Interleukin 1α Increases the Susceptibility of Rabbits to Experimental Viridans Streptococcal Endocarditis

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Major predisposing conditions for infective endocarditis (IE) are the presence of a cardiac platelet-fibrin vegetation and of circulating bacteria with relatively low susceptibility to microbicidal activity of blood platelets. The influence of proinflammatory conditions on development of IE is unknown. We studied the effects of the presence of a catheter, inserted to induce platelet-fibrin vegetations, and of the proinflammatory cytokine interleukin-1α in rabbit experimental IE. Leaving the catheter in place after challenge with viridans streptococci predisposed for experimental IE. IE susceptibility rapidly decreased between 0 to 6 h after catheter removal. The catheter did not predispose for IE by providing a site for bacterial adherence, as almost all explanted catheters were culture negative. To mimic the proinflammatory influence of the catheter, rabbits were injected with interleukin-1α at 24 h after catheter removal and at 0, 1, and 3 h before bacterial challenge. Interleukin-1α injected 3 h prior to challenge significantly increased IE incidence due to a platelet releasate-susceptible Streptococcus oralis strain, with rapidly increasing numbers of bacteria within the vegetations. IE due to the Streptococcus sanguis strain less susceptible to platelet releasate was not enhanced. We conclude that proinflammatory stimuli, either a catheter or interleukin-1α, enhanced susceptibility to IE due to the platelet releasate-susceptible S. oralis. As with rabbits, temporary intravascular proinflammatory conditions may predispose for IE in humans at risk for this serious infection.

Infective endocarditis (IE) denotes infection of the endocardial surface of the heart (3). The presence of sterile vegetations at the damaged surface of the endocardium (1) and bacteria and fungi circulating in the bloodstream that are able to adhere to and to colonize the vegetation (3, 9, 13, 29, 35) are prerequisites in the pathogenesis of IE. A great diversity of microorganisms cause IE, with viridans streptococci (VS) as the predominant microorganisms encountered in native valve IE (3, 14, 16, 24, 27, 30, 33, 34).

Another major determinant for the development of IE is the susceptibility of the circulating microorganisms to platelet microbicidal activity (2, 7–9) due to cationic proteins such as thrombocidin or platelet microbicidal proteins, released after thrombin stimulation (7, 9, 26, 36, 39). Neutralization of this defense mechanism enhanced susceptibility of rabbits to IE due to platelet releasate-susceptible VS (8). Platelet microbicidal activity is very likely to operate in humans as well, as VS and Staphylococcus aureus isolated from vegetations of endocarditis patients are generally less susceptible than oral isolates (7, 8, 38). Despite an apparent selective advantage, the VS isolates from IE patients are presumably still subject to the platelet defense system, since these isolates are only slightly less susceptible to purified thrombocidins in vitro (9, 41).

In the rabbit model of experimental IE, a catheter inserted retrograde across the aortic valve induces platelet-fibrin vegetations (18, 19, 32). The rabbits are then challenged with an intravenous (i.v.) inoculum of bacteria (13) colonizing the vegetations. The presence of the catheter enhanced the production and the further development of IE due to various bacterial species (7, 8, 17, 19, 40) and delayed spontaneous sterilization of infected vegetations (19, 40).

The infection-enhancing effect of the catheter has been explained by impairment of the host phagocytic defense (19, 21). However, in monocytic (37) and granulocytic (28) rabbits, susceptibility to IE due to VS was not different from controls. The catheter as a foreign body has been suggested to provide bacteria with a site for adherence, allowing subsequent colonization of fibrin-platelet vegetation (40), but no data to support this have been published. An alternative hypothesis would be that the catheter provokes a continuous proinflammatory condition. Such a condition has been shown to compromise innate immune efficacy (25) and might be an additional risk factor for development of IE in persons at risk. We therefore studied whether the catheter in the rabbit model was a site for bacterial adherence and assessed the effects of cytokine IL-1α mimicking a proinflammatory condition on susceptibility to IE. By challenging the rabbits with VS differing in their susceptibility to platelet microbicidal activity, a possible relationship between a proinflammatory condition and the effectivity of the platelet-mediated defense in IE was investigated.

**MATERIALS AND METHODS**

**Viridans streptococcal test strains and inoculum preparation.** For experimental IE studies with rabbits, three VS strains used in previous studies were selected (9, 26). Streptococcus oralis strain J30 (platelet releasate susceptible) (9) was isolated from the oral cavity of a patient with a non-IE cardiac disease. Both Streptococcus mitis strain S224 (platelet releasate susceptible) and Streptococcus sanguis strain U108 (less platelet releasate susceptible) were isolated from a blood culture of an IE patient (9). The strains were stored in skim milk (Oxoid, Basingstoke, United Kingdom) at −20°C, cultured on sheep blood (5% [vol/vol]) agar (Oxoid) plates at 37°C in 5% CO₂ for 48 h, and maintained at 4°C for 1 week. Before each test, bacteria were freshly grown in Mueller-Hinton broth (pH 7.4; Difco, Detroit, MI) on a rotary shaker at 90 rpm for 24 h without aeration.
After centrifugation (4,000 × g; 4°C; 10 min), the bacteria were washed three times with phosphate-buffered saline (8.1 mM Na2HPO4, 1.5 mM KH2PO4, 140 mM NaCl, 3 mM KCl [pH 7.2]) and resuspended in 0.9% (wt/vol) NaCl. Suspensions were sonicated for 30 s at 50 kHz (Branson 32; Branson Power Co., Danbury, CT) and adjusted to an optical density at 540 nm of 1.0 (model 24 spectrophotometer; Beckman Instruments, Inc., Palo Alto, CA) with 0.9% NaCl. These standardized suspensions contained approximately 10⁹ CFU/ml and were diluted in 0.9% NaCl to 10⁵ CFU/ml for rabbit challenges.

**Rabbit model of experimental IE.** Experiments were performed in accordance with the animal experimentation guidelines of the Universities of Amsterdam and Groningen, The Netherlands. Sterile left-sided vegetations were induced in New Zealand White rabbits each weighing 2.1 to 3.1 kg. A polyethylene catheter (external diameter, 0.5 mm; internal diameter, 0.4 mm) was inserted into the left carotid artery and placed retrograde across the aortic valve into the left ventricle, as previously described (22). The catheter either remained in place or was removed prior to bacterial challenge (8).

**Infection of IL-1α.** Rabbits received an injection of 1 ml of saline containing IL-1α (1.25 μg/kg of body weight [8,000 U/mg; Genzyme, Cambridge, MA; R&D Systems, Abingdon, United Kingdom]) in a marginal ear vein at 0, 1, and 3 h prior to challenge. Standardized bacterial inocula were prepared as previously described (9) and checked by plating.

**Evaluation of bacterial adherence to vegetations and development of IE.** Rabbits were injected with 1 ml of 0.9% NaCl containing 10⁵ CFU of the VS test strain in a marginal ear vein 24 h after placement of the catheter. Rabbits from which the catheter was removed were challenged 24 h after the removal of the catheter, unless otherwise indicated. Rabbits were sacrificed by i.v. injection of pentobarbital (20 mg/kg) at 5 min, 30 min, 4 h, or 48 h after challenge. The heart was removed under aseptic conditions, and vegetations were excised immediately and rinsed three times, each time with 5 ml of 0.9% NaCl. The total weight of the vegetations from each rabbit heart was determined, and the vegetations were homogenized and quantitatively cultured as previously described (9). 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.** Blood from rabbits was 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 previously described (9).

**Culturing of catheter-adherent bacteria.** After the heart was opened, three segments were cut from the catheter: the part embedded in the vegetation, the intracardiac part not contacting the vegetation, and the first 5 cm residing in the aorta-carotid artery. The segments were aseptically removed and rinsed twice, each time with 20 ml of sterile saline, to remove traces of blood. The rinse was discarded, the washed segments were individually placed in empty petri dishes, and then pour plates with sheep blood agar were made and incubated at 37°C in a 5% CO₂ atmosphere. Colonies were counted after 48 h.

**Statistics.** The incidence of IE in different experimental groups of rabbits was compared by Fisher’s exact test or the chi-square test. The significance of differences between numbers of CFU cultured from vegetations or between numbers of CFU cultured from catheter segments was assessed with the Wilcoxon rank sum test.

**RESULTS**

**Incidence of IE due to VS test strains in rabbit with catheters present or removed.** More than 90% of the rabbits with catheters left in place during challenge with 10⁵ CFU of one of the three VS test strains developed IE (Table 1). When the catheter was removed 24 h before challenge, none of the rabbits injected with the platelet releasate-susceptible strains *S. mitis* S224 or *S. oralis* J30 developed IE; three rabbits (30%) inoculated with the less-releasate-susceptible *S. sanguis* strain U108 developed IE (Table 1).

**Incidence of IE in relation to interval between catheter removal and bacterial challenge.** The interval between catheter removal and intravenous injection of 10⁵ CFU of strain J30 determined the incidence of IE 48 h later. At an interval of 30 min, five of six (83%) rabbits developed IE, which was not significantly different from the incidence of IE in rabbits with the catheter left in place (11/12 [90%]) (Table 1). At an interval of 2 h, four of seven (57%) developed IE; at an interval of 6 h, only two of seven rabbits (28.5%) developed IE. The latter incidence was significantly less (*P* = 0.01) than in the rabbits with the catheter left in place (11/12 [90%]) (Table 1).

**Early course of IE, number of CFU adherent to vegetations, and development of IE in rabbits with catheters present or removed.** Rabbits with catheters in place (catheterized rabbits) or with catheters removed 24 h prior to challenge (excatheterized rabbits) were sacrificed at 5 min, 30 min, 4 h, or 48 h after challenge with 10⁵ CFU of *S. oralis* strain J30. The weight of the vegetations, ranging from 26 to 166 mg, was not different in the two models. The number of circulating CFU and the number of CFU adherent to the cardiac vegetations at 5 min and 30 min after inoculation were similar in both models (Fig. 1). The average numbers of circulating CFU were 28 (range 16 to 58) and 34 (range, 21 to 34) CFU/ml at 5 min and decreased to 3 (range, 1 to 11) and 3 (range, 1 to 9) CFU/ml at 30 min for rabbits with catheters in place and removed, respectively. At 4 h after inoculation, the vegetations of the eight excatheterized rabbits were all culture positive, with high numbers of adherent CFU ranging from 1,700 to 8,238 CFU per vegetation (Fig. 1A). Blood cultures yielded an average of 13 (range, 2 to 23) CFU/ml. In contrast, at 4 h after inoculation only five (55%) of nine excatheterized rabbits had culture-positive vegetations (Fig. 1B), with significantly lower numbers of CFU per vegetation (2 to 112 CFU; mean, 26 CFU) (Fig. 1A, 4-h time point; *P* = 0.008), and blood cultures were negative. After 48 h, IE had been induced in 11 of 12 rabbits with the catheter in place, while none of the 8 rabbits from which the catheter had been removed developed IE due to strain J30.

**Catheter colonization.** After sacrifice at 5 min, 30 min, 4 h, or 48 h after challenge with strain J30, the intravascular part of the catheter, the intracardiac but not vegetation-contacting part, and the next 5 cm localized in the aorta and carotid artery were cultured. Only 3 (12%) of the intravascular catheter segments collected at 5 min after challenge were culture positive, and only low numbers of CFU were recovered, ranging from 1 to 20 CFU per segment (Table 2).

**Influence of IL-1α on susceptibility to IE.** In these experiments, we used the low-susceptibility IE model of the ex-
catheterized rabbits. The catheter was removed 24 h prior to i.v. injection of 1.25 μg (10 U) of IL-1α per kg. The inoculum of 10⁵ CFU of the VS test strains was injected immediately or 1 or 3 h after IL-1α injection. After simultaneous injection of IL-1α and VS, rabbits had no IE due to strain S224 or strain J30 (Table 3). In rabbits that received IL-1α 1 h prior to inoculation, no (S224) or only a slight increase (J30) in the incidence of IE was observed. However, in rabbits injected with IL-1α at 3 h before challenge with strains S224 and J30, IE incidence increased up to 25% (not significant) and 50% (P = 0.04), respectively (Table 3). Susceptibility to IE due to the less-platelet releasate-susceptible strain U108 was not increased after injection of IL-1α or at 0, 1, or 3 h prior to challenge.

Quantitative cultures of vegetations excised from rabbits that received IL-1α at 3 h prior to challenge with strain J30 showed that the numbers of adherent CFU at 5 min, 30 min, and 4 h were not different but after that time point increased to reach high values at 48 h in rabbits developing IE (Fig. 2). In contrast, in rabbits that had not received IL-1α, the numbers of CFU strongly decreased at 30 min; vegetations were culture negative at 48 h (Fig. 1B). The average level of bacteremia in the IL-1α-injected rabbits decreased from 26 (range, 17 to 43) CFU/ml at 5 min to 2 (range, 1 to 2) CFU/ml at 30 min after challenge; at 4 h after challenge, all blood cultures were negative. This was not different from the level and period of bacteremia in excatheterized rabbits not injected with IL-1α (see above).
The catheter does not function as a site for VS adherence. Rabbits with the catheter left in place developed IE after challenge with 10^5 CFU of any of the three VS test strains, whereas no (strains J30 and S224) or 30% (strain U108) IE was produced in rabbits from which the catheter was removed 24 h prior to challenge (excatheterized rabbits) (Table 1). Susceptibility to IE due to strain J30 was shown to decline rapidly within the first 6 h after catheter removal. The enhancing effect of the catheter on the induction and course of experimental IE due to platelet releasate-susceptible VS strains has been recognized in earlier studies. Adherence to the catheter allowing subsequent colonization of the adjacent fibrin-platelet vegetation has been suggested as a mechanism, but catheter colonization was not quantified (40). We found that at 5 min after challenge only a few intravegetational catheter segments of rabbits challenged with 10^5 CFU of strain J30 were culture positive, yielding only small numbers of VS CFU (Table 2). At later time points, all catheter segments were culture negative, whereas at 48 h the vegetations of >90% of the rabbits yielded high numbers of J30 CFU. We therefore conclude that adherence of the bacteria to the catheter is not a mechanism for the induction and development of experimental IE due to VS.

**IE due to platelet releasate-susceptible VS strains is enhanced by the proinflammatory cytokine IL-1α.** As the catheter in combination with the streptococci will provoke a proinflammatory response, we studied the influence of the proinflammatory cytokine IL-1α on susceptibility to experimental IE. IL-1α is one of the predominant cytokines expressed in rabbit aorta endothelium in response to injection of bacterial components such as lipopolysaccharide (6). In excatheterized rabbits, IL-1α significantly increased susceptibility to IE due to *S. oralis* J30 when IL-1α was given 3 h before challenge. IE incidence due to the releasate-susceptible *S. mitis* S224 was also increased, although not significantly. The incidence of IE due to the less-releasate-susceptible *S. sanguis* U108 was not increased by IL-1α. Remarkably, a period of 3 h between injection of IL-1α and bacterial challenge was required for the increase in susceptibility to IE due to releasate-susceptible VS strains. A possible explanation for this lag time is discussed below.

**IE is enhanced due to reduced bacterial clearance in the presence of the catheter or after IL-1α injection.** As initial adherence to vegetations, duration of bacteremia, and numbers of circulating VS during the first hours after challenge were not different in catheterized and excatheterized rabbits, the enhanced incidence of IE in the presence of the catheter must be explained by reduced clearance of adherent bacteria from the vegetations after their initial adherence. In rabbits challenged with 10^5 CFU of VS, the number of CFU cultured from vegetations decreased between 5 and 30 min but increased again from 2 h after challenge onwards, resulting in development of IE (Fig. 1). In previous studies, we found that at lower levels of inocula, VS were fully cleared from the vegetations at 2 to 4 h after challenge (7, 9). As this clearance is not due to serum bactericidal activity or phagocytosis by polymorphonuclear phagocytes (9), clearance is most likely mediated by microbicidal proteins released from platelets after thrombin stimulation (7, 9, 26, 36, 39). This protective role of platelet microbicidal activity is underlined by the fact that neutralization of this microbicidal activity increased susceptibility of rabbits to IE (8) and that clinical VS IE isolates generally have a reduced susceptibility to platelet microbicidal activity (8).

In the present study, the platelet releasate-susceptible strains S224 and J30 did not cause IE in rabbits from which the catheter had been removed 24 h prior to challenge (Table 1). Since bacteria were found to adhere to the VGs at 5 min after challenge, they apparently were effectively cleared, most likely involving an effective platelet microbicidal activity (see above). The high frequencies of IE in the presence of the catheter, 90% for S224 and 92% for J30, strongly suggest that the clearance by the platelet microbicidal activity was impaired. In rabbits challenged with the less-releasate-susceptible strain U108, removal of the catheter also led to a reduction in the incidence of IE, although 30% infection was still recorded. This suggests that 24 h after removal of the catheter platelet microbicidal activity is sufficiently high to reduce the incidence of IE due to a less-releasate-susceptible strain such as U108.
Although less susceptible to crude platelet releasate (9), strain U108 is killed by purified antimicrobial proteins from platelet releasate (41). For Staphylococcus aureus, the importance of susceptibility to platelet microbicidal activity has been shown with an isogenic mutant with reduced susceptibility to rabbit platelet microbicidal proteins (10). For viridans streptococci, no such isogenic mutants are yet available.

In vivo, VS may be exposed to high local concentrations of antimicrobial proteins released from platelets in close contact with the bacteria. Platelets carrying their full content of antimicrobial proteins will even be capable of killing less-susceptible strains. However, a (temporary) reduction of platelet microbicidal capacity due to premature release of their antimicrobial proteins will allow VS strains to colonize the vegetation, particularly VS with reduced susceptibility to platelet microbicidal factors (8).

Irrespective of the presence of the catheter, the number of CFU cultured from vegetations excised at 30 min was significantly lower than from the vegetations excised at 5 min post-challenge (Fig. 1). In absence of the catheter, no growth of the bacteria at later time points was recorded; in the presence of the catheter, the numbers of CFU increased again at 2 and 4 h after challenge. In rabbits with catheters in place, the clearance mechanism apparently is of limited capacity and not capable of complete clearance of the initially adherent VS. As a consequence, at 4 h the mean number of adherent CFU at the cardiac vegetations of rabbits with the catheter in place was >180-fold higher than in excatheterized rabbits (Fig. 1). When excatheterized rabbits received an injection with IL-1α at 3 h prior to challenge with S. oralis J30, IE frequency increased significantly (Table 3). Furthermore, the bacterial numbers increased rapidly in the rabbits developing IE; at 4 h, the bacterial numbers were similar to those in rabbits with the catheters in place (compare Fig. 2 and Fig. 1A). Apparently, IL-1α caused a reduction in bacterial clearance similar to that caused by the continuous presence of the catheter.

Hypothesis to explain IL-1α-mediated reduction of bacterial clearance. IL-1α increased susceptibility of excatheterized rabbits to IE due to S. oralis J30 but only when IL-1α was given 3 h before and not immediately or 1 h prior to challenge (Table 3). This period of 3 h required to enhance susceptibility to IE might be explained as follows. Three hours has been reported to be the time period required for IL-1 to induce maximal endothelial tissue factor activity and subsequent thrombin generation in rabbits (31). The first response of platelets to low (nanomolar) concentrations of thrombin is the aggregation-independent release of α-granule content, whereas higher thrombin concentrations are needed for further activation, characterized by processes such as release of dense and lysosomal granule content (4, 31). Since the α-granules store the major microbicidal proteins of platelets (7, 26), the platelets may become “disarmed” in the presence of low concentrations of thrombin. Such disarmed circulating platelets would be impaired in their ability to clear adherent VS upon their deposition onto the colonized vegetation surface and not be capable of preventing development of IE. The only other occasion where we observed development of IE due to platelet releasate-susceptible VS in rabbits challenged after removal of the catheter was with rabbits that had antibodies neutralizing their platelet antimicrobial activity (8). This supports the hypothesis that platelet microbicidal activity may be compromised in the IL-1-injected rabbits.

A proinflammatory stimulus as a risk factor for development of IE. Our data provide new insights into the pathogenesis of rabbit experimental IE and also into the pathogenesis of native IE in human patients. The frequency of native IE is low, ranging from 0.68 to 6.5 cases per 100,000 person-years in the general population (3, 11, 23, 30). A higher frequency would be expected, based on the findings that cardiac vegetations are present in 2 to 3% of elderly patients (5) and that low-grade bacteremia occurs daily in 30 to 70% of persons analyzed (3, 12, 15). We have now shown that circulating VS alone are not sufficient to cause IE in rabbits with preexisting vegetations;
but with catheters removed. IE developed only when the catheter was still present or when the rabbits had received an injection with the proinflammatory cytokine IL-1α 3 h prior to challenge. In humans, persons with cardiac vegetation would be predominantly at risk when bacteria gain access to their circulation several hours after an event inducing an intravascular proinflammatory response. Conversely, the need for coincidental low-grade bacteremia and a proinflammatory stimulus several hours prior to this could explain the low frequency of IE, despite the relatively high frequency of vegetations and low-grade bacteremias. Such a time window of enhanced susceptibility to IE would have serious consequences for considerations of IE prophylaxis.

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