adherent colony-forming units also were similar in nonvaccinated and vaccinated rabbits (table 2). Thus, vaccination did not influence the number of circulating bacteria shortly after challenge or the initial adherence of circulating VS to vegetations.

**Development of experimental IE in nonvaccinated and vaccinated rabbits in 2 experimental IE models.** In the nonvaccinated rabbits with the catheter in place or in animals from which the catheter had been removed, only 2 of 22 and 0 of 8, respectively, developed IE due to strain J30 (table 3). Conversely, 10 of 12 vaccinated rabbits with the catheter in place (*P* < .001, vs. nonvaccinated rabbits) and 4 of 6 from which the catheter had been removed (*P* = .015, vs. nonvaccinated rabbits) developed IE due to strain J30. Thus, vaccination apparently rendered the rabbits susceptible to IE due to this releasatesusceptible VS strain, regardless of the presence of the catheter during challenge.

**Table 1.** Microbicidal activity of releasates from thrombin-activated human and rabbit platelets supplemented with serum from rabbits vaccinated with sonicate supernatants of human platelets against *Bacillus subtilis* ATCC 6633 and *Streptococcus oralis* J30.

<table>
<thead>
<tr>
<th>Releasates supplemented with DMEM-C or serum</th>
<th>Human platelet release, mean reductionb (%) of 10⁴ cfu of</th>
<th>Rabbit platelet release, mean reductionb (%) of 10⁴ cfu of</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-C</td>
<td>&gt;99.9 ± 4</td>
<td>&gt;99.9 ± 4</td>
</tr>
<tr>
<td>Preimmune serum</td>
<td>&gt;99.9 ± 4</td>
<td>&gt;99.9 ± 4</td>
</tr>
<tr>
<td>Serum after vaccination 1</td>
<td>&gt;99.9 ± 4</td>
<td>&gt;99.9 ± 4</td>
</tr>
<tr>
<td>Serum after vaccination 2</td>
<td>&gt;99.9 ± 4</td>
<td>&gt;99.9 ± 4</td>
</tr>
<tr>
<td>Serum after vaccination 3</td>
<td>28 ± 4 ± 5</td>
<td>25 ± 3 ± 3</td>
</tr>
<tr>
<td>Serum after vaccination 4</td>
<td>14 ± 3 ± 4</td>
<td>11 ± 2 ± 3</td>
</tr>
</tbody>
</table>

* Aliquots (0.3 mL) of releasates were supplemented with 0.3 mL of Dulbecco’s modified Eagle medium (DMEM)-C or indicated serum and were incubated at 37°C for 30 min, before assessment of microbicidal activity against *B. subtilis* and *S. oralis* J30.

b Percentage ± SD reduction after exposure for 30 min at 37°C.
Table 2. No. of colony-forming units of viridans streptococci per milliliter of blood, number of culture-positive vegetations, and no. of colony-forming units per vegetation 5 min after intravenous challenge of nonvaccinated and vaccinated rabbits with the catheter in place or removed 24 h before challenge.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Streptococcus test strain</th>
<th>S. oralis 330 (releasate susceptible)</th>
<th>S. sanguis U108 (releasate resistant)</th>
<th>S. mitis S224 (dx', releasate susceptible)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catheter in place, inoculum size 10^4 cfu</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonvaccinated Blood, cfu/mL (range)</td>
<td>5 (2–12)</td>
<td>1 (0.2–5)</td>
<td>5 (3–7)</td>
<td></td>
</tr>
<tr>
<td>No. of culture-positive vegetations/no. of</td>
<td>19/25 (76)</td>
<td>4/18 (22)</td>
<td>8/9 (89)</td>
<td></td>
</tr>
<tr>
<td>vegetations evaluated (%)</td>
<td>12 (1–38)</td>
<td>4 (3–5)</td>
<td>2 (1–4)</td>
<td></td>
</tr>
<tr>
<td>Vaccinated Blood, cfu/mL (range)</td>
<td>5 (3–14)</td>
<td>2 (0.2–3)</td>
<td>5 (2–7)</td>
<td></td>
</tr>
<tr>
<td>No. of culture-positive vegetations/no. of</td>
<td>13/18 (72)</td>
<td>3/18 (17)</td>
<td>7/8 (88)</td>
<td></td>
</tr>
<tr>
<td>vegetations evaluated (%)</td>
<td>4 (1–12)</td>
<td>4 (2–7)</td>
<td>3 (1–7)</td>
<td></td>
</tr>
<tr>
<td>Catheter removed, inoculum size 10^5 cfu</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonvaccinated Blood, cfu/mL (range)</td>
<td>34 (21–43)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>No. of culture-positive vegetations/no. of</td>
<td>6/6 (100)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>vegetations evaluated (%)</td>
<td>41 (6–130)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Vaccinated Blood, cfu/mL (range)</td>
<td>30 (22–40)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>No. of culture-positive vegetations/no. of</td>
<td>6/7 (86)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>vegetations evaluated (%)</td>
<td>37 (16–80)</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. ND, not done; dx’, dextran negative.

The incidence of IE due to releasate-resistant strain U108 was not significantly different in nonvaccinated (1 of 18) and vaccinated rabbits (2 of 8) with the catheter in place, nor in nonvaccinated (3 of 10) and vaccinated rabbits (3 of 8) from which the catheter was removed (table 3). This illustrates that vaccination as such did not alter the susceptibility of the rabbits for IE [5].

We used a limited number of vaccinated rabbits in an additional experiment with releasate-susceptible S. mitis S224. Catheters were left in place during challenge. The incidence of IE was significantly higher among vaccinated rabbits (7 of 10) than among nonvaccinated rabbits (1 of 8; P = .07; table 3).

There was no difference in the weights of vegetations harvested from vaccinated and nonvaccinated rabbits 48 h after challenge in any experiment. Mean bacterial densities in infected vegetations were 10^5–10^10 cfu/g vegetation and were not significantly different in nonvaccinated and vaccinated rabbits (data not shown).

Discussion

Microbicidal proteins released by platelets after thrombin stimulation are thought to be an important innate host defense factor against experimental IE due to VS in rabbits [3, 17]. These platelet proteins probably are responsible for the clearance of adherent VS from vegetations within 2 h after adherence in the standard rabbit experimental IE model [3]. In the same experimental IE model, Sullam et al. [34] observed that vegetations of thrombocytopenic rabbits were colonized with more VS than were nonthrombocytopenic controls. In addition, an S. aureus mutant strain with reduced susceptibility to rabbit PMPs proliferated to higher densities in vegetations and, in contrast to the susceptible parent strain, also was cultured from kidneys and spleens after hematogenous dissemination [35].

In this study, the proportion of VS isolates resistant to human platelet microbicidal activity was much higher among isolates from blood of patients with IE than from their gingival sulcus, in VS blood and gingival sulcus isolates from neutropenic bacteremic patients without IE, and of VS isolated from the gingival sulcus of patients with non-IE cardiac disease at risk for IE. This selective distribution of resistance to platelet microbicidal activity suggests that resistant VS have a selective advantage after gaining access to the bloodstream and adherence to vegetations. Apparently resistant VS can persist after adherence, propagate, and finally cause IE. The finding that a high proportion of VS isolates from the blood of patients with IE are resistant to platelet releasate is in accordance with our observation that VS resistant to platelet microbicidal activity are not cleared from vegetations in the rabbit experimental IE model [3].

The species distribution of the IE-causing VS isolates also highlights the selectivity for platelet releasate resistance. S. sanguis represented only 13% of all 103 VS sulcus isolates and 5% of the blood isolates of bacteremic patients without IE. In contrast, this species, which appeared to be intrinsically resistant to platelet microbicidal activity, accounted for 29% of the 73 bacteremic VS blood isolates from patients with IE (P =
S. mutans, S. intermedius, and the bacteremic isolates of this species from patients without IE were susceptible to platelet microbicidal activity, whereas most gingival sulcus isolates of these species were resistant. Finally, IE were susceptible to platelet microbicidal activity, whereas most gingival sulcus isolates of these species were resistant. A similar selectivity for reduced susceptibility to platelet microbicidal activity suggested that resistant to platelet microbicidal activity suggested that resistance to platelet-release microbial activity may be a VS trait that contributes to their ability to cause IE. To test this hypothesis, we attempted to induce antibodies neutralizing the rabbit platelet microbicidal activity to subsequently test the susceptibility of these rabbits to IE due to VS. Human platelets contain 10 antimicrobial proteins (TCs) [18, 19], and rabbit platelets contain 7 different PMPs [21]. Because of the high numbers of microbial proteins in human and rabbit platelets and because there are insufficient quantities of individual proteins to prepare separate vaccines, rabbits were vaccinated with supernatants of sonicated human platelets containing the antimicrobial peptides. Sonicate supernatants and releasates from thrombin-stimulated platelets had similar microbicidal activity. Releasates from thrombin-stimulated platelets were not used as the antigen to avoid induction of anti–thrombin antibodies [30], which might influence the development of IE by interfering with coagulation and formation of vegetations.

The rabbit immune serum obtained after 4 vaccinations neutralized the microbicidal activity released from human and rabbit platelets. A number of studies have shown that platelets of various mammalian species are capable of releasing microbial activity, often related to cationic proteins [3, 17, 19–21, 39–43]. The serum from the vaccinated rabbits in our study contained antibodies against the majority of the proteins in the human platelet sonicate that had been used as the antigen and also against purified TC-1 and TC-2. TC-1 and TC-2 are the major cationic microbial proteins in human platelets [18, 19]. Since the rabbit immune serum neutralized the microbicidal activity of human and rabbit platelet releasates, it may well be that antibodies raised against the human TCs neutralized the equivalent rabbit microbial proteins. Of note, the antibodies raised against other constituents in the platelet sonicate also may have contributed to the neutralization of the platelet microbicidal activity.

In the rabbit experimental IE model with the catheter left in place during challenge, vaccination with pooled human platelet sonicate supernatants rendered the rabbits more susceptible to IE due to the releasate-susceptible strains J30 and S224. Even in a low susceptibility IE rabbit model [24], where the catheter was removed before challenge with 10^4 cfu of strain J30, vaccination enhanced the susceptibility for IE. The enhanced susceptibility to IE of the vaccinated rabbits was not due to an increased adherence to or growth of VS at the vegetations, nor were the magnitude and duration of bacteremia or the size of the vegetations different in vaccinated and nonvaccinated rabbits. The susceptibility of the rabbits for IE due to the platelet releasate resistant strain U108 was not altered by the vaccination. Collectively, these data show that neutralization of platelet microbicidal activity substantially enhanced the susceptibility of rabbits for IE due to the platelet releasate-susceptible VS, even in the absence of the catheter during challenge.

Although a role for platelet microbicidal activity in the pathogenesis of experimental IE has been suggested [3, 17, 28, 34, 35], our study is the first to present in vivo experimental evidence for such a role. In humans, platelet microbicidal activity also may be a relevant factor in the innate host defense against IE. In the general population, the presence of cardiac vegetations is not rare. Vegetations were found in 2.4% of 3404 autopsies of hospitalized patients without IE [44]. The incidence of low-level transient bacteremia after daily activities such as chewing candy and eating (17%–51%) [1, 45] or tooth brushing (7%–50%) [10], is high. However, the incidence of IE is low (0.68–6.5 cases/100,000 person-years in the general population [1, 46] and 20–180/100,000 person years in patients with cardiac diseases at risk for IE [47]). Thus, only a small proportion of the persons at risk actually develop IE. Assuming that during daily low-level bacteremias

<table>
<thead>
<tr>
<th>Streptococcus test strain</th>
<th>Percentage of survival of test strains in rabbit platelet releasate in vitro</th>
<th>No. of colonized vegetations/no. vegetations evaluated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Catheter in place, inoculum size 10^8 cfu</td>
<td>Catheter removed, inoculum size 10^8 cfu</td>
</tr>
<tr>
<td></td>
<td>Nonvaccinated</td>
<td>Vaccinated</td>
</tr>
<tr>
<td>S. oralis 330 (releasate susceptible)</td>
<td>50</td>
<td>2/22 (9)</td>
</tr>
<tr>
<td>S. sanguis U108 (releasate resistant)</td>
<td>90</td>
<td>1/18 (6)</td>
</tr>
<tr>
<td>S. mitis S224 (dx, releasate susceptible)</td>
<td>30</td>
<td>1/8 (12.5)</td>
</tr>
</tbody>
</table>

NOTE. Vaccinated rabbits had received 4 intramuscular injections with 1 mL of pooled human platelet sonicate supernatant. ND, not determined; dx, dextran negative.

* Nonvaccinated vs. vaccinated rabbits.
VS will frequently adhere to the vegetations of the majority of the persons at risk for IE, a mechanism must exist that usually clears the adherent VS from vegetations.

We hypothesize that, much like in experimental IE, this clearance is due to the platelet microbialic activity. This hypothesis predicts that VS causing IE are less susceptible to platelet microbialic activity. Indeed, the vast majority of VS blood isolates from patients with IE were resistant to the cidal activity of a standard platelet releasate in contrast to VS blood isolates from bacteremic patients without IE and oral VS isolates, which were almost all susceptible. Therefore, the microbialic activity of blood platelets may explain, at least in part, why IE is rare.

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