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Enhancement of Generation of Monocyte Tissue Thromboplastin by Bacterial Phagocytosis: Possible Pathway for Fibrin Formation on Infected Vegetations in Bacterial Endocarditis

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The deposition of fibrin on infected vegetations and the presence of mononuclear phagocytes that have phagocytized bacteria are remarkable features in experimental bacterial endocarditis. In a study in vitro, we show that phagocytosis of bacteria by human monocytes enhances thromboplastin generation by these cells. Maximal enhancement of the generation of thromboplastin by monocytes was about six times compared with that in the control experiment without bacteria, and it was obtained by preincubation of the monocytes with 5 to 10 bacteria per monocyte. No quantitative difference was observed between *Staphylococcus epidermidis* and *Streptococcus sanguis* as to the enhancement of the monocyte thromboplastin generation. An enhancement of the procoagulant activity generation was also observed after addition of bacteria to human or rabbit whole blood. Probably, this generation was also due to synthesis of thromboplastin by monocytes. It is conceivable that fibrin deposition on infected vegetations during bacterial endocarditis is mediated by thromboplastin synthesis by monocytes.

Several morphological studies have demonstrated that fibrin deposition is a prominent feature of bacterial endocarditis (1-3). This deposition of fibrin might be important for the pathogenesis of bacterial endocarditis. Vegetations formed at areas of endocardial lesions (the so-called nonbacterial thrombotic endocarditis) consist predominantly of fibrin-platelet thrombi. These vegetations provide a suitable surface for adherence of bacteria (1, 3), as also do artificial platelet-fibrin thrombi (13). If bacteria are released into the bloodstream, they adhere specifically to these vegetations (4, 14, 15). Subsequently, the settled bacteria become covered by a fibrin layer, enabling the bacteria to multiply, almost unhindered by host defense mechanisms (2, 3).

The significance of fibrin formation for the development of bacterial endocarditis has been demonstrated by studies that showed that the anticoagulant warfarin had an inhibitory effect in vivo on the development of experimental endocarditis produced by *Staphylococcus epidermidis* (15). However, earlier studies with warfarin failed to demonstrate an effect in vivo on the development of experimental *Streptococcus sanguis* endocarditis (4, 14). This different response to anticoagulant treatment between rabbits infected with *S. sanguis* and those infected with *S. epidermidis* might be due to a difference between the microorganisms to induce local blood clotting.

Hitherto, it was unknown which factors (humoral, cellular, bacterial) induce the deposition of fibrin on infected endocardial lesions. Immune complexes and activated complement stimulate the generation of procoagulant activity (PCA) by human mononuclear leukocytes (9, 12). According to Durack, fibrin and mononuclear phagocytes that have phagocytized bacteria are simultaneously present on the surface of the infected vegetations in an early stage of infection (3). The critical step leading to fibrin formation on infected endocardial vegetations has never been defined. We postulate that monocytes that have been activated by phagocytosis of bacteria synthesize tissue thromboplastin (TP) which induces local formation of fibrin.

In this study in vitro, we demonstrate that phagocytosis by monocytes of *S. sanguis* and *S. epidermidis* enhances the generation of monocyte TP. To elucidate the different response of

anticoagulant treatment on the course of endocarditis in rabbits infected with *S. sanguis* and those infected with *S. epidermidis,* we examined whether the enhancement of such generation by phagocytosis differs quantitatively for *S. sanguis* and *S. epidermidis.*

MATERIALS AND METHODS

Isolation of leukocytes. Monocytes were isolated from normal human venous blood by centrifugation with Ficoll-Isopaque (density = 1.079 g/ml) as described previously (16). Of the leukocytes, 23 ± 4.5% ($\bar{x}$ ± standard deviation, n = 56) were monocytes and the remainder were lymphocytes, with less than 2% granulocytes. The leukocytes were resuspended in RPMI-1640 (GIBCO Laboratories, Grand Island, N. Y.) to which 1% (wt/vol) human albumin, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Mycopharm, Delft, The Netherlands) had been added. This suspension has been henceforth indicated as monocyte suspension despite the large contamination with lymphocytes. The suspension containing 10^6 monocytes per ml was used within 1 h after isolation.

Leukocytes were counted by a Coulter Counter (model ZF), and the percentage of monocytes was determined by electronic analysis of the leukocytes (8).

Lymphocytes were isolated from peripheral human blood by nylon wool filtration (11); purity was ≥98%.

Whole blood. Blood used for incubation experiments was collected as follows: 9 parts blood + 1 part 3.8% (wt/vol) trisodium citrate–3.7% (wt/vol) glucose. Human blood was obtained from the antecubital vein, and rabbit blood from the central artery of the ear. Immediately after blood collection, penicillin (100 U/ml) and streptomycin (100 μg/ml) were added.

Bacteriology. The microorganisms used were *S. sanguis* and *S. epidermidis* type S6 according to Baird Parker. These were the same strains as used previously in anticoagulation studies of experimental bacterial endocarditis (14, 15). *S. sanguis* was maintained in Tarozzi medium and cultured overnight in Todd-Hewitt medium, giving an average of 3.4×10^8 colony-forming units per ml (range, 0.5×10^8 to 10×10^8). *S. epidermidis* was maintained in diagnostic sensitivity test (DST; Oxoid, London) agar medium and cultured overnight in glucose broth, giving an average of 1.7×10^7 colony-forming units per ml (range, 10^7 to 3×10^7). Heat-killed bacteria were prepared by incubating the bacterial strains at 60°C for 2 h.

To determine bacterial numbers of the overnight cultures, 10-fold serial dilutions were made in 0.15 M NaCl, and 0.1-ml samples were plated on sheep blood agar plates (*S. sanguis*) or on DST-agar plates (*S. epidermidis*). After 24 h of incubation at 37°C, plates with 6 to 500 colonies were counted with an electric colony counter. Bacterial numbers were calculated from the means of two consecutive dilutions.

Effect of bacteria on the generation of monocyte TP. Overnight cultures of bacteria were preopsonized by incubation for 30 min at 37°C in 100% fresh human serum and washed three times in 0.15 M NaCl. Monocytes (10^6/ml) and various numbers of preopsonized bacteria were incubated for 10 min at 37°C in a shaking water bath. Thereafter, non-cell-associated bacteria were removed by repeated (3×) low-speed centrifugation (180 × *g*, 10 min). Next, the sedimented monocytes with adherent and intracellular bacteria were resuspended in the starting volume of RPMI-1640 supplemented with 1% human albumin, penicillin (100 U/ml), and streptomycin (100 μg/ml). A sample of this suspension was used for cytospin preparations and for electron microscopical examination. The residual monocyte suspension was incubated at 37°C in plastic tubes in a shaking water bath for periods up to 5 h. The pH remained 7.4 throughout this incubation. At the start and at given intervals, samples were taken for the determination of the TP activity.

Effect of bacteria on PCA generation by whole blood. PCA generation in whole blood was determined after heat-killed bacteria had been added to freshly collected human or rabbit blood. The blood with the bacteria was incubated for up to 5 h at 37°C under 5% (vol/vol) CO_2 in a shaking water bath. PCA was determined at zero time and at given intervals during the incubation.

Clotting assay. Experimental details of the assay of TP activity are described elsewhere (16). In short, TP activity of cell suspensions was measured by a modified one-stage recalcification time with normal human plasma as the substrate, except when otherwise indicated. The clotting times were converted into units of TP by using a calibration curve and a brain TP standard of our laboratory. This standard has an activity of 10^6 arbitrary units of TP per ml.

The PCA of whole blood samples were assayed by incubating 0.1 ml of sample with 0.1 ml of normal plasma (human or rabbit) for 1 min at 37°C, followed by the addition of 0.2 ml of $CaCl_2$ solution (0.025 M $CaCl_2$ + 0.075 M NaCl). Thereafter, the clotting time was recorded.

Anti-TP serum. Anti-TP serum was raised in rabbits against purified human brain TP. The purification of the antigen and the properties of the antiserum will be described elsewhere (Van Ginkel, in preparation). This antiserum blocked >99% of the clotting activity of brain TP and monocyte TP.

Electron microscopy. Cells were fixed with Karnovsky fixative, washed during 1 h with 0.1 M sodium phosphate (pH 7.4) at 4°C, and postfixed with 1% OsO_4 for 1 h at 4°C, by the method of Millonig. After dehydration in a series of increasing ethanol concentrations, the cells were embedded in Epon. Ultrathin sections were mounted on copper grids, stained with saturated aqueous uranyl acetate and lead citrate, and examined with a Philips EM-300 electron microscope.

Reagents. Cycloheximide and rabbit brain TP were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Dade Diagnostics (Miami, Fla.), respectively.

Lactate dehydrogenase (EC 1.1.1.27) was measured by the method of Wacker et al. (17).

Statistics. Statistical evaluation was performed on paired observations by Student's t test.

RESULTS

Enhancement of human monocyte TP generation by phagocytosis of bacteria. After 10-min preincubation of monocytes with

both strains of bacteria, a substantial number of bacteria was ingested by or adherent to monocytes. This was shown by electron microscopy (Fig. 1A and B) and by light microscopy of cytospin preparations stained with methylene blue. At this time, the PCA of the monocytes was still negligible. However, after a lag period of about 2 h, this activity gradually increased during further incubation of the cells. In control experiments without bacteria, an increased PCA

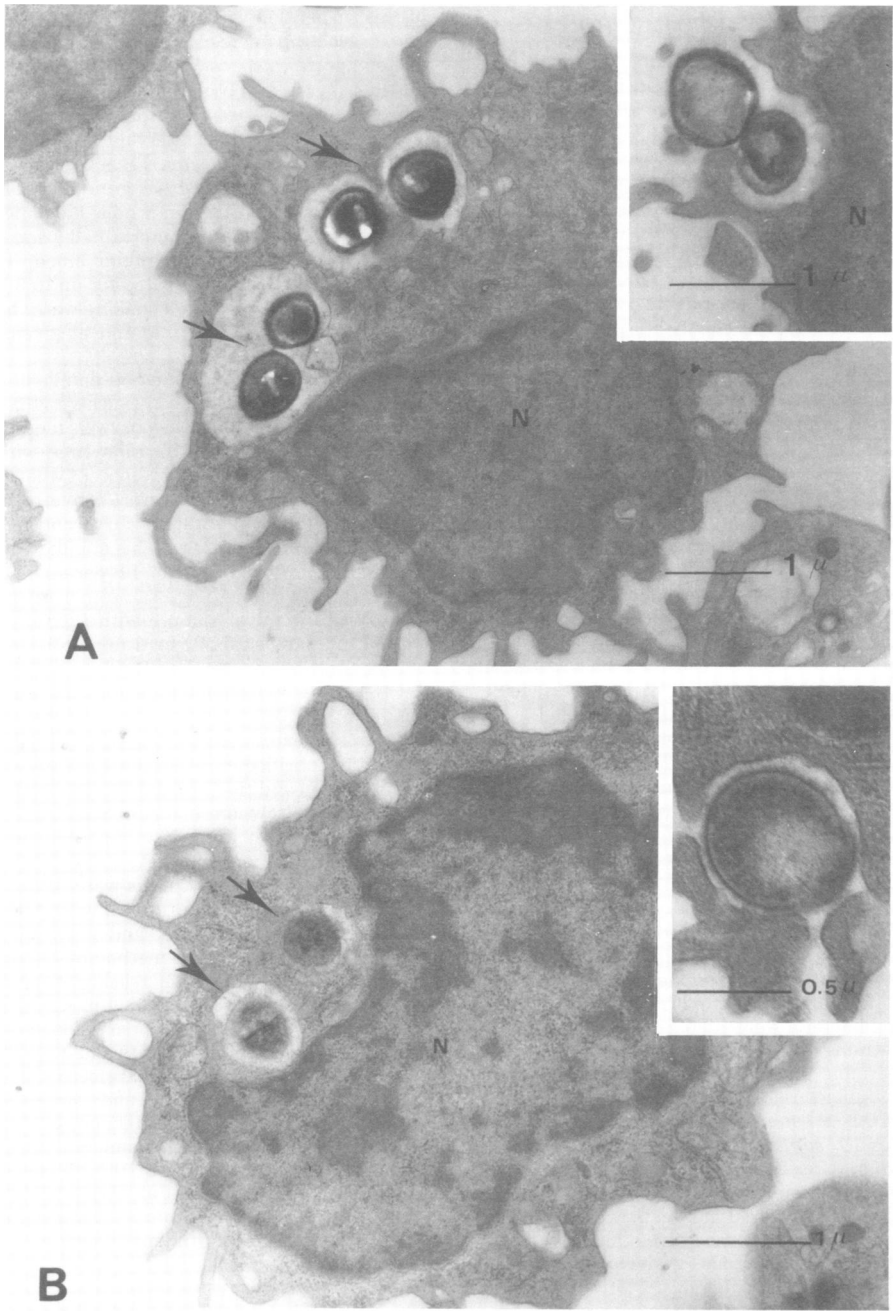

FIG. 1. *Electron micrographs of human monocytes that had been incubated with serum-preopsonized bacteria for 10 min (A, S. epidermidis; B, S. sanguis). The stage of adherence is demonstrated in the inset figures, and the central figures show the probable ingestion stage.*

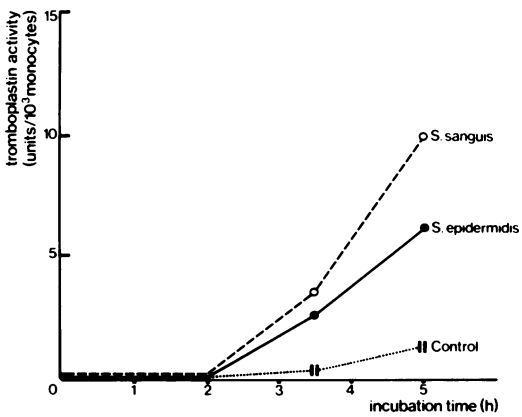

FIG. 2. *Enhancement of monocyte thromboplastin generation by phagocytosis of bacteria. After preincubation of human monocytes with serum-preopsonized bacteria (B/M ratio, about 10) the non-cell-associated bacteria were removed and the incubation of monocytes was continued. At intervals, the thromboplastin activity of the intact suspension was determined. The control experiment represents monocytes that had been preincubated with 0.15 M NaCl or with the supernatant of the serum-preopsonized bacteria. The results shown are representative for five individual experiments.*

was detectable only after 4 to 5 h (Fig. 2). The PCA of the monocytes that had been preincubated with bacteria for 5 h was about six times as much as that in the control experiment.

When purified lymphocytes were similarly incubated with bacteria, the PCA after incubation for 5 h was only 0.1% of the amount generated by monocytes. This demonstrates that in the cell preparation used presumably only monocytes contribute to PCA generation.

To determine the identity of the monocyte PCA, two sets of experiments were performed. By using plasma congenitally deficient in factor VIII and factor VII in the assay of the PCA, this activity was shown (Table 1) to be dependent on factor VII to shorten the recalcification time. Furthermore, the addition of heterologous antihuman TP serum almost completely (>99%) inhibited monocyte PCA (Table 2). These results demonstrate that monocytes generate TP-like PCA after phagocytosis of bacteria.

Enhancement of monocyte TP generation by phagocytosis of bacteria: dependency on B/M ratio. To investigate whether *S. epidermidis* and *S. sanguis* differ quantitatively in their capacity to enhance monocyte TP generation, monocytes were preincubated with these bacteria at ratios of about 0.1 to 100 bacteria per monocyte (B/M). After removal of the free bacteria, the monocytes were incubated for another 5 h, and the TP generation was measured. Figure 3A shows that, for both bacterial strains, the TP generation increased as the B/M ratio rose. However, at B/M ratios of more than 10, the TP generation decreased. A similar relationship between monocyte TP generation and B/M ratio was also observed after incubation times of 2.5 and 3.5 h.

The results of six separate experiments, with incubation periods of 5 h, are summarized in Table 3. These results show that no difference exists between *S. sanguis* and *S. epidermidis* as to the B/M ratio at which the TP generation is maximal. There was only a minor difference between the two bacterial strains for the maximally generated TP activity.

To investigate why monocyte TP activity was decreased at the highest B/M ratio tested (about

TABLE 1. *Effect of PCA of monocytes on the recalcification time*[a]

Sample	Recalcification time (s)		
	Factor VIII-deficient plasma	Factor VII-deficient plasma	Normal plasma
Buffer	>250	>250	>250
Brain TP	50	>250	51
Monocytes + *S. epidermidis*	48	223	52
Monocytes + *S. sanguis*	46	220	51
Monocytes	133	>250	123

[a] Substrates were normal plasma and plasmas deficient in factor VIII and deficient in factor VII. Monocytes were activated by phagocytosis of *S. epidermidis* and *S. sanguis* as described in the text. In control experiments, monocytes were treated in an identical way but in the absence of bacteria. Brain TP refers to an aqueous extract of acetone-dried human brain. The buffer was RPMI-1640.

TABLE 2. *Neutralization of the monocyte PCA by an antiserum raised in rabbits against purified human brain TP*[a]

Sample	Recalcification time (s)	
	Anti-TP serum	Normal rabbit serum
Brain thromboplastin	>250	33
Monocytes + *S. epidermidis*	184	34
Monocytes + *S. sanguis*	195	34
Monocytes	199	76

[a] The samples were incubated with the antiserum for 1.5 h at 37°C: 8 volumes of sample + 1 volume of antiserum. After incubation the procoagulant activity was determined by the recalcification time, with normal plasma as substrate. Normal rabbit serum was used as control.

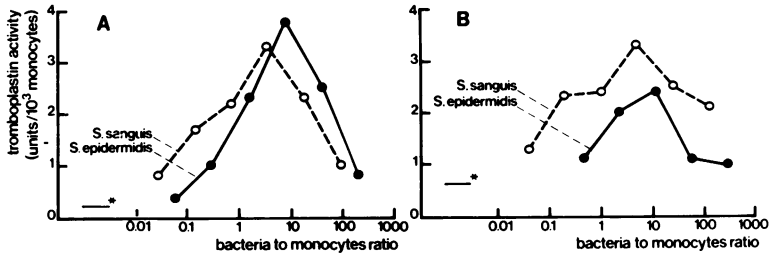

FIG. 3. *Effect of B/M ratio on generation of monocyte thromboplastin. Human monocytes were preincubated at various ratios with either viable (A) or heat-killed serum-opsonized bacteria (B). Subsequent experimental procedures were similar to those described in the legend to Fig. 2. The results shown are representative for six individual experiments with viable bacteria and for two experiments with heat-killed bacteria. *, Control experiments with monocytes preincubated without bacteria.*

TABLE 3. *Effect of B/M ratio on generation of monocyte TP*[a]

Organism	B/M ratio with maximal TP generation[b]	Maximal monocyte TP generation[b] (TP units/10^3 monocytes)
S. epidermidis	5.7 ± 1.0	6.9 ± 0.8
S. sanguis	5.8 ± 2.2	8.4 ± 1.4
	$P > 0.1$	$0.05 < P < 0.1$

[a] Monocytes were preincubated with viable bacteria at various B/M ratios. The free bacteria were removed, and TP activity was measured after subsequent incubation for 5 h.

[b] $\bar{x}$ ± standard error, $n = 6$.

100), the following experiments were performed.

(i) To test whether viable bacteria at B/M ratios of 100 are toxic for the TP-generating capacity of the monocytes, the experiment was repeated with heat-killed bacteria. Figure 3B shows that similar results were obtained as with viable bacteria. The possibility that monocytes incubated with bacteria at high B/M ratios are damaged was also investigated by measuring the release of the cytoplasmic enzyme lactate dehydrogenase. Release of this enzyme by monocytes after incubation with bacteria at a ratio of about 6 was lower than after incubation with bacteria at a ratio of 100 (Table 4). This indicates a decreased viability of the monocytes after incubation with bacteria at high B/M ratios.

(ii) To check whether anticlotting activity, or TP inactivators, or both are released at high B/M ratios, monocyte TP was incubated with supernatants from monocytes that had been preincubated with bacteria at B/M ratios of 6 or 100 for 10 min, followed by subsequent incubation of the monocytes for 5 h. Monocyte TP was obtained from monocytes activated as previously described (16). These supernatants had neither anticlotting activity nor TP-inactivating capacity.

Enhancement of PCA generation in whole blood by bacteria. Addition of bacteria to isolated human monocytes stimulated monocyte TP generation. Figure 4 shows that addition of bacteria to whole human blood also enhanced PCA generation. In these experiments, heat-killed bacteria were used, because during incubation with whole blood viable *S. sanguis* induced a notable decrease in pH, which might interfere with monocyte TP generation. Because it was difficult to isolate a sufficient number of monocytes from rabbit blood, PCA generation was only measured in rabbit whole blood. Addition of bacteria to rabbit whole blood resulted in an increase of PCA that was comparable to that found in whole human blood.

To investigate whether this effect in whole rabbit blood was due to generation of monocyte TP, we performed the following experiments (Fig. 5). (i) *S. epidermidis* was added to rabbit whole blood and to plasma derived from the same animal. The final number of *S. epidermidis* used in this experiment was 10^9/ml. After incubation, PCA generation was detectable only in whole blood. This indicates that a cellular component is necessary for the PCA generation in rabbit whole blood. (ii) After incubation of whole

TABLE 4. *Viability of the monocytes after phagocytosis*[a]

Incubation mixture	B/M ratio	Lactate dehydrogenase release	Significance
Control		5.6 ± 0.7 (4)	
S. epidermidis	6	8.4 ± 0.9 (3)	$0.05 < P < 0.1$
S. epidermidis	100	27.9 ± 6.1 (3)	
S. sanguis	6	10.7 ± 0.9 (4)	$0.01 < P < 0.025$
S. sanguis	100	15.6 ± 2.2 (4)	

[a] Monocytes were preincubated for 10 min without bacteria (control) or with bacteria at a B/M ratio of about 6 or 100. After removal of the non-cell-associated bacteria, the incubation of monocytes was continued for 5 h. Subsequently, lactate dehydrogenase was determined in the supernatant and expressed as percentage of the total enzyme content of the suspension ($\bar{x}$ ± standard error). The number of experiments is indicated in parentheses.

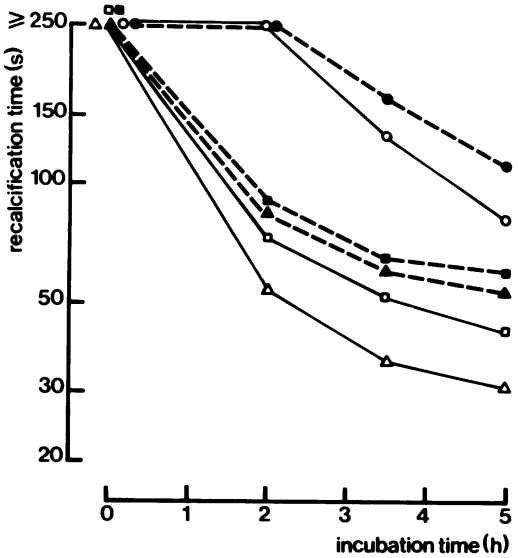

FIG. 4. *Generation of PCA by whole human or rabbit blood during incubation with heat-killed bacteria. The final number of bacteria in human blood was 2 × 10^8 colony-forming units per ml, and that in rabbit blood was 10 × 10^8 colony-forming units per ml. The recalcification time was determined after incubation with pooled human and rabbit plasma, respectively, as the substrate. Symbols: ●, human blood (control); ○, rabbit blood (control); ■, human blood + S. sanguis; ▲, human blood + S. epidermidis; □, rabbit blood + S. sanguis; △, rabbit blood + S. epidermidis.*

blood with *S. epidermidis* (10^9/ml), PCA was determined in whole blood as mentioned above and in the corresponding supernatant plasma. Because only minor PCA was found in the plasma, it can be concluded that PCA remained cell associated. (iii) Whole blood with *S. epidermidis* (10^9/ml) was incubated in the presence of the protein synthesis inhibitor cycloheximide (25 μg/ml). By using serial dilutions of rabbit brain thromboplastin, it was calculated that after 5 h of incubation, the generation of cycloheximide was decreased to 4% compared with the control without inhibition. This decrease indicates that protein synthesis is involved in the PCA generation in rabbit whole blood.

Similar experiments performed with *S. sanguis* gave results comparable to those obtained with *S. epidermidis*.

DISCUSSION

This study demonstrates that monocyte TP generation is stimulated by the interaction between bacteria and human monocytes. This interaction leads, via attachment, to ingestion and presumably to intracellular killing and digestion of the bacteria. Our results do not allow a conclusion as to which step in this chain of events is actually responsible for the stimulation of monocyte TP generation. Other investigators have shown that immunoglobulin G, antigen-antibody complexes, and C_{3b} stimulate TP generation by human leukocytes (9, 12). Therefore, one might speculate that C_{3b} and immunoglobulin G, which are possibly bound to serum-opsonized bacteria, contribute to the enhancement of monocyte TP generation. Endotoxin can also enhance monocyte TP generation (7, 10), but in our experiments only gram-positive bacterial strains were used and all the reagents were sterile. Moreover, the supernatant of the serum-preopsonized bacteria did not affect the TP generation by monocytes. This observation rules out the possibility that the enhancing effect is due to a humoral factor (e.g., endotoxin).

Recent studies have shown that phagocytizing monocytes and macrophages produce prostaglandins (5, 6). However, the contribution of prostaglandins to the TP generation by monocytes is unlikely because indomethacin (2 to 20 μg/ml), a known inhibitor of prostaglandin synthesis, has no effect on monocyte TP generation

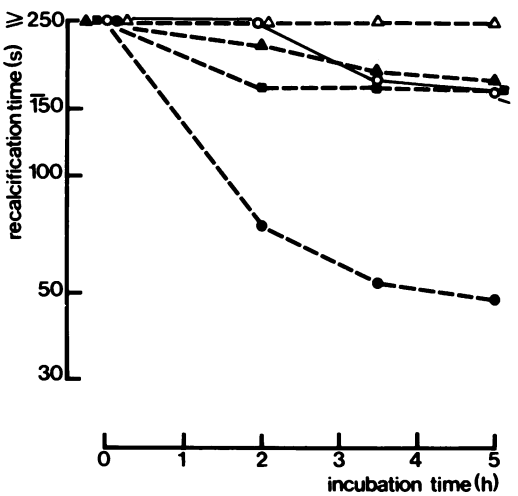

FIG. 5. *Generation of PCA during incubation of rabbit whole blood and plasma with heat-killed S. epidermidis. Blood and plasma were incubated with S. epidermidis for various intervals. Afterwards the recalcification time was determined, with pooled rabbit plasma as the substrate. In addition, the recalcification time of the supernatant (8,000 × g, 2 min) from whole blood that had been incubated with S. epidermidis was determined with pooled rabbit plasma as the substrate. Symbols: △, plasma + S. epidermidis; ▲, supernatant from rabbit blood + S. epidermidis; ■, rabbit blood + S. epidermidis + cycloheximide; ○, rabbit blood (control); ●, rabbit blood + S. epidermidis.*

(Van Ginkel, in preparation).

There was an optimal B/M ratio at which maximal TP generation occurred (Fig. 3). The cause of the decrease of TP generation at B/M ratios above the optimal ratio is not clear. Release of anticlotting or TP-inactivating activity, or both, to account for this effect could be excluded. Moreover, the comparable results of TP generation by monocytes enhanced by viable and heat-killed bacteria exclude a toxic effect of the bacteria on the moncytes, an effect specific for viable bacteria. It is conceivable, however, that the monocytes are less viable at high B/M ratios, resulting in a decreased capacity to generate TP. This possibility is supported by the increased lactate dehydrogenase release from the monocytes at this B/M ratio (Table 4). However, in the experiments with *S. epidermidis,* lactate dehydrogenase from the bacteria may have interfered with the results because the lactate dehydrogenase content of *S. sanguis* is negligible (unpublished data).

The enhancement of monocyte TP generation by phagocytosis of bacteria not only occurred with isolated human monocytes, but also with human or rabbit whole blood (Fig. 4). In this study, we did not prove that PCA generation in rabbit whole blood is due to TP generation by rabbit monocytes. However, the following observations (Fig. 5) are compatible with the assumption that monocytes in rabbit whole blood are responsible for TP synthesis: (i) a cellular component is necessary for the PCA generation in rabbit whole blood; (ii) the generated PCA remains cell associated; (iii) the PCA generation can be inhibited by cycloheximide, which indicates the involvement of protein synthesis; and (iv) the PCA generation can be enhanced by bacteria. Because these four characteristics of PCA generation applied to TP synthesis by human monocytes, it is reasonable that our observations with human monocytes are also valid for rabbit monocytes.

Morphological studies by Durack have shown that bacteria injected intravenously into rabbits adhere specifically to the surface of the preexisting vegetations (2, 3). At 30 min after bacterial challenge, *S. sanguis* was found predominantly inside mononuclear phagocytes on the vegetations, whereas *S. epidermidis* was located extracellularly on the surface of the vegetations. After 24 h, colonies of bacteria are observed in the vegetations covered by fibrin layers in a sandwich-like manner (2, 3). Which critical step induces local fibrin formation on the surface of infected vegetations has never been defined. Combining the observations of Durack with our findings, we hypothesize that phagocytosis of bacteria by monocytes, which are numerously present on infected vegetations shortly after bacterial challenge, stimulates these cells to generate TP. This TP remains associated with the outside of the monocyte and locally induces fibrin formation. Some bacteria, associated with or close to monocytes on the surface of vegetations, survive and multiply while protected against host defense mechanisms by a fibrin layer.

Hook and Sande (4) and Thompson et al. (14) demonstrated that experimental *S. sanguis* endocarditis is not influenced by anticoagulant treatment, and Thörig et al. (15) observed an inhibitory effect of warfarin treatment on experimental *S. epidermidis* endocarditis. However, these divergent effects of warfarin treatment on the induction and course of endocarditis caused by *S. sanguis* and *S. epidermidis* cannot be explained by the results of this study in vitro. We found no difference between the bacteria in relation to the enhancement of the monocyte TP generation.

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