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Influence of Monocytes and Antibiotic Treatment on Tissue Factor Activity of Endocardial Vegetations in Rabbits Infected with *Streptococcus sanguis*

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A main feature in the pathogenesis of bacterial endocarditis is the activation of the coagulation system via the extrinsic pathway, resulting in the formation of infected endocardial vegetations. Earlier studies gave indirect evidence that monocytes play an important role in the procoagulant response during the course of the disease. In this study, we assessed the role of monocytes more directly. We compared weights and tissue factor activities (TFA) of endocardial vegetations of normal rabbits infected with *Streptococcus sanguis* with those of rabbits which were treated with the cytostatic drug etoposide (Vepesid; Bristol-Myers Squibb B.V.) to induce a selective monocytopenia. Furthermore, the importance of the presence of bacteria was determined through the influence of antibiotic treatment on TFA, vegetational weight, and infection of the vegetations. The TFA of the vegetations was measured chromogenically by monitoring the factor VII-dependent activation of factor X with an amidolytic assay for factor Xa. We found that the degree of infection and the weight of vegetations of rabbits treated with the cytostatic drug etoposide did not differ from that of untreated rabbits. Their TFA, however, was significantly lower than the TFA of vegetations of rabbits not treated with etoposide. We also found that, as with the monocytopenic rabbits, the weight of the vegetations was not reduced in penicillin G-treated rabbits. The degree of infection and TFA, however, were significantly lower. We conclude that monocytes indeed are involved in the activation of the coagulation system during the course of bacterial endocarditis and that the degree of infection is positively correlated to the TFA of the vegetations.

The activation of the coagulation system, which ultimately leads to the formation of a fibrin-platelet matrix, is a major event in the onset and course of bacterial endocarditis. Infecting microorganisms as well as monocytes, granulocytes, and thrombocytes are embedded in this matrix, which is called the endocardial vegetation (17). The coagulation cascade can be initiated via the intrinsic or the extrinsic pathway. In bacterial endocarditis, this activation most probably occurs via the extrinsic route (5). Since this pathway requires cell-associated tissue factor (TF), endothelial cells, fibroblasts, or monocytes could be involved.

Of these, endothelial cells and monocytes, which normally lack tissue factor activity (TFA), can be induced by various stimuli to express TF (4, 11), while fibroblasts constitutively express TF (16). Studies in our laboratory have shown that endocardial vegetations, which were carefully removed from the heart during the early stages of a *Streptococcus sanguis* endocarditis, did contain factor VII (FVII)-dependent procoagulant activity. Endothelial cells and fibroblasts were not present in these vegetations (3). We also found that the TFA of infected vegetations is higher than the TFA of sterile catheter-induced vegetations (3, 18). These infected vegetations contain a significantly higher number of monocytes, making these cells the most probably source for the increased TFA.

Through an in vitro study, we could show that phagocytosis of bacteria is a stimulus for monocytes in a cell suspension to express TFA. A bacterium/monocyte ratio of 10 to 1 was found to result in maximal induction of TFA (18). Recently, we found that in an in vitro model for bacterial endocarditis, in which monocytes and bacteria are attached to a fibrin surface, an interaction between these cells and the bacteria results in expression of TFA on their cell surface (1).

In the present study, we set out to further investigate the role of monocytes as well as of bacteria in the activation of the coagulation system in the endocardial vegetations. We compared the TFA of endocardial vegetations that were isolated from rabbits treated with the cytostatic drug etoposide (Vepesid; kindly donated by Bristol-Myers Squibb B.V., Woerden, The Netherlands) to induce a selective monocytopenia with the TFA of vegetations from untreated rabbits. Etoposide treatment reduces the number of monocytes in endocardial vegetations (18). To assess the role of the bacteria, TFA was measured in vegetations from rabbits that were either sterile or infected with *S. sanguis*. In addition, bacterial numbers in the vegetations were reduced by treating rabbits infected with *S. sanguis* with penicillin G, and the TFA was measured in these vegetations.

MATERIALS AND METHODS

Microorganism. *S. sanguis* NCTC 7864 was the same strain as that used in previous studies (2, 14, 18). Streptococci from an overnight culture in Todd-Hewitt broth (Oxoid, London, England) were washed three times in pyrogen-free saline and diluted to a concentration of approximately 10^8 CFU/ml. For this strain, the minimal inhibitory concentration of penicillin G was 0.022 mg/liter, as determined in Todd-Hewitt broth in a twofold dilution series. The minimal bactericidal concentration was 0.045 mg/liter as determined by serial dilution and plating of aliquots of the minimal inhibitory concentration dilutions (2, 14).

Experimental design. Bacterial endocarditis was induced in male New Zealand White rabbits as described previously (3, 6). In short, rabbits weighing about 2 kg were anesthetized by intramuscular injection of 1.5 ml of fentanyl-citrate and fluanisone (Hypnorm; Janssen Pharmaceutica, Tilburg, The Netherlands), and a polyethylene catheter (Portex, Hythe, England) was introduced into the

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left ventricle of the heart via the left carotid artery. The catheter was left in situ for the duration of the experiment. After 48 h, 10^8 CFU of live streptococci in 1 ml of pyrogen-free saline were injected intravenously into a marginal ear vein. One or 2 days later, the rabbits were sacrificed by intravenous injection of sodium pentobarbital (Euthesate; Apharma, Arnhem, The Netherlands). The heart was removed, and the endocardial vegetations were isolated aseptically. Care was taken that no material except the vegetations was removed (2). Control rabbits were catheterized but received 1 ml of saline instead of streptococci at 48 h.

Cytostatic drug. The cytostatic drug etoposide was used to induce a selective monocytopenia, as described elsewhere with a slight modification (13). Briefly, a daily dose of 12.5 mg per rabbit was injected intravenously on 6 consecutive days, starting 3 days before catheterization (see Fig. 1).

Antibiotic treatment. On 2 consecutive days, a dose of 3×10^4 U of penicillin G was given subcutaneously at 8-h intervals. The first dose was given 20 h after injection of the streptococci. Rabbits were sacrificed 12 h after the last injection of penicillin G. The concentration of penicillin G in serum was determined as described earlier (14).

Quantitation of blood monocytes. Blood samples, drawn daily from a marginal ear vein, were collected in plastic vials containing 10 mg of crystalline potassium EDTA (Sherwood Medical, 's Hertogenbosch, The Netherlands). Total leukocytes were counted with a Coulter Counter (model ZF; Coulter Electronics Ltd., Luton, England). The total number of monocytes per ml of blood was calculated from the total number of leukocytes per ml of blood and four differential counts of 100 leukocytes in Giemsa-stained blood smears.

Handling of vegetations. Isolated vegetations were weighed and homogenized (5% [wt/vol]) in buffer A, which contained 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Sigma, St. Louis, Mo.), 137 mM NaCl (Merck, Darmstadt, Germany), 11 mM α-D-glucose (BDH Chemicals Ltd., Poole, England), 4 mM KCl (Merck), 5 mg of bovine serum albumin per ml (Sigma) (pH 7.45). Part of the homogenate was used for the determination of bacterial numbers, and part was used for the assessment of the TFA. The degree of infection of the vegetations was determined as described previously (4) and is expressed as log CFU per gram of vegetation. The part of the homogenate to be used for assessment of TFA was frozen and thawed three times to lyse intact cells and then stored at −70°C.

TFA assay. The TFA of the vegetations was measured by monitoring the FVII-dependent factor X (FX) activation with an amidolytic assay for activated factor X (FXa). FVII and FX were purified as described earlier (1).

Of the vegetation homogenate (5% [wt/vol]), 25 μl was mixed with 10 μl of FVII (final concentration, 1.05 nM), 5 μl of 0.1 M $CaCl_2$, and 50 μl of buffer A in a 96-well microtiter plate, and the mixture was incubated for 20 min at 37°C. Fifteen microliters of FX (final concentration, 0.1 μM) was then added, and the mixture was incubated for 20 min at 37°C. Twenty-five microliters of the chromogenic substrate S2337 (Chromogenix, Mölndal, Sweden; 20 mM in 50 mM EDTA) was added. The colorimetric reaction was stopped with 50 μl of 50% acetic acid (Merck), and the optical density at 405 nm was measured. The absorption value was converted to nanomolar FXa by use of a calibration curve from 0 to 30 nM FXa. The FXa calibrator was prepared by complete activation of isolated FX with the FX activator from Russel's viper venom (Chromogenix). The results are expressed as picomolar FXa per gram of vegetation per minute.

Blood cultures. Immediately before the rabbits were sacrificed, blood was drawn from their marginal ear veins and collected in vials containing EDTA, and 100 μl was plated on sheep blood agar plates. CFU were counted after overnight incubation at 37°C.

Statistical analysis. For determination of significance of differences between the TFAs of control rabbits, penicillin G-treated rabbits, and etoposide- and/or streptococcus-treated rabbits, analysis of variance with Newman-Keuls correction and the Kruskal-Wallis test for nonparametric analysis of variance were used when appropriate. The degree of infection of the vegetations was analyzed by analysis of variance.

RESULTS

Effect of etoposide on the number of peripheral blood monocytes and granulocytes. The course of the numbers of peripheral blood monocytes was monitored in each rabbit, starting on day −3. Etoposide was injected at the time points indicated in Fig. 1. On day 0, there was a reduction of 95% of the numbers of peripheral blood monocytes. The mean number of blood granulocytes in both untreated and etoposide-treated rabbits on day −3 was $9.97 \times 10^2/mm^3$. The number of granulocytes did not change significantly during drug treatment.

Effects of monocytopenia on infection and vegetation weights. All vegetations of streptococcus-injected rabbits were colonized with *S. sanguis*. The degree of infection was between 7.7 and 9.7 log CFU/g for all infected vegetations. Bacterial numbers in the vegetations were not significantly different on days 3 and 4. Monocytopenia did not influence the bacterial numbers of *S. sanguis*-infected vegetations. All control rabbits had sterile vegetations.

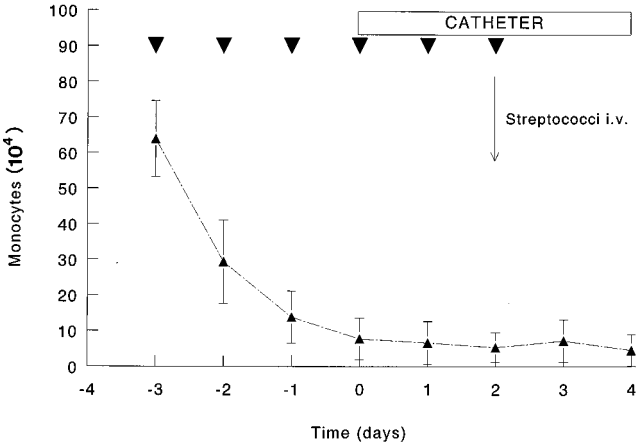

FIG. 1. Effect of etoposide on the number of peripheral blood monocytes. Results shown are mean numbers of monocytes ± standard deviations for at least four rabbits.

On day 3, there were no significant differences in vegetational weight between infected and noninfected rabbits. On day 4, the mean weight of vegetations of *S. sanguis*-infected rabbits was significantly higher than the mean weight of vegetations of noninfected control rabbits (40.7 ± 23.2 mg versus 14.2 ± 10.8 mg, $P < 0.001$). Although vegetational weights in infected rabbits were lower after etoposide treatment, this difference was not significant (Fig. 2).

Effect of infection and monocytopenia on TFA of vegetations. Comparison of vegetations on days 3 and 4 of *S. sanguis*-infected and noninfected control rabbits showed higher TFAs for infected rabbits (Table 1), but the difference was only significant on day 4. The TFA of vegetations of monocytopenic rabbits was found to be lower than that of nonmonocytopenic rabbits, but this difference was only significant on day 4. This was true in both infected and sterile vegetations.

Effect of penicillin G treatment on infection and vegetation weights. Vegetations of both penicillin G-treated and saline-treated rabbits were infected with *S. sanguis*. On day 4, the degree of infection in the vegetations of penicillin G-treated

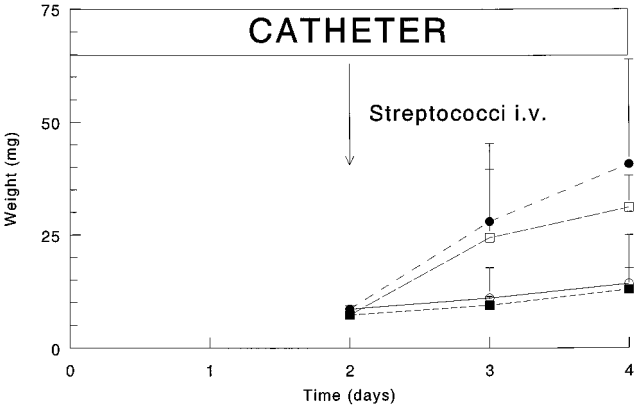

FIG. 2. Effect of infection and monocytopenia on vegetation weights. Results shown are mean weights ± standard deviations for at least four rabbits. Symbols: ○, sterile; ■, sterile, monocytopenic; ●, *S. sanguis* infected; □, *S. sanguis* infected, monocytopenic.

TABLE 1. Effect of infection and monocytopenia on TFA of endocardial vegetations of *S. sanguis*-infected rabbits

Treatment	TFA[a]	
	Day 3	Day 4
Sterile	99 ± 37	392 ± 100.5[b]
Sterile plus etoposide	99 ± 37.5	249 ± 93
S. sanguis	255 ± 68.5	735.5 ± 156[b,c]
S. sanguis plus etoposide	179 ± 25	278.5 ± 65[c]

[a] Picomolar FXa per gram of vegetation activated per minute. Results are means ± standard deviations for at least four rabbits. Significant differences are indicated.
[b] $P < 0.001$ (sterile versus *S. sanguis* treatment).
[c] $P < 0.005$ (*S. sanguis* versus *S. sanguis* plus etoposide treatment.

rabbits was about 15-fold lower than that of control rabbits (8.7 ± 0.7 log CFU/g of vegetation for control rabbits versus 7.1 ± 1.0 log CFU/g of vegetation for penicillin G-treated rabbits, $P < 0.001$) (Table 2). There was no difference between the penicillin G-treated and control groups of rabbits in vegetational weight, i.e., weight of total removable vegetational mass. Serum penicillin G concentrations were found to vary between 3.9 ± 1.4 mg/liter at 0.5 h (after first injection) and 0.134 ± 0.08 mg/liter at the time of death of the rabbits.

Effect of penicillin G treatment on TFA of vegetations. Vegetations of penicillin G-treated *S. sanguis*-infected rabbits were compared with those of *S. sanguis*-infected control rabbits. The penicillin treatment resulted in a significantly lower TFA of the vegetations on day 5 (Table 2).

DISCUSSION

The main conclusions from the present study are that vegetations from rabbits infected with *S. sanguis* have higher TFAs than vegetations from noninfected control rabbits, that an increased vegetational TFA (measured as picomolar FXa per gram per minute) is correlated with increased vegetational weights, and that a reduction of circulating monocytes results in a decrease of the vegetational TFA. Etoposide treatment did not influence infection of the vegetations, whereas penicillin G treatment led to a decrease in the TFA as well as an approximately 97% reduction in the infection of the vegetations.

TF is responsible for the activation of the coagulation system in infected endocardial vegetations (3). Of the three cell types (monocytes, endothelium, and fibroblasts) that could be responsible for the expression of the TF needed to initiate this activation in bacterial endocarditis (2), only monocytes are present in the vegetations during the stage in which an increased TFA could be demonstrated (9, 12, 18). Since fibroblasts are not present in the vegetation in this early stage of the disease and endothelial cells are not present because the vegetations were removed very carefully from the underlying endothelium (3), monocytes are the most probable source of vegetational TFA.

The TFA of vegetations of streptococcus-infected rabbits was significantly higher than the TFA of vegetations of noninfected rabbits. This is in agreement with our previous findings (3). Furthermore, in monocytopenic rabbits, infected vegetations had a significantly lower TFA than that of nonmonocytopenic rabbits. In an earlier study, we found that in etoposide-treated rabbits, *S. sanguis*-infected vegetations contained significantly fewer monocytes than vegetations of rabbits with normal monocyte numbers (18). Combined with the results of the present study, this is additional evidence that the TFA of endocardial vegetations is related to the vegetation-associated monocytes.

Several stimuli can induce TF expression in monocytes. With an in vitro model of bacterial endocarditis, we have shown that binding of the monocyte to fibrin is in itself a sufficient stimulus to induce TFA expression on the monocyte. However, stimulation in the presence of *S. sanguis* leads to a significantly higher level of TF expression on the monocytes, which depends on the bacterium-to-cell ratio (1). When rabbits were treated with antibiotics, the TFA of the vegetations as well as their degree of infection decreased. The lower numbers of *S. sanguis* in the vegetation may lead to a lower stimulation of the monocytes to express TF. However, the bacteremia was also interrupted. Thus, stimulation of monocytes on the vegetational surface by blood-borne bacteria, which during the course of the disease may contribute significantly to the vegetational TFA, no longer occurs.

These findings lead to the question whether the enhanced TFA of infected vegetations is the result of a direct interaction between bacteria and monocytes, for instance, by phagocytosis of the bacteria on the vegetational surface, or is mediated indirectly by humoral or other cellular factors. The bacteria are attached to a fibrin surface or trapped in the fibrin matrix, which impedes the monocytes to phagocytose the bacteria. Therefore, an indirect effect might be more important, such as interactions of bacteria with monocytes or adherence of monocytes to the infected vegetation, which, in addition, contains fibronectin (putative ligand for the very late antigens 4 and 5 [VLA-4, VLA-5]) and which may bind to the bacteria. Cross-linking of these receptors may be required for the upregulation of the procoagulant activity. It has been reported that leukocyte accumulation plays a role in fibrin deposition mediated by P selectin on adherent platelets (15). Also, TF expression by monocytes is enhanced via an interaction with platelets and granulocytes (10), and cross-linking of the β1 integrin VLA-5 by fibronectin leads to activation of the β2 integrin CR3, which in turn may be involved in the upregulation of TF expression on the surface of the monocyte (7, 19), while engagement of the β1 integrin VLA-4 leads to a direct upregulation of TF (8). Future investigations, therefore, will be directed toward TF expression resulting from platelet interactions with and fibronectin binding by streptococcus-stimulated monocytes.

TABLE 2. Effect of penicillin G treatment on TFA, infection, and weight of *S. sanguis*-infected endocardial vegetations on day 4[a]

Penicillin G treatment	TFA (pM FXa/g/min)[b,c]	Log CFU/g of vegetation[c]	Vegetational wt (mg)
+	250 ± 50	7.1 ± 0.1	21.0 ± 5.8
−	602 ± 56	8.7 ± 0.7	22.4 ± 4.2

[a] Results are means ± standard deviations of at least four rabbits.
[b] Values are picomolar FXa per gram of vegetation activated per minute.
[c] $P < 0.001$ (untreated versus penicillin G treated).

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