Role of Monocytes in Experimental *Staphylococcus aureus* Endocarditis

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In the pathogenesis of bacterial endocarditis (BE), the clotting system plays a cardinal role in the formation and maintenance of the endocardial vegetations. The extrinsic pathway is involved in the activation of the coagulation pathway with tissue factor (TF) as the key protein. *Staphylococcus aureus* is a frequently isolated bacterium from patients with BE. We therefore investigated whether *S. aureus* can induce TF activity (TFA) on fibrin-adherent monocytes, used as an in vitro model of BE. We also assessed in vivo in rabbits with catheter-induced vegetations, the effect of *S. aureus* infection on vegetational TFA. In vitro experiments showed that adherent *S. aureus* induced TFA on fibrin-adherent monocytes which was optimal at a bacterium/monocyte ratio of 1 to 1. Monocyte damage occurred when this ratio exceeded 4 to 1 (visually) or 6 to 1 (propidium iodide influx) Consequently, TFA decreased. In vivo *S. aureus* led to very high bacterial numbers in the vegetations and a significant increase of their weight. However, TFA of infected vegetations was the same as of sterile ones. This may be due to the high bacteria to monocyte ratio as well as bacterium-induced monocyte damage. Teicoplanin treatment of infected rabbits reduced bacterial numbers in the blood and in the vegetations. Two-day treatment resulted in an increase of vegetational TFA, but after four-day treatment vegetational TFA dropped, most probably due to a suboptimal bacterium/monocyte ratio. *S. aureus* endocarditis in endoside (Vepesid)-treated rabbits, leading to a selective monocytopenia, caused a rapid death of the animals. In these rabbits no vegetations were found at all. We conclude that, like *Streptococcus sanguis* and *Staphylococcus epidermidis*, *S. aureus* is able to induce TFA in fibrin-adherent blood monocytes. In addition, monocytes have a protective effect during the course of *S. aureus* endocarditis.

An inflammatory process resulting in the formation of so-called endocardial vegetations characterizes bacterial endocarditis (BE). These vegetations consist of a fibrin clot, which contains the infecting microorganisms embedded in a matrix of proteins and blood cells (17). For their formation the coagulation system has to be activated which, as shown earlier, occurs via the extrinsic pathway (4, 11). Vegetations have a procoagulant activity which is factor VII (FVII) dependent, indicating the involvement of tissue factor (TF), a transmembrane glycoprotein which serves as the central point in the extrinsic clotting pathway (1, 2, 5, 9, 11). In earlier studies we have shown that monocytes account for the TF activity (TFA) of vegetations infected with *Streptococcus sanguis* or *Staphylococcus epidermidis* (2, 3, 4).

Another frequently isolated bacterium from patients with BE is *Staphylococcus aureus* (10). It often causes an acute and massive valvular destruction in patients with previously intact heart valves (6, 14), in contrast to *S. sanguis*, which causes subacute endocarditis on natural valves, and *S. epidermidis*, which causes prosthetic valve endocarditis (6, 16). Because of these differences, we investigated whether *S. aureus* can also induce TFA on fibrin-adherent monocytes, used as an in vitro model of BE (1). We assessed in rabbits with catheter-induced vegetations the effect of *S. aureus* infection on vegetational TFA in vivo.

*S. aureus* 5558, the same strain as used in a previous study (21), was grown overnight in brain heart infusion (BHI) broth (Oxoid, London, England) at 37°C. Before use, bacteria were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS; pH 7.45), and diluted to the concentration to be used. Staphylococcal numbers in the overnight cultures were measured by colony count in serial dilutions incubated overnight at 37°C on blood agar.

Adherence of the staphylococci to fibrin plates, prepared from fibrinogen (Sigma, St. Louis, Mo.) in 24-well culture plates (Costar, Cambridge, England) was measured as described previously (4) and cultured overnight in RPMI 1640 (Gibco BRL, Paisley, Scotland) in Teflon bags (19) at 37°C in 5% CO2 before use. The TFA of monocytes used as described by Bancsi et al. (1, 2). Briefly, each well was incubated with purified factor VII (FVII) and CaCl2 (Merck, Darmstadt, Germany) for 15 min to allow formation of TF-FVII-Ca complex. Next, purified factor X (FX) was added and, after 5 min, achieved FX formation (FXa) was measured. These monocytes were isolated from a fresh buffy coat as described previously (4) and cultured overnight in RPMI 1640 (Gibco BRL, Paisley, Scotland) in Teflon bags (19) at 37°C in 5% CO2 before use. The TFA of monocytes was assessed as described by Bancsi et al. (1, 2). Briefly, each well was incubated with purified factor VII (FVII) and CaCl2 (Merck, Darmstadt, Germany) for 15 min to allow formation of TF-FVII-Ca complex. Next, purified factor X (FX) was added and, after 5 min, achieved FX formation (FXa) was stopped by the addition of EDTA (Boehringer, Mannheim, Germany). Then, PefachromeFXa (Kordia, Leiden, The Netherlands), a chromogenic substrate for FXa, was added. After 20 min, the addition of acetic acid (Merck) stopped the conversion of the substrate. The optical density at 405 nm was measured and converted to FXa concentrations. For this calculation a calibration curve was used from purified FX that was fully activated with Russel Viper Venom (Chromogenix, Mölndal, Sweden). Data are expressed as picomoles of FXa/106 monocytes.

The effect of the bacterium/monocyte ratio on the TFA of the monocytes was determined by incubating different numbers of adherent bacteria with a standard number of 1.5 × 106 monocytes, as described earlier (3). Because bacterial constit-
unteers such as peptidoglycan and lipoteichoic acid can activate endothelial cells and monocytes (8, 13, 20), we investigated whether such constituents, generated during overnight incubation of *S. aureus* in the presence of 10 μg of teicoplanin (Gist-brocades NV, Delft, The Netherlands) per ml, i.e., “the supernatant,” could activate monocytes to generate TFA. Supernatants were collected from *S. aureus* cultures grown for 5 h at 37°C in BHI broth, after which 10 μg of teicoplanin per ml was added to the medium, and then the bacteria were cultured for an additional 20 h. Monocytes were then incubated for 4 h on fibrin plates with these supernatants diluted 1 to 10 with RPMI 1640.

Cytotoxicity for monocytes of staphylococci or their breakdown products was determined by measuring cell permeability with propidium iodide (PI) influx. Monocytes were recovered from the fibrin plates after 4 h of incubation with bacteria or bacterial supernatant, incubated for 10 min with 1 μg of PI per ml, and then analyzed in a fluorescence-activated cell sorter (Becton Dickinson). Results are expressed as mean arbitrary units (AU) of fluorescence.

For the in vivo study, BE was induced in male New Zealand White rabbits as described elsewhere (4, 12). A polyethylene catheter (Portex, Hythe, England) was introduced into the left ventricle of the heart via the left carotid artery and left in situ during the experiment. Animals were sacrificed by intravenous injection of sodium pentobarbital (Euthesate; Apharmo, Arnhem, The Netherlands). Hearts were removed from which vegetations were aseptically isolated. Care was taken that vegetations were isolated without underlying endocardial tissue to avoid isolation of other potential sources of TF, such as adjacent endothelial cells. Isolated vegetations were handled as described previously (2). They were weighed and homogenized (5% [wt/vol]) in saline. Portions (100 μl) of serial dilutions of the homogenate were plated on blood agar to determine the number of CFU per gram of vegetation. The remainder of the homogenate was three times frozen in liquid nitrogen and thawed at 37°C in a water bath to lyse cells. To measure TFA, 25 μl of the homogenate with lysed cells was used. TFA was expressed as picomoles of FXa/gram of vegetation. The effect of *S. aureus* on vegetational TFA was assessed by comparing results of vegetations of noninfected control rabbits with those of infected rabbits either treated or not treated with teicoplanin. The MIC and minimal bactericidal concentration (MBC) of teicoplanin for *S. aureus* were determined as described earlier (3). The MIC was 0.25 μg/ml, while the MBC was 0.5 μg/ml. Serum concentrations of teicoplanin were determined at several time points with the Innufluor Reagent Set for the quantitative determination of teicoplanin (International Bioclinical, Inc., Portland, Ore.). Two hours after administration of the first teicoplanin dose, serum concentrations were already 16 times the MBC value. At 8 h the serum levels of teicoplanin were maximal, after which they dropped but at the time of administration of the second dose they were still 20 times larger than the MBC. For determination of bacterial numbers in the circulation, blood was drawn immediately before sacrifice and collected in vials containing crystalline potassium EDTA (Sherwood Medical, S-H Hertogenbosch, The Netherlands). Bacterial numbers were measured by colony count in serial dilutions of 200 μl of blood plated on blood agar and incubated overnight at 37°C.

The role of monocytes on vegetational TFA in rabbits with *S. aureus* BE was investigated by induction of selective monocytes with the cytostatic drug etoposide (Vepesid; kindly donated by Bristol-Meyers Squibb B.V., Woerden, The Netherlands) as described previously (2, 3). Etoposide treatment resulted in a significant decrease in the numbers of blood monocytes and of vegetational monocytes (18) without affecting other white blood cells or platelets, as shown by Meddens et al. (15).

Unpaired Student's t test was used to calculate the significance of difference of the TFA of monocytes by the different bacterium/monocyte ratios. To calculate the significance of the differences in the vegetational TFA, weight, and infection, the Bonferroni test was used.

In vitro the adherence of staphylococci to fibrin plates with inocula ranging from 10⁶ to 10⁸ CFU/ml was approximately 10%. This was somewhat higher than the 5% of *S. sanguis* NCTC 7864 (2) and the 7% of *S. epidermidis* ATCC 149900 (3). Fibrin-bound *S. aureus* stimulated monocytes to generate TFA. At a bacterium/monocyte ratio of about 1:1, this stimulation was maximal (Fig. 1), with the TFA being twofold higher than that of monocytes cultured without bacteria. At lower as well as higher ratios, the TFA was lower (Fig. 1). These findings were different from those with *S. sanguis* (2) and *S. epidermidis* (3). With these microorganisms an increase in the TFA was found with an increasing ratio, reaching a maximum at bacterium-to-cell ratios of 7:1 and 9:1, respectively, while at levels above these ratios the TFA remained at the high level. Supernatants of *S. aureus* cultured in the presence of teicoplanin induced monocytes to express TFA at the same level as was found for monocytes incubated on *S. aureus*-infected fibrin matrixes (Table 1).

This decline in monocytic TFA at a ratio exceeding 1:1 is probably due to progressive cell damage caused by *S. aureus*, which was apparent from less-adherent monocytes and many irregularly shaped cells in the supernatants when ratios exceeded 4:1. Moreover, PI influx in monocytes increased with increasing bacterium/monocyte ratios. The mean fluorescence intensity of monocytes adherent to noninfected fibrin matrix and of monocytes adherent to infected fibrin matrix at a ratio of 1:1 was approximately 34 AU. This fluorescence intensity increased approximately 14-fold at a bacterium/monocyte ratio of 6:1 and 35-fold when this ratio was increased to 60:1, indicating an increase in cell permeability, which is a measure of cell damage. This increase of PI influx coincided with a decrease of monocyte TF antigen expression (data not shown).
TABLE 1. Effect of teicoplanin on the TFA of monocytes incubated with S. aureus or staphylococcal constituents

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean fold increase in FXa formation ± SD</th>
<th>Without teicoplanin</th>
<th>With teicoplanin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.00c</td>
<td>1.25 ± 0.02d</td>
<td>0.59 ± 0.03e</td>
</tr>
<tr>
<td>S. aureus</td>
<td>1.59 ± 0.36a</td>
<td>1.65 ± 0.00D</td>
<td>1.63 ± 0.35f</td>
</tr>
<tr>
<td>Supernatant</td>
<td>1.13 ± 0.20c</td>
<td>2.12 ± 0.02g</td>
<td>1.07 ± 0.35f</td>
</tr>
</tbody>
</table>

\( ^{a} \) N = 10; \( ^{b} \) mean of N = 4; \( ^{c} \) mean of N = 3; \( ^{d} \) mean of N = 2; \( ^{e} \) mean of N = 1; \( ^{f} \) mean of N = 0.5

As with intact bacteria, the supernatant of S. aureus cultures exposed to teicoplanin induced an increase of PI influx in a concentration-dependent manner. In contrast to the findings for S. aureus, cell damage was not observed with either S. sanguis or S. epidermidis, even at the highest ratio tested (2, 3).

Control rabbits infected with S. aureus had bacterial numbers in the blood ranging from \( 8 \times 10^{2} \) to \( 7 \times 10^{3} \) CFU/ml. Infected vegetations were different in texture compared to sterile vegetations. They were easy to remove and were loose and flabby. The weight of the infected vegetations was significantly higher than that of sterile vegetations (44.26 ± 14.21 and 11.19 ± 5.07 mg, respectively; \( P = 0.002, n = 4 \)). Bacterial titers in the infected vegetations were more than \( 10^{13} \) CFU/g of vegetation. This was markedly higher than found with S. sanguis (5 \( \times 10^{12} \) CFU/g) and S. epidermidis (1 \( \times 10^{12} \) CFU/g) (2, 3). However, the TFA of the S. aureus-infected vegetations was similar to that of sterile vegetations (Table 2).

To investigate in vivo the importance of the bacterium/monocyte ratio on the vegetational TFA, catheterized infected rabbits were treated with teicoplanin. This treatment resulted in reduction of the bacterial numbers in the blood from up to \( 7 \times 10^{3} \) CFU/ml to a maximum of 30 CFU/ml. Moreover, bacterial numbers were reduced in vegetations to 8.63 ± 0.59 log CFU/g of vegetation after two doses and to 7.27 ± 0.5 log CFU/g of vegetation after four doses (Table 2). The TFA of vegetations from infected rabbits after two doses of teicoplanin was higher than that of vegetations from infected non-teicoplanin-treated rabbits. After four doses of teicoplanin, the vegetational TFA dropped below that of infected non-teicoplanin-treated rabbits, although the difference was not significant (Table 2). Teicoplanin treatment had no effect on vegetational weight (Table 2).

During etoposide treatment, blood monocyte numbers fell to 5 to 10% of the initial values within 2 days. At day 6 of etoposide treatment, 10^6 CFU of staphylococci were injected. Six of eight monocyteplenic rabbits died within 20 h. The two remaining rabbits were in a very poor condition and were consequently sacrificed. Bacterial counts in the blood were 5 \( \times 10^{6} \) CFU/ml. Most surprisingly, S. aureus-infected etoposide-treated rabbits had no vegetations at all neither on the valves nor the mural endocard. Valves were completely destroyed. Most probably the vegetations were released as septic emboli into the circulation as a result of the massive destruction of the valvular tissue. These findings were markedly different from those in etoposide-treated rabbits infected with S. sanguis or S. epidermidis (2, 3). These rabbits always had valvular vegetations. In rabbits with S. sanguis BE, monocytopenia led to a reduction of vegetational weight and a decrease of vegetational TFA, whereas with S. epidermidis neither vegetational weight nor TFA were affected. With both microorganisms, monocytopenia had no effect on the bacterial counts in the vegetation (2, 3).

In the non-etoposide-treated rabbits, infection with S. aureus had no effect on vegetational TFA. This result was different from the increase of vegetational TFA caused by S. sanguis and S. epidermidis. However, with the latter microorganisms in vitro monocytic TFA remained maximal above given bacterium/monocyte ratio, whereas for S. aureus monocytic TFA was maximal at a bacterium/monocyte ratio of 1:1 and decreased at higher ratios. This decrease can be accounted for by monocyte damage caused by S. aureus. Although infection of vegetations results in monocyte recruitment (18), the overwhelming bacterial numbers in the S. aureus-infected vegetations resulted in bacterium/monocyte ratios most probably exceeding the optimal ratio for induction of monocytic TFA. Also, monocytopenia, caused by S. aureus as shown by PI influx, could be a factor contributing to the lower vegetational TFA. Although not specifically considered here, an effect of the overwhelming bacteremia on platelet numbers could be an additional factor in the defective vegetation formation. This might also explain the difference in texture of the vegetations compared to those found with S. sanguis (2) or S. epidermidis (3) infection. Teicoplanin treatment reduced bacterial numbers in the vegetations and led to an increase of vegetational TFA after 2 days, but after 4 days of treatment the TFA again dropped. Apparently, reduction of bacterium titers initially lead to a more effective bacterium/monocyte ratio with respect to the TFA, but with a further decrease of the bacterial numbers in the vegetation this ratio again became suboptimal. Moreover, supernatants of S. aureus, incubated in the presence of teicoplanin, induced monocytes to express TFA. Thus, due to the decline in bacterial numbers induced by teicoplanin, not only the bacterium/monocyte ratio may have become more effective with respect to TFA but also the bacterial breakdown products, such as peptidoglycan or lipoteichoic acid, may have stimulated monocyte TFA. However, further reduction of the bacterial numbers after prolonged teicoplanin treatment apparently was more important than accumulation of bacterial breakdown products with regard to the TFA.

In conclusion, S. aureus can induce TFA on fibrin-adherent monocytes. Furthermore, monocytes play a protective role in

TABLE 2. Effect of S. aureus 5558 infection and teicoplanin treatment on TFA, infection, and weight of rabbit endocardial vegetations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infection (log CFU of vegetation)</th>
<th>TFA (pmol of FXa of vegetation)</th>
<th>Wt (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile</td>
<td>&gt;10^6</td>
<td>116 ± 4D</td>
<td>11.19 ± 5.07H</td>
</tr>
<tr>
<td>S. aureus</td>
<td>8.43 ± 0.59B</td>
<td>157 ± 1F</td>
<td>30.80 ± 11.07g</td>
</tr>
<tr>
<td>S. aureus plus 2× teicoplanin</td>
<td>7.27 ± 0.50C</td>
<td>85 ± 4G</td>
<td>36.63 ± 7.90d</td>
</tr>
</tbody>
</table>

\( ^{a} \) Endocarditis was induced as described in the Text. All values represent the mean ± the SD of four rabbits. \( P \) values (superscript letters): A versus B, \( P = 0.004 \); A versus C, \( P < 0.001 \); B versus C, \( P = 0.005 \); D versus E, \( P > 0.05 \); E versus F, \( P = 0.032 \); F versus G, \( P < 0.001 \); H versus J, \( P = 0.002 \); I versus K, \( P > 0.05 \); J versus L, \( P > 0.05 \).

\( ^{b} \) Teicoplanin treatment started 24 h after injection of staphylococci and was given at 24-h intervals.
S. aureus BE, which is a finding comparable to the findings with S. epidermidis BE made by Meddens et al. (15).

REFERENCES


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