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Involvement of Bactericidal Factors from Thrombin-Stimulated Platelets in Clearance of Adherent Viridans Streptococci in Experimental Infective Endocarditis

J. DANKERT,[1]* J. vAN DER WERFF,[2] S. A. J. ZAAT,[1] W. JOLDERSMA,[2] D. KLEIN,[2] AND J. HESS[3]

Department of Medical Microbiology, University of Amsterdam, 1105 AZ Amsterdam,[1] Department of Hospital Epidemiology, University Hospital Groningen, 9700 RB Groningen,[2] and Department of Pediatric Cardiology, Sophia Children's Hospital, 3038 GE Rotterdam,[3] The Netherlands

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Platelets activated with thrombin release bactericidal factors. We studied the role of the susceptibility of viridans streptococci to these bactericidal factors in the development of infective endocarditis (IE). By using the experimental endocarditis rabbit model, the initial adherence and the development of IE were assessed for 10 viridans streptococcal strains differing in their susceptibilities to releasate (material released) from thrombin-activated platelets. Six strains were susceptible and four strains were resistant to these releasates. The numbers of vegetations (VGs) colonized at 5 min and 48 h after intravenous challenge with 10^4 CFU were determined. At 5 min after challenge, significantly more VGs were colonized with bacteria of the six platelet releasate-susceptible strains than with bacteria of the four releasate-resistant strains ($P < 0.005$). In the releasate-susceptible group of strains, the number of colonized VGs decreased significantly between 5 min and 48 h after intravenous inoculation ($P < 0.001$). Such a decrease was not observed with the releasate-resistant strains. As a result, the final developments of IE due to releasate-susceptible and -resistant strains were not significantly different. The releasate-susceptible strain 1 and the releasate-resistant strain 2 were selected for more detailed experiments. Rabbits were killed at 5 and 30 min and 2, 4, and 48 h after inoculation. The number of culture-positive VGs as well as the number of adherent bacteria on the individual VGs were determined. The 90% infective dose for each strain was 10^5 CFU. At low inoculum concentrations (10^3 and 10^4 CFU) a larger proportion of the inoculated bacteria of both strains was found to be adherent on VGs than at higher challenge doses. The number of culture-positive VGs as well as the number of adherent bacteria per VG decreased rapidly in the first 30 min after challenge with strain 1 but not after challenge with strain 2. Additional experiments with the platelet releasate-susceptible strain S224 and the platelet releasate-resistant strain S182 confirmed the data obtained with strains 1 and 2 and indicated that releasate-susceptible strains disappeared from the VGs with time, whereas releasate-resistant strains persisted. In vitro studies with VGs excised 5 min after challenge with strain 1 or 2 showed that clearance of the releasate-susceptible strain 1 was not caused by complement bactericidal activity or surface phagocytosis by polymorphonuclear cells. Bacterial cells of strain 1 adherent on excised VGs were rapidly cleared by exposure to fresh clotting blood or to releasates from thrombin-stimulated platelet suspensions. In contrast, strain 2 bacteria adherent on VGs were hardly affected by these treatments. These data strongly indicate that bactericidal factors released from platelets upon thrombin stimulation are involved in the clearance of bacteria early after their adherence to VGs. Therefore, development of IE is the combined result of the abilities of viridans streptococci to adhere to VGs and to resist the activity of platelet-released bactericidal factors.

The ability of circulating bacteria to adhere to preexisting cardiac vegetations (VGs) at the damaged surface of endothelium (1) and the ability of bacteria to survive after adherence to VGs are major determinants in the pathogenesis of infective endocarditis (IE) (2, 14, 18, 34). In vitro studies have demonstrated a relationship between the ability of bacteria to adhere to isolated strips of heart valves (20), damaged heart valves (32), and artificial fibrin-platelet layers (35) and the capacity of bacteria to induce IE. Experimental IE studies revealed that viridans streptococci adhered to the surfaces of preformed VGs within 15 min (8, 12, 26, 27, 31) but disappeared with time (8, 31).

The mechanisms involved in the clearance of adherent bacteria from VGs are presently unknown. Possible mechanisms are complement-dependent bactericidal activity (13), phagocytosis by polymorphonuclear cells (PMNs) (30), and detachment, although bacteria adhere tightly to artificial fibrin-platelet layers (27, 31, 35). Alternatively, clearance might be caused by antimicrobial factors derived from thrombin-stimulated platelets. Such factors have been reported by several authors and are designated β-lysins (9, 25), platelet bactericidal protein (45), thrombodefensins (7), and platelet microbicidal protein (48). Although these platelet-derived factors with antimicrobial activity are not necessarily identical, they share several important characteristics. They have low molecular weights, are cationic and heat stable, and require thrombin to be released from the platelet alpha-granules (7).

Morphologic studies of VGs early after intravenous injection of bacteria have shown that bacterial deposition onto VGs is followed by rapid accumulation of platelets and fibrin at the surfaces of VGs (2, 16, 21, 33). The fibrin deposition indicates thrombin activity at the site of the VG. Therefore, platelet

* Corresponding author. Mailing address: Dept. Medical Microbiology, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Phone: 31-20-5664861. Fax: 31-20-6979271.

TABLE 1. Viridans streptococcal strains, their initial adherence to VGs, and development of IE after 48 h in rabbits

Strain	Species[a]	Origin	Dextran in MH broth ($\mu g/10^9$ CFU)	% Survival in releasate	No. of colonized VGs/ total no. of VGs after:	
					5 min	48 h
Group 1						
S267	*S. mitior* dx+	Oral	31.3	10	1/8	0/6
S294	*S. mitior* dx+	Blood (IE)	26.1	30	5/9	1/7
S224	*S. mitior* dx−	Blood (IE)	13.5	30	8/9	2/8
NCTC7864	*S. sanguis*		17.0	30	5/8	3/9
S189	*S. sanguis* II	Blood (non-IE)	24.8	40	8/8	5/7
J30 (strain 1)	*S. sanguis* II	Oral	25.2	50	10/11	0/9
Total					37/53[b]	11/46[c]
Group 2						
U108 (strain 2)	*S. sanguis* II	Blood (IE)	13.0	90	3/10	1/7
J274	*S. sanguis* II	Oral	22.4	90	6/17	2/8
S182	*S. mutans*	Blood (IE)	22.6	100	2/8	2/8
S277	*S. mitior* dx+	Blood (IE)	13.2	100	4/9	6/7
Total					15/44	10/30[d]

[a] dx+, dextran producing; dx−, dextran negative.
[b] $P < 0.005$ versus totals for group 2 strains after 5 min by chi-square test with Yates correction.
[c] $P < 0.001$ versus totals for group 1 strains after 5 min by chi-square test with Yates correction.
[d] No significant difference versus totals for group 2 strains after 5 min or versus those for group 1 strains after 48 h.

alpha-granule release will also be induced at the VG surface, and the released bactericidal factors may well play an important role in the removal of bacteria adherent on VGs.

To study the role of platelet-released bactericidal factors in the pathogenesis of experimental endocarditis, we have analyzed the initial colonization of VGs, the persistence of adherent bacteria, and final development of IE in the rabbit model, using viridans streptococcal strains differing in their susceptibilities to releasate (material released) from thrombin-activated platelets.

MATERIALS AND METHODS

Test organisms. From a collection of 81 viridans streptococcal isolates (7), 10 strains were selected on the basis of their susceptibilities to bactericidal factors released from thrombin-stimulated platelets (Table 1). *Streptococcus sanguis* type II strains 1 and 2 were used for most of the more detailed studies. Both strains have been used in earlier experimental IE studies (8, 15, 22). *Micrococcus lysodeikticus* was obtained from the National Institute of Public Health and Environmental Hygiene (Bilthoven, The Netherlands).

Strains were stored in skim milk (Oxoid, Unipath Ltd., Basingstoke, England) at −20°C and maintained on sheep blood (5%, vol/vol) agar (Oxoid) plates at 4°C for one week. For each experiment bacteria were freshly grown in Mueller-Hinton (MH) broth (pH 7.4) (Difco Laboratories, Detroit, Mich.) at 90 rpm for 24 h without aeration. Numbers of CFU were routinely determined by plating appropriate dilutions on sheep agar plates and counting after incubation in 10% CO_2 at 37°C for 48 h. Doubling times for strains 1 and 2 in MH broth were 32 and 52 min, respectively. For strains S224 and S182, doubling times in MH broth were 46 and 48 min, respectively. *M. lysodeikticus* was grown in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 24 h in a rotary shaker-incubator (New Brunswick Scientific Co., Inc., Edison, N.J.).

Dextran assay. The abilities of the strains to produce dextran were routinely evaluated after growth of the bacteria on mitis salivarius agar (Oxoid Ltd.). Cell-associated dextran from bacteria grown in MH broth (without added sucrose) for 24 h was quantified with a tryptophan assay (6). After the reaction, the optical densities at 500 nm of the test samples were compared with values for a dilution series of commercial dextran 250 (Sigma Chemical Co., St. Louis, Mo.) and expressed as micrograms of dextran per 10^9 CFU.

LTA extraction and quantification. Whole cells of strains 1 and 2 from stationary-phase cultures (4 h after cessation of exponential growth) were harvested by centrifugation (10,000 × *g*; 15 min; 4°C). Freeze-dried cells (10 g [dry weight]) were extracted with 50% phenol in water at 68°C (46). The aqueous phases were dialyzed against distilled water. Lipoteichoic acid (LTA) was isolated from the crude extract by using Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, Calif.), purified by using phosphatidyl choline vesicles (38), and lyophilized. After reconstitution in an appropriate volume of distilled water, portions were quantitatively analyzed. Analysis was carried out by an enzyme-linked immunosorbent assay (4) with LTA as the antigen and rabbit antiserum raised against LTA of *Lactobacillus casei* (kindly provided by K. W. Knox and A. J. Wicken, Sydney, Australia).

Preparation of bacterial suspensions. After centrifugation (4,000 × *g*; 10 min; 4°C), bacteria were washed three times with PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl; pH 7.2) and resuspended in 0.9% (wt/vol) NaCl. Suspensions were aspirated and ejected twice through a sterile 25-gauge steel needle and then filtered through an 8-μm (pore-size) membrane filter (type SC; Millipore Corp., Bedford, Mass.) to remove bacterial aggregates. Suspensions were adjusted to an optical density at 540 nm of 1.0 (model 24 spectrophotometer; Beckman Instruments Inc., Palo Alto, Calif.) with 0.9% NaCl and contained approximately 10^9 CFU/ml. These standardized suspensions were diluted in 0.9% NaCl to the appropriate inocula as noted for individual experiments. In each experiment the inocula were checked by serial plating.

Rabbit model of nonbacterial thrombotic VGs. Left-sided nonbacterial thrombotic 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 (22).

Production and evaluation of bacterial adherence, colonization, and infection. Twenty-four hours after introduction of the catheter, rabbits were injected in a marginal ear vein with 1 ml of bacterial inoculum in 0.9% NaCl. The catheter was left in place during the challenge. Rabbits were sacrificed by intravenous injection of pentobarbitone at 5 or 30 min or 2, 4, or 48 h after injection with the test organisms. The heart was removed, and the VG was excised immediately with aseptic precautions and subsequently rinsed in three washes of 5 ml of 0.9% NaCl to remove nonadherent bacteria. In order to assess detachment of bacteria adherent on VGs due to the rinsing, the total volume of the washings of each VG was cultured quantitatively by preparing pour plates.

Immediately after the rinsing, VGs were weighed and homogenized in 1 ml of 0.9% NaCl with Teflon tissue grinders (Tamson, Zoetermeer, The Netherlands). The tissue grinder and the tube used to homogenize VGs removed at 5 or 30 min or 2 h after challenge were rinsed with 0.5 ml of 0.9% NaCl. The rinse fluid was pipetted in 0.1-ml portions onto sheep blood agar plates. The total homogenate, either undiluted (5- and 30-min and 2-h VGs) or appropriately diluted (4- and 48-h VGs) was plated onto sheep blood agar plates (22). CFU were counted after incubation in 10% CO_2 at 37°C for 48 h. The number of bacteria recovered from a VG represents the sum of CFU cultured from the homogenate and from the rinse fluid of the tissue grinder system. The method effectively detected low numbers of bacteria in homogenates. Culturing of the homogenates of excised sterile VGs (mean weight, 57 ± 36 mg) from unchallenged rabbits, which were spiked with 10^2 CFU, and rinse fluids of the tissue grinder yielded 93% ± 6% of the original inoculum. Numbers of bacteria per VG at 48 h are given as $\log_{10}$ CFU per gram of VG. If the undiluted homogenate (representing approximately 90% of the original VG) showed no growth, the VG was counted as sterile.

Because the weights of VGs and the inoculum sizes varied, results were also expressed as an adherence ratio, defined as the number of CFU adherent to 1 g

of VG divided by the original inoculum (CFU per milliliter) and multiplied by 10^4. IE was defined by positive cultures of VGs at 48 h.

Blood cultures. Blood (5 ml) was drawn from the right ventricle immediately after killing of the rabbits, using syringes containing 0.1 ml of 1% (wt/vol) sodium polyanethol sulfonate (SPS) ("liquoid"; Hoffmann-La Roche, Basel, Switzerland) to prevent clotting. For each blood sample, five pour plates containing 1 ml of blood and 20 ml of glucose agar broth (Oxoid) were made. Numbers of CFU were counted after incubation in 10% CO_2 at 37°C for 48 h. Bacteremia was expressed as the number of CFU per milliliter of blood.

Serum bactericidal activity. Strain 1 or strain 2 (10^3 CFU) was incubated in 1.5 ml of fresh whole rabbit serum at 37°C for 24 h (with shaking). Duplicate 0.1-ml samples were drawn at 0, 5, and 30 min and 2, 4, 16, and 24 h. The number of CFU was determined by plate counting.

VGs, excised at 5 min after challenge of rabbits with 10^5 CFU of strain 1 or strain 2, were rinsed and incubated in 3 ml of fresh whole rabbit serum at 37°C for 30 min or 2 h (with shaking). Numbers of bacteria in the homogenates were determined.

Isolation of rabbit leukocytes. Blood of healthy New Zealand White rabbits drawn into syringes containing heparin (final amount, 10 U/ml) was allowed to settle by gravity in 0.9% NaCl containing 6% dextran (molecular weight, 200,000; Pharmacia Fine Chemicals AB, Uppsala, Sweden) for 45 min at 37°C. The leukocyte-rich plasma was withdrawn and centrifuged ($500 \times g$; 10 min). Residual erythrocytes in the pellet were lysed with ice-cold 0.832% (wt/vol) NH_4Cl–0.084% (wt/vol) $NaHCO_3$–43.2 mg of EDTA per liter of sterile water (44). After centrifugation ($500 \times g$; 5 min), leukocytes were washed twice with Hanks' balanced salt solution with 0.1% (wt/vol) gelatin (Gel-HBSS). Leukocytes were adjusted to a concentration of 10^7/ml of Gel-HBSS. The viability of leukocytes was always greater then 95% as determined by trypan blue exclusion. Leukocytes were always more than 96% PMNs.

Phagocytosis of *S. sanguis* 1 and 2 in vitro by rabbit PMNs. Bacteria (10^6 CFU in 0.5 ml of Gel-HBSS) were added to 0.5 ml of Gel-HBSS containing 2×10^6 PMN and supplemented with 10% (vol/vol) fresh pooled rabbit serum as a source of complement. The reaction mixtures were incubated under rotation (30 rpm) at 37°C for 30 min. Phagocytosis was stopped with 9 ml of ice-cold deionized water followed by mixing for 30 s with a Whirlimixer (Cenco, Breda, The Netherlands). The number of viable bacteria was determined. Each strain was tested in five independent experiments.

To assess phagocytosis of bacteria adherent on VGs in vitro, VGs were removed from the hearts of rabbits 5 min after challenge with 10^5 CFU. These VGs were incubated in 1 ml of Gel-HBSS supplemented with 10% (vol/vol) fresh pooled rabbit serum, either with or without 2×10^6 rabbit PMNs, for 30 min at 37°C with shaking. After incubation, the VGs were rinsed twice in 10 ml of ice-cold 0.9% NaCl to stop phagocytosis. VGs were homogenized in 1 ml of deionized water to facilitate osmotic disruption of PMNs, and numbers of CFU were determined.

To determine whether strain 1 in vivo was entrapped by leukocytes present in the VGs, VGs were excised from rabbits killed 30 min after challenge with 10^5 CFU and were incubated in 1 ml of Gel-HBSS containing 50 U of penicillin G (PPG) and 25 μg of streptomycin (SM) (both from Brocades Pharma, Delft, The Netherlands) and 2 mg of phenylbutazone (Geigy, Basel, Switzerland). Extracellular bacteria are rapidly killed by PPG-SM (22), whereas phenylbutazone prevents killing of phagocytized bacteria by the PMNs (39). VGs were rinsed with saline containing 100 U of penicillinase per ml (Mycofarm, Delft, The Netherlands) and homogenized. Numbers of CFU were determined on penicillinase-containing (50 U/ml) blood agar plates.

Preparation of fresh platelet suspensions. Blood (9 ml) of healthy New Zealand White rabbits was collected into siliconized glass tubes (Vacutainer Systems, Becton Dickinson, Meylan, France) containing 1 ml of 3.8% buffered citrate solution (0.11 M sodium citrate · $2H_2O$ and 0.002 M citric acid · H_2O, pH 5.5), and centrifuged at $225 \times g$ for 20 min at 37°C. The upper two-thirds of the supernatant was recentrifuged. Subsequently the upper three-fourths of the supernatant was carefully withdrawn, and platelets were pelleted ($2,000 \times g$; 10 min; 37°C) and separated from the supernatant (to give platelet-poor plasma [PPP]). The platelet pellet was washed twice in Dulbecco modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) containing 10 mM (final concentration) sodium citrate (pH 7.2) (citrated medium [CM]) and resuspended either in CM or in PPP to prepare platelet-rich plasma (PRP) at final concentrations of 2×10^8 to 4×10^8 platelets per ml. Suspensions were maintained at 37°C and used within 2 h. Siliconized glassware was used throughout all platelet-handling procedures.

Exposure of *S. sanguis* 1 and 2 adherent on VGs to clotting blood or platelet suspensions. VGs excised from rabbits killed 5 min after injection with 10^5 CFU of strain 1 or strain 2 were added to 1 ml of fresh sterile rabbit blood, which was allowed to clot at 37°C for 30 min. For controls VGs were added to 1 ml of fresh sterile rabbit blood containing 0.1% (wt/vol) SPS to prevent clotting. In addition, VGs were added to 1 ml of PRP, and clotting was induced by adding human thrombin (2 U). The influence of thrombin on *S. sanguis* was tested by exposure of VGs to 1 ml of fresh whole rabbit serum supplemented with human thrombin (2 U). After incubation at 37°C for 30 min, the VGs and clots were homogenized, and CFU were determined.

Preparation of platelet releasates. Platelets (2×10^8 to 4×10^8 per ml of CM) were stimulated with human thrombin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands; final concentration, 1 NIH unit per ml) for 15 min at 37°C. The releasate of the platelets was collected after centrifugation ($2,000 \times g$; 10 min; 37°C) and used within 2 h. Supernatants of non-thrombin-stimulated platelets in CM were used as controls. In addition, PRP and PPP were treated with thrombin (1 U/ml; 15 min; 37°C). The resulting sera were designated PRP serum and PPP serum, respectively.

Bactericidal activity of platelet releasates. Bacteria were incubated in duplicate in 1.5 ml of each of three independent platelet releasates at a final concentration of 10^3 CFU/ml. Incubations in CM served as controls. Releasates supplemented with 0.1 to 10 μg of egg white lysozyme (Difco Laboratories) were used to test the effect of lysozyme on the test strains. All tests were carried out in siliconized glass tubes (Vacutainer Systems). Test suspensions were incubated in a moist 10% CO_2 atmosphere at 37°C. At 0, 5, 15, 30, and 60 min, aliquots of 0.3 ml were transferred to 0.7 ml of PBS containing 0.01% (wt/vol) SPS ("liquoid"), which immediately abolishes the bactericidal activity of the platelet releasates (7). The viability of the test organisms was not affected by incubation in PBS with up to 0.2% (wt/vol) SPS for 30 min. After incubation, the samples were treated ultrasonically (50 kHz) (Bransonic 32; Bransonic Power Co., Danbury, Conn.) for 60 s to disperse bacterial clumps (5), and numbers of CFU were determined. The ultrasonic treatment did not affect the viability of the test strains. The number of CFU before incubation (at 0 min) was used to calculate the decrease or increase of CFU.

Lysozyme activity of platelet releasates. Brain heart infusion (BBL Microbiology Systems) agar plates were inoculated with 0.1 ml of PBS containing 10^8 CFU of *M. lysodeikticus*. Wells (5-mm diameter) were cut and filled with 0.3 ml of test sample. Lysozyme standard (Difco Laboratories) dissolved in Bacton lysozyme phosphate buffer (pH 6.2) (Difco Laboratories) or in CM at 0.1 to 10 μg/ml was used as a reference. Plates were read for lysis of *M. lysodeikticus* after 24 h at 37°C.

Statistical evaluation. The significance of differences between the numbers of culture-positive and culture-negative VGs was calculated with Fisher's exact test or the chi-square test with the Yates correction. The significance of differences between the numbers of bacteria adherent on VGs was determined by using the Wilcoxon rank sum test. The variances reported are standard deviations.

RESULTS

Susceptibility of viridans streptococci to bactericidal activity in releasates of thrombin-activated rabbit platelets. The numbers of surviving bacteria of 10 viridans streptococcal isolates exposed to thrombin-activated rabbit platelet releasates were monitored over time. Within 10 min of exposure, the maximum bactericidal effect of the releasates was observed. The susceptibilities of the isolates, determined as the level of killing after 30 min, varied between 0 and 90% (±5%). Strains were grouped according to their susceptibilities to platelet releasate, with group 1 comprising the six susceptible strains (10 to 50% survival) and group 2 comprising the four resistant strains (90 to 100% survival) (Table 1). Lysozyme activity in the releasates was compared with that of an egg-white lysozyme standard and was equivalent to less than 0.1 μg/ml. The viabilities of the isolates were not affected even in the presence of 5 μg of egg white lysozyme per ml.

All isolates except one had the ability to produce dextran when grown on mitis salivarius sucrose-containing agar. Inocula for all experiments were prepared from cultures grown in MH broth without added sucrose. In this medium, amounts of cell-adherent dextran were small, not significantly different among the test strains, and not correlated with susceptibilities to platelet releasate bactericidal activity (Table 1). Supplementation of MH broth with 5% sucrose increased the amount of cell-associated dextran of the dextran-positive strains 10- to 30-fold (data not shown).

Initial adherence and final development of experimental IE due to streptococci differing in susceptibility to platelet releasate. VGs from the hearts of rabbits challenged with 10^4 CFU of the 10 test strains were rinsed three times immediately after excision. The first rinse of VGs removed at 5 min postchallenge contained a relatively high number of bacteria, but the third rinse always was sterile. The percentage of colonized VGs at 5 min after challenge ranged from 12.5 to 100% for the different test strains. Statistical analysis of the summarized colonization data at 5 min and 48 h (Table 1) revealed (i) that in group 1

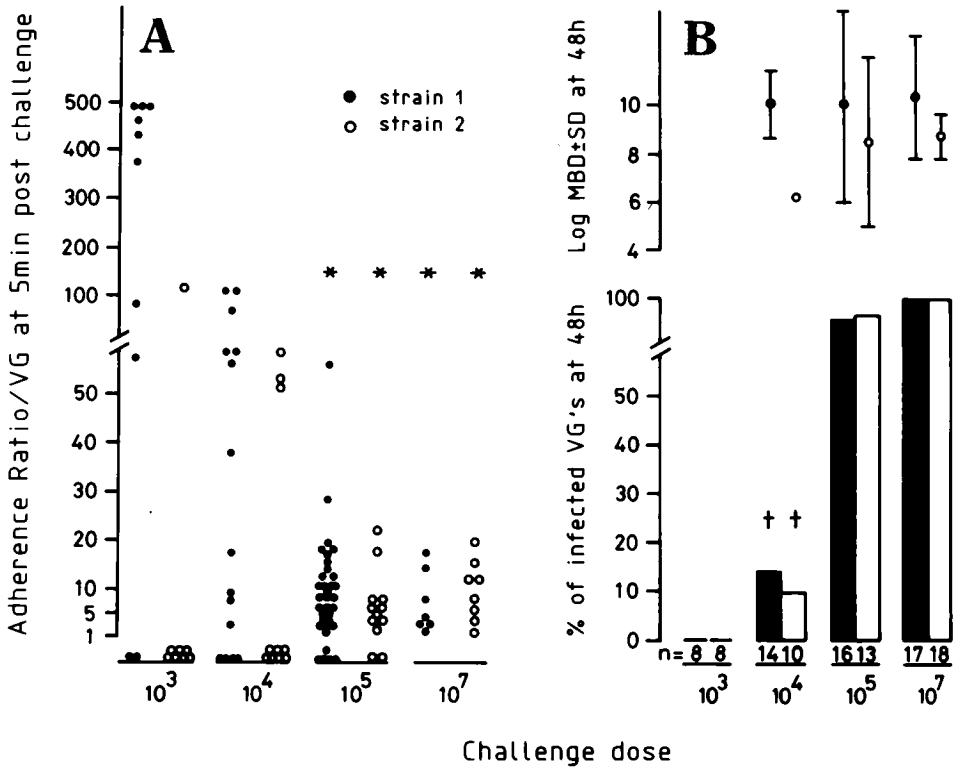

FIG. 1. Density of *S. sanguis* type II on VGs at 5 min after intravenous injection with various inoculum sizes (10^3, 10^4, 10^5, or 10^7 CFU) and development of endocarditis. Strain 1 or strain 2 was injected 24 h after insertion of left-heart catheters in rabbits. (A) The adherence ratio per VG at 5 min after challenge represents the absolute number of bacteria adherent to 1 g of VG divided by the original inoculum for each experiment and multiplied by 10^4. (B) In the lower part, the incidence of endocarditis (percent) due to strain 1 or strain 2 48 h after intravenous challenge with various inoculum sizes is shown. The numbers at the bottom of each column represent the numbers of rabbits injected. In the upper part, the $\log_{10}$ mean bacterial densities (MBD) (± SD [bars]) per gram of infected VG are shown. Asterisk, $P < 0.005$ compared with inoculum sizes of 10^3 and 10^4 by the Wilcoxon rank sum test; dagger, $P < 0.001$ compared with inoculum sizes 10^5 or 10^7 CFU by Fisher's exact test.

(platelet releasate-susceptible) strains significantly more VGs were colonized at 5 min after inoculation than in group 2 (releasate-resistant) strains ($P < 0.005$), (ii) that in group 1 the number of colonized VGs significantly decreased between 5 min and 48 h after inoculation ($P < 0.001$), and (iii) that in group 2 no such decrease was observed. As a result, the final developments of IE due to group 1 and 2 strains were not significantly different.

Adherence of *S. sanguis* II to VGs shortly after challenge and development of IE with various challenge doses. Strains 1 and 2 were selected as representative strains for groups 1 and 2 (Table 1), respectively. LTA preparations of both strains contained approximately 10 mg of LTA. An enzyme-linked immunosorbent assay using 1 mg of the preparations per ml detected LTA of strain 1 in dilutions of 1:10,240 and LTA of strain 2 in dilutions of 1:40,960. Rabbits injected intravenously with 10^3, 10^4, 10^5, or 10^7 CFU of these strains were killed at 5 min or 48 h after challenge. The mean weight of the excised VGs was 54 ± 22 mg, ranging from 26 to 186 mg. Quantitative cultures of blood samples at 5 min as well as at 48 h after challenge yielded similar numbers of circulating bacteria of either strain at the different inoculum concentrations. After injection of each inoculum, the respective median numbers of bacteria per milliliter of blood were 0.4 (range, 0.2 to 5), 2 (range, 0.4 to 13), 25 (range, 0.6 to 91), and 270 (range, 56 to 714). No correlation was found between the number of circulating bacteria and the number of adherent bacteria on VGs at 5 min after injection for any of the challenge doses (data not shown).

In order to express the results independent of variations in inoculum sizes and the sizes of the VGs, we determined the adherence ratio of the VGs for each experiment. The high adherence ratio of VGs at low inocula demonstrated that the proportion of the circulating bacteria adhering to VGs was significantly greater at low inocula than at high inocula ($P < 0.005$) (Fig. 1A). Despite the fact that 5 min after challenge the number of strain 1 bacteria adherent to VGs was significantly greater than that of strain 2 bacteria ($P < 0.005$), the development of endocarditis after 48 h was not different (Fig. 1B).

Number of culture-positive VGs over time after challenge with 10^4 or 10^5 CFU of *S. sanguis* II. The mean weights of VGs from rabbits injected with strain 1 or strain 2 were 44 ± 21 mg and 50 ± 17 mg, respectively. Quantitative cultures of blood samples collected at 5 min after challenge with 10^4 CFU yielded mean numbers of 3 ± 3 CFU/ml of blood in rabbits injected with strain 1 and of 2 ± 2 CFU/ml of blood in rabbits injected with strain 2. All blood cultures collected at 30 min, 2 h, and 4 h after challenge with 10^4 CFU were sterile. After injection with 10^5 CFU, at 5 and 30 min the mean number of CFU per milliliter of blood was 1 ± 1. Cultures from blood obtained at 2 and 4 h were sterile.

Strain 1 was recovered from the majority of VGs at 5 min after injection, irrespective of the challenge dose (Table 2). With time bacteria of strain 1 disappeared from VGs of rabbits infected with 10^4 CFU (Table 2). Strain 2, in contrast, adhered to only a few VGs after challenge with 10^4 CFU, but no significant decrease of the number of infected VGs over time was

TABLE 2. Number of culture-positive VGs at various times after intravenous challenge with *S. sanguis* type II strains 1 and 2 and incidence of IE

Strain	Inoculum (CFU)	No. of culture-positive VGs[a]/total no. of rabbits at:				No. of rabbits with IE/total no. of rabbits at 48 h[b]
		5 min	30 min	2 h	4 h	
1	1×10^4	19/25	13/33[c]	3/19[d]	2/14[e]	2/22[d]
	3×10^5	30/35	28/30	13/14	8/8	15/16
2	1×10^4	4/18[f]	2/15	3/8	1/10	1/18
	3×10^5	10/12	12/13	ND[g]	9/9	12/13

[a] Times after the bacterial injections.
[b] For each strain, $P < 0.001$ for 1×10^4 versus 3×10^5 CFU by Fisher's exact test.
[c] $P < 0.06$ versus value at 5 min by chi-square test with Yates correction.
[d] $P < 0.001$ versus value at 5 min by Fisher's exact test.
[e] $P = 0.001$ versus value at 5 min by Fisher's exact test.
[f] $P = 0.001$ versus value at 5 min after challenge with 3×10^5 CFU by Fisher's exact test.
[g] ND, not done.

observed (Table 2). The incidence of IE in rabbits challenged with 10^4 CFU of either strain was low. Only 2 of 22 (9%) and 1 of 18 (6%) rabbits injected with strains 1 and 2, respectively, had infected VGs. Conversely, approximately 90% of the rabbits injected with a 30-fold-higher inoculum dose (3×10^5 CFU) of either strain developed endocarditis.

The mean bacterial densities recovered from infected VGs at 48 h after challenge with 10^4 or 10^5 CFU were not different. Mean $\log_{10}$ values of 9.9 and 8.2 CFU per g of VG were obtained from culture of infected VGs of rabbits after challenge with strains 1 and 2, respectively.

Numbers of platelet releasate-susceptible and -resistant viridans streptococci adherent to VGs at different times after challenge. To investigate whether the decrease in the number of culture-positive VGs over time was also reflected in a decrease of the numbers of bacteria adherent to individual VGs, the numbers of CFU of the platelet releasate-susceptible *S. sanguis* type II strain 1 and the platelet releasate-resistant *S. sanguis* type II strain 2 on VGs excised from rabbits at different times after inoculation were determined. At an inoculum dose of 10^4 CFU, the number of bacteria of strain 1 adherent on VGs decreased within 30 min. The number of adherent bacteria at 5 min, ranging from 1 to 38 (median, 10), was significantly higher than the number at 30 min (median, 3; range, 1 to 14) ($P < 0.025$). Almost all VGs of rabbits injected with 10^4 CFU of strain 2 were culture negative. The few culture-positive VGs after challenge with strain 2 always had a low number of bacteria adherent on VGs (Fig. 2A).

After challenge with 3×10^5 CFU of strain 1, the numbers of bacteria adherent on VGs ranged from 3 to 63 (median, 13) at 5 min (Fig. 2B). After 30 min, a significant drop in the number of adherent bacteria was observed (median, 1; range, 1 to 77) ($P < 0.025$), after which growth of the still-adherent bacteria occurred, resulting in 10- and 2,500-fold multiplication after 2 and 4 h, respectively, compared with the numbers of bacteria adherent on VGs at 30 min.

In contrast to the case for strain 1, no reduction in the number of adherent strain 2 bacteria at 30 min after challenge was observed (Fig. 2B). The number of bacteria on VGs at 4 h after injection increased only 20-fold compared with the number of bacteria on VGs at 30 min after challenge. This is in accordance with the finding that strain 2 multiplies at a slower rate than strain 1 in MH broth. Thus, strain 1 adhered to VGs more readily than strain 2 but was (partially) cleared from the VGs early after challenge.

The experiments were partially repeated with the platelet

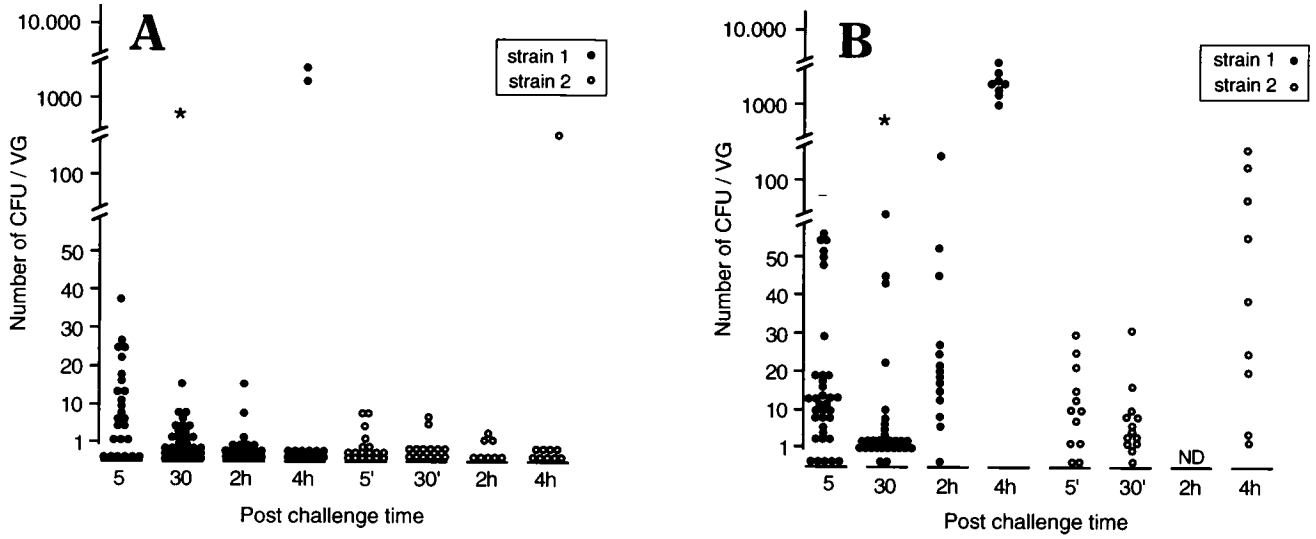

FIG. 2. Number of *S. sanguis* type II bacteria recovered from cardiac VGs induced by left-heart catheterization in rabbits. (A) Twenty-four hours after catheterization, rabbits were injected intravenously with 1×10^4 CFU (A) or 3×10^5 CFU (B) of strain 1 or strain 2. Each point represents the absolute number of bacteria adherent on the VG of a single rabbit early after challenge. Asterisk, $P < 0.025$ compared with the 5-min value by the Wilcoxon rank sum test; ND, not done.

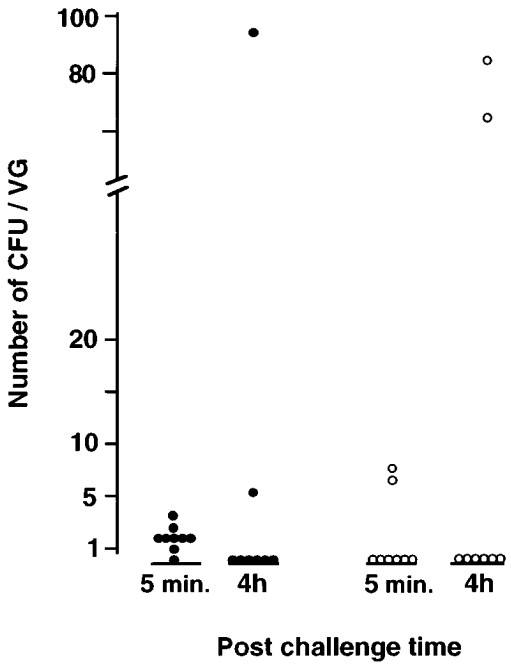

FIG. 3. Numbers of *S. mitior* S224 (●) and *S. mutans* S182 (○) bacteria recovered from VGs induced by left-heart catheterization in rabbits. Twenty-four hours after catheterization, rabbits were injected intravenously with 10^4 CFU. Each point represents the absolute number of bacteria adherent on the VG of a single rabbit.

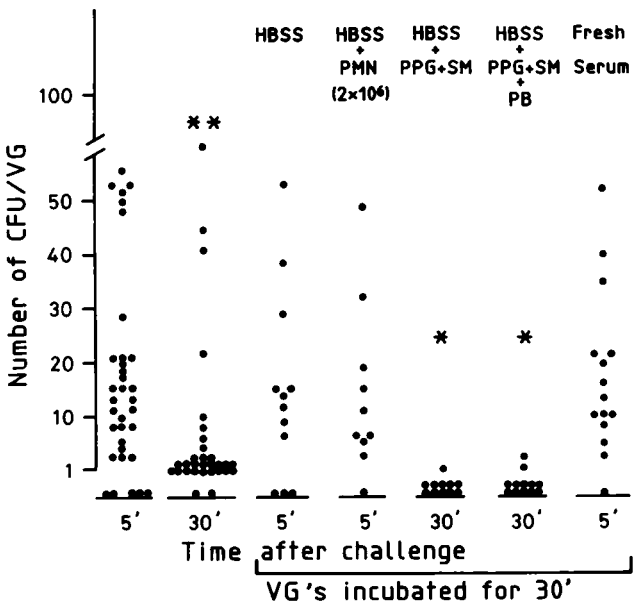

FIG. 4. Number of bacteria recovered from VGs 5 or 30 min after intravenous injection with 10^5 CFU of *S. sanguis* type II strain 1 immediately after removal (controls) or after subsequent incubation of VGs at 37°C for 30 min in Gel-HBSS containing 10% (vol/vol) fresh pooled rabbit serum (HBSS), in HBSS containing 2×10^6 rabbit PMNs to determine surface phagocytosis by PMNs, or in HBSS with 50 U of PPG and 25 μg of SM per ml or with 50 U of PPG, 25 μg of SM, and 2 mg of phenylbutazone (PB) per ml to assess phagocytosis and killing of adherent bacteria by PMNs entrapped in VGs. To study the effect of the complement-dependent serum bactericidal activity on the number of adherent bacteria, VGs excised at 5 min after bacterial challenge were incubated in fresh whole rabbit serum at 37°C for 30 min. Each point represents the absolute number of viable bacteria cultured from the VG. The Wilcoxon rank sum test was used to determine the significance of differences in numbers of bacteria on VGs. Asterisk, $P < 0.005$ compared with results for VGs excised at 5 min after challenge; double asterisks, $P < 0.025$ compared with results for VGs excised at 5 min after challenge.

releasate-susceptible strain *Streptococcus mitior* S224 (30% survival [Table 1]) and the releasate-resistant strain *Streptococcus mutans* S182 (100% survival [Table 1]). Rabbits inoculated with 10^4 CFU of either strain were sacrificed at 5 min and 4 h. The data shown in Fig. 3 match the data obtained with inocula of 10^4 CFU of *S. sanguis* type II strains 1 and 2 (Fig. 2A). The releasate-susceptible strains 1 and S224 initially colonized most of the VGs (5 min), although the numbers of CFU of strain S224 (1 to 4; median, 2) were lower than those of strain 1. Like strain 1 bacteria, bacteria of strain S224 disappeared from the VGs with time. At 4 h, six of eight VGs were sterile, whereas the numbers of CFU on culture-positive VGs had increased. The releasate-resistant strain S182 initially adhered to only two of eight VGs (6 and 7 CFU), but at 4 h two of eight VGs were still culture positive and the bacteria had multiplied (48 and 77 CFU) (Fig. 3).

Mechanisms causing disappearance of adherent bacteria. (i) Serum bactericidal activity. To verify that bacteria adherent to VGs are indeed cleared by bactericidal factors from platelets, an effect of serum bactericidal activity or phagocytosis had to be ruled out. No serum bactericidal activity against either strain in fresh whole rabbit serum was noted. The doubling times of each of the strains in fresh whole rabbit serum were correspondingly similar to those in MH broth. The number of bacteria of strain 1 or strain 2 adherent on VGs excised 5 min after challenge and subsequently incubated for 30 min in fresh whole rabbit serum did not decrease compared with the number of bacteria adherent on VGs at 5 min after challenge (Fig. 4). Homogenates prepared from VGs incubated for 2 h in fresh whole serum showed an increase in the number of CFU.

(ii) Role of PMNs. Bacteria of strains 1 and 2 incubated in rabbit PMN suspensions in Gel-HBSS at a bacterium/PMN ratio of 1:1 were phagocytized and killed. The number of viable bacteria of strains 1 and 2 decreased 44% ± 6% and 52% ± 8%, respectively, after 30 min of exposure to PMNs. Surface phagocytosis of strain 1 bacteria adherent on VGs by PMNs in Gel-HBSS, however, did not occur within 30 min, despite the low bacterium/PMN ratio (Fig. 4). No change in the number of bacteria adherent on VGs excised at 5 min after challenge and subsequently incubated in Gel-HBSS for 30 min compared with the number of bacteria adherent on VGs at 5 min after challenge was observed (Fig. 4). In addition, PMNs entrapped in VGs rarely contained bacteria, since cultures of almost all VGs excised 30 min after challenge and incubated in Gel-HBSS supplemented with PPG and SM or with PPG, SM, and phenylbutazone yielded no growth (Fig. 4).

(iii) Effect of thrombin-stimulated platelets on *S. sanguis* II strains 1 and 2 adherent on VGs in vitro. Since platelets constitute a major part of VGs and since they are known to secrete bactericidal substances after stimulation by thrombin (7, 48), we investigated the role of thrombin-activated platelets in the clearance of bacteria adherent on VGs. For this purpose, VGs excised from rabbits 5 min after challenge with strain 1 were rinsed and incubated in fresh clotting rabbit blood. Most VGs were sterilized within 30 min (Fig. 5). The number of bacteria recovered from culture-positive VGs after exposure to fresh clotting blood was significantly lower than the number of adherent bacteria on VGs excised at 5 min or the number of adherent bacteria on VGs incubated in fresh blood prevented from clotting by SPS ($P < 0.005$). The number of viable bacteria adherent on VGs was also reduced significantly in clotting

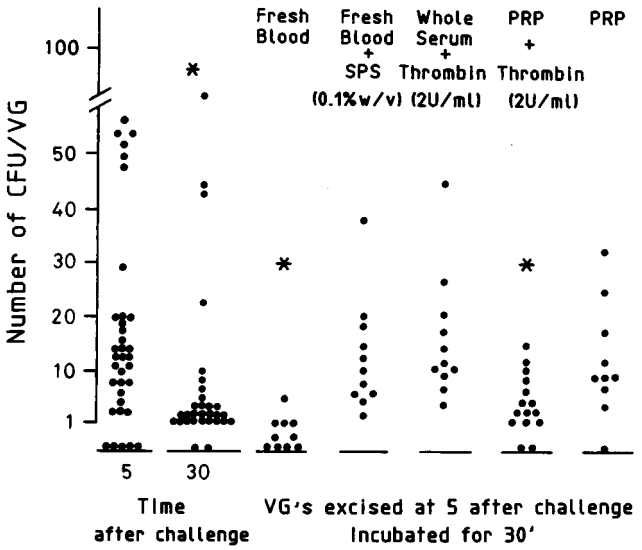

FIG. 5. Number of bacteria recovered from VGs excised 5 or 30 min after intravenous injection with 10^5 CFU of *S. sanguis* type II strain 1. The number of bacteria was quantitated immediately after removal and rinsing of VGs (controls) or after subsequent incubation of the rinsed VGs at 37°C for 30 min in fresh clotting rabbit blood, in fresh rabbit blood containing 0.1% (wt/vol) SPS, in whole fresh rabbit serum supplemented with human thrombin (2-U/ml final concentration), in clotting rabbit PRP plus 2 U of thrombin per ml, or in PRP. Each point represents the absolute number of viable bacteria cultured from the VG. Asterisk, $P < 0.005$ compared with results for VGs excised at 5 min after challenge. The Wilcoxon rank sum test was used to calculate the significance of differences in numbers of bacteria recovered from VGs.

platelet suspensions (Fig. 5) compared with the number of adherent bacteria on VGs at 5 min after challenge ($P < 0.005$). No reduction in the number of viable adherent bacteria on VGs subsequently incubated in fresh whole rabbit serum supplemented with thrombin or in platelet suspensions without thrombin was observed. The number of viable bacteria of strain 2 adherent on VGs excised from rabbits and subsequently incubated in fresh clotting blood or in clotting platelet suspensions showed no significant decrease (data not shown). Thus, thrombin-activated platelets in fresh clotting blood and platelet suspensions mediated the elimination of strain 1 bacteria adherent on VGs, whereas strain 2 bacteria, which are not susceptible to platelet releasate, remained viable on VGs.

DISCUSSION

Bacterial adherence on VGs is considered to be the first essential step in the pathogenesis of IE. In order to cause disease, however, the bacteria have to be able to persist at the VG after adherence. Adherent bacteria are subject to various defense mechanisms of the host, among which are bactericidal factors released from platelets upon thrombin activation (7, 48, 49). Using a panel of 10 viridans streptococcal isolates differing in their susceptibilities to these platelet factors, we analyzed the relative importance of early bacterial adherence to VGs and persistence in rabbits with catheter-induced VGs. The strains were either susceptible (group 1; six strains, 10 to 50% survival) or resistant (group 2; four strains, 90 to 100% survival) to bactericidal factors released from platelets, as tested by exposure to rabbit platelet releasates.

We found a remarkable discrepancy between the initial high number of culture-positive VGs due to releasate-susceptible bacteria and the rather low number of rabbits developing IE after 48 h after challenge with such strains. The releasate-susceptible strains appeared to be removed from the VGs over time ($P < 0.001$), in contrast to the releasate-resistant strains. Because of a significantly lower level of initial colonization by the releasate-resistant strains compared with that by the releasate-susceptible strains ($P < 0.005$), there was no significant difference between the two groups in the final development of IE (Table 1). These data show that a high level of initial adherence of viridans streptococci on VGs per se is not decisive for the development of IE and that resistance to bactericidal factors released from platelets is important for persistence of viridans streptococci on VGs in vivo.

There were significant differences between the test strains in the ability to produce dextran when they were grown on sucrose-containing mitis salivarius agar. In our experiments, however, the strains were grown in MH broth without added sucrose. Since all strains produced low and similar amounts of cell-associated dextran in this medium (Table 1), a role for dextran in either the high initial adherence of the group 1 strains or the resistance of group 2 strains to bactericidal factors released from thrombin-activated platelets can be excluded for these experiments.

To further characterize the initial events and the role of susceptibility to platelet releasate in the development of IE, *S. sanguis* type II strain 1 and strain 2, two strains which have been used in earlier experimental IE studies (8, 15, 22), were selected. Strain 1 is moderately susceptible to the standard preparation of releasate, adheres avidly after 5 min, and is very efficiently removed from VGs with time. Strain 2 is resistant to the releasate preparation, shows a relatively low level of initial adherence to VGs, and is not significantly removed from VGs with time. The strains have similar amounts of dextran when grown in MH broth (Table 1).

The development of IE due to strains 1 and 2 was dependent on the number of bacteria injected intravenously (Fig. 1). Both strains showed a 90% infective dose of 10^5 CFU, which matches data from similar studies (19). At the high inoculum concentrations (10^5 or 10^7 CFU), the proportions of the circulating bacteria that adhered to the VGs were similar for strains 1 and 2. With a small inoculum (10^3 or 10^4 CFU), however, a significantly higher proportion of strain 1 bacteria adhered on VGs, even though the magnitude of bacteremia in the rabbits was the same for both strains (Fig. 1).

The difference in initial adherences of the two strains was not caused by different amounts of dextran (Table 1). Since adherence of viridans streptococci to platelet-fibrin matrices is influenced by the LTA content of the cell wall (27), cell wall preparations of strains 1 and 2 were assayed for LTA content. The amount of LTA appeared to be inversely related to the level of adherence, with strain 2 containing approximately four times as much LTA as strain 1. Therefore, the question of which factors provide the different capacities of the strains to adhere to VGs remains.

After challenge with 10^3 or 10^4 CFU, the releasate-susceptible *S. sanguis* type II strain 1 bacteria were removed from VGs with time, resulting in sterilization of all or the majority of the initially colonized VGs, respectively (Tables 1 and 2; Fig. 1 and 2A). After challenge with 10^4 CFU of *S. sanguis* type II strain 2, the bacteria adhered to only a few VGs but were not removed with time. Experiments with inocula of 10^4 CFU of the releasate-susceptible *S. mitior* S224 and the releasate-resistant *S. mutans* S182 parallelled the results for strain 1 and strain 2, respectively. Again, the releasate-susceptible strain but not the releasate-resistant strain disappeared from the VGs with time (Fig. 3).

When challenged with 3×10^5 CFU of strain 1 bacteria, VGs were not sterilized with time (Table 2), but monitoring

the number of adherent CFU on individual VGs revealed a significant reduction of numbers of adherent bacteria at 30 min postchallenge. This initial reduction was insufficient to prevent the development of IE (Table 2), since surviving bacteria started to multiply within the VG within 2 h after challenge (Fig. 2B).

The reduction of the numbers of bacteria with time was not caused by detachment of bacteria due to the washing procedure, although the first rinse of the VGs excised at 5 min postchallenge always contained bacteria. These bacteria were most likely derived from blood, since at times postchallenge when blood cultures were sterile (and VGs were culture positive), only 6 (7.5%) of the first rinses of 80 VGs yielded growth. Sequential washings of VGs failed to remove significant numbers of bacteria. Although the role of detachment in vivo cannot effectively be studied, our in vitro data with VGs excised from rabbits are in accordance with those of other studies, in which no detachment of either *Streptococcus intermedius* (31) or *S. sanguis* (27, 35) from platelet-fibrin matrices was observed.

On the basis of the association of the releasate susceptibilities of the strains and their removal from VGs with time (Table 1), we hypothesized that bactericidal factors released from platelets were involved in this clearance. To eliminate the possibility of clearance by other mechanisms, we studied the role of complement-dependent serum bactericidal activity and surface phagocytosis of adherent bacteria by PMNs. Streptococci are known to be resistant to lysis by the complement-dependent serum bactericidal system (3). Indeed, killing of neither bacteria in suspension nor those adherent on VGs during incubation in fresh whole rabbit serum was observed for either strain 1 or 2 (Fig. 4).

The role of PMNs in the clearance of *S. sanguis* type II attached to VGs also was negligible (Fig. 4). Although cells of both test strains were phagocytized and effectively killed by rabbit PMNs in vitro, the number of bacteria adherent on VGs after exposure to PMNs was not reduced. This finding indicates that surface phagocytosis of adherent bacteria by PMNs (47) either did not occur or occurred only marginally. Additionally, we demonstrated that PMNs entrapped in the VGs rarely had engulfed bacteria, and if they had, only small numbers of bacteria were present in the PMNs (Fig. 4). The limited role of PMNs in clearance of *S. sanguis* adherent on VGs is in accordance with the findings (i) that only few PMNs are present in VGs (11, 17, 28), (ii) that circulating PMNs are unable to penetrate the fibrin-platelet meshwork of the VGs to reach the sites where the bacteria are attached (11, 14, 18), and (iii) that susceptibility to IE due to *S. sanguis* in granulocytopenic rabbits is not different from that in controls (29, 43). In summary, *S. sanguis* 1 and 2 are not cleared from VGs by detachment, serum bactericidal activity, or surface phagocytosis by PMNs.

The fact that the releasate-susceptible strain 1 was cleared from the VGs only when incubated in fresh clotting blood or in thrombin-stimulated platelet suspensions (Fig. 5) strongly supports the hypothesis that factors released from thrombin-activated platelets are involved in this clearance. Upon thrombin activation, platelets release lysozyme as well as bactericidal cationic proteins designated platelet-associated bactericidal substances (23), cationic bactericidal protein (45), β-lysin (9, 25, 41), thrombodefensin (7), or platelet microbicidal protein (48). Since we found no detectable lysozyme activity in the supernatant of thrombin-stimulated platelets, it is likely that the observed clearance in vitro (Fig. 5) as well as in vivo (Tables 1 and 2; Fig. 2) is due to platelet-derived cationic proteins with bactericidal activity as mentioned above.

The role of platelets and platelet-released bactericidal factors in experimental IE is further supported by recent studies by Sullam et al. (40). VGs of rabbits that were made thrombocytopenic 1 h after challenge contained significantly higher densities of *S. sanguis* 1 bacteria, showing that depletion of platelets reduces the clearance of bacteria from VGs.

A prerequisite for the release of these bactericidal proteins from platelets is the presence of thrombin. Since thrombin is required for the conversion of fibrinogen to fibrin and since fibrin is one of the main constituents of VGs, thrombin activity certainly is present at sites of VG formation. The amount of thrombin generated at the VG surface is unknown, but it has been noted that minute amounts (0.01 to 0.1 U/ml) of thrombin activate platelets (37) and initiate release of bactericidal activity (unpublished observation). The fact that anticoagulants did not influence the susceptibility of rabbits to infection with viridans streptococci (11, 14, 19, 24, 42) seems to argue against a role of platelet-released bactericidal proteins in IE. Even in rabbits intensively treated with anticoagulants, however, layers of fibrin covering adherent bacteria have been observed (14, 42). This means that sufficient procoagulant activity, including thrombin, is generated in the microenvironment of the damaged endothelium with adherent bacteria (10). Additionally, the release of bactericidal proteins by thrombin-stimulated platelets occurs independent of blood coagulation (41, 45). Even in heparinized blood, low concentrations of thrombin have been measured (36) and the presence of bactericidal proteins has been shown (41).

In conclusion, we have shown that at least two characteristics of viridans streptococci are decisive in the early pathogenesis of endocarditis. The first is the ability of bacteria to adhere to VGs early after challenge. The second, even more important characteristic is the ability of the adherent bacteria to persist on VGs after adherence. Our studies strongly indicate that the persistence of viridans streptococci adherent on VGs is related to their susceptibility to bactericidal factors released from platelets by thrombin stimulation. Studies are in progress to characterize the platelet bactericidal factors and to further delineate their role in the pathogenesis of experimental IE due to viridans streptococci.

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