Cellular origin and procoagulant properties of microparticles in meningococcal sepsis


Patients with meningococcal sepsis generally suffer from disseminated intravascular coagulation (DIC). The aim of this study was to address whether these patients have elevated numbers of circulating microparticles that contribute to the development of DIC. Plasma samples from 5 survivors, 2 nonsurvivors, and 5 healthy volunteers were analyzed for the presence of microparticles by flow cytometry. Ongoing coagulation activation in vivo was quantified by enzyme-linked immunosorbent assay of plasma prothrombin fragment F$_{1+2}$, and procoagulant properties of microparticles in vitro were estimated by thrombin-generation assay. On admission, all patients had increased numbers of microparticles originating from platelets or granulocytes when compared with controls (P = .004 and P = .008, respectively). Patients had elevated levels of F$_{1+2}$ (P = .004), and their microparticles supported thrombin generation more strongly in vitro (P = .003) than those of controls. Plasma from the patient with the most fulminant disease course and severe DIC contained microparticles that expressed both CD14 and tissue factor, and these microparticles demonstrated extreme thrombin generation in vitro. We conclude that patients with meningococcal sepsis have elevated numbers of circulating microparticles that are procoagulant. These findings may suggest a novel therapeutic approach to combat clinical conditions with excessive coagulation activation. (Blood. 2000;95:930-935)

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Introduction

Meningococcal sepsis is a life-threatening disease that occurs most frequently during childhood and is characterized by excessive activation of many cells and cascades, which results in disseminated intravascular coagulation (DIC). Although it is clear that the cascade of inflammatory and clotting reactions is triggered by the meningococcal bacteria, particularly by their release of endotoxin, the precise mechanisms underlying these reactions are not well understood. For example, the mechanism underlying the development of DIC, a typical threatening feature of meningococcal sepsis, is unknown.

Clotting requires the presence of phospholipid cofactors that serve as a surface to assemble the various complexes to activate the clotting factors. In vitro studies have shown that activated platelets, and in particular microparticles generated from them, contain a large number of binding sites for activated factor IX (IXa),3 factor Va,4 and factor VIII5 and support both factor Xa activity6,7 and prothrombinase activity.5,7 Increased numbers of platelet-derived microparticles are present in the circulation of patients who have an increased risk for thromboembolic complications, such as patients undergoing cardiac surgery8 or plasmapheresis9 and in patients suffering from diabetes,10 heparin-induced thrombocytopenia,11 myocardial infarction,12 uremia,13 idiopathic thrombocytopenic purpura,14 or thrombotic thrombocytopenic purpura.15 Functional studies of these microparticles were not performed. Other studies about the presence of microparticles of nonplatelet origin in the circulation have not been reported thus far. We have shown recently that elevated levels of platelet- and erythrocyte-derived microparticles are present in wound blood collected directly from the pericardial cavity in patients undergoing cardiac surgery.16 These in vivo–generated microparticles strongly bind annexin V, a protein known for its interaction with negatively charged phospholipids such as phosphatidylserine, one of the essential lipid cofactors for clotting. Upon addition to normal plasma, these microparticles supported the generation of thrombin by a tissue factor–factor VII-mediated pathway. Hence, microparticles may be involved in activation of the systemic coagulation in vivo.

In the present study, we investigated the presence, cellular source, and function of circulating microparticles in patients suffering from meningococcal sepsis. Plasma samples of these patients were analyzed for the presence of microparticles (flow cytometry) and their procoagulant activity (thrombin generation assay) and were compared with the number and properties of microparticles in plasma from 5 healthy volunteers. Our results show that patients with meningococcal sepsis have elevated numbers of circulating microparticles derived from various blood cells and that these microparticles support clotting. We suggest that extreme DIC is strongly linked to circulating tissue factor–expressing microparticles in this disease and possibly also in other clinical conditions with excessive coagulation activation.
Materials and methods

Reagents and assays
Reptilase was obtained from Boehringer Mannheim (Mannheim, Germany), thrombin chromatographic substrate S2238 from Chromogenix AB (Möln达尔, Sweden), and normal mouse serum and fluorescein isothiocyanate (FITC)-labeled anti-CD4 (anti-CD4–FITC) from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands). Anti-lymphocytoma virus (anti-LYV) and anti-CD61–FITC were obtained from Dakopatts (Glostrup, Denmark). Mouse IgG1-FITC, IgG2a-FITC, IgG2b-FITC, IgG3-FITC, IgG2a–phycoerythrin (PE; all used as controls), anti-CD14–PE, anti-CD8–FITC, and anti-CD20–FITC monoclonal antibody (mAb) were from Becton Dickinson (San Jose, CA). Annexin V–FITC was from Nexins Research B.V. (Hoeven, The Netherlands), anti-factor VII and anti-factor XII were from CLB, and anti–E-selectin–FITC (anti-CD62E–FITC) was from Serotec Ltd (Kidlington, UK). Anti-CD14–FITC was from Biosource (Camarillo, CA), anti-C66b–FITC and IgG2a–PE (control mAb) were from Immuno Quality Products (Groningen, The Netherlands), and anti–tissue factor–FITC and polyclonal rabbit anti-human tissue factor were from American Diagnostics (Greenwich, CT). Annexin V–PE was from Pharmingen (San Jose, CA). F1 (1–132 mg/L) was determined by enzyme-linked immunosorbent assay (Enzymognost F1 from PharMingen (San Jose, CA). F1 was preincubated separately for 30 minutes at room temperature before the addition. Plasma samples were stored in aliquots at −70°C until use. All plasma samples from a single patient were tested in the same experiment to avoid day-to-day variation of the flow cytometer between samples from 1 patient. Healthy volunteer samples, collected in the same period and stored identically, were used for comparison.

Flow cytometric analysis
For flow cytometry, 250 µL of plasma was centrifuged for 15 minutes at 15 500 g at room temperature. Plasma samples were stored in aliquots at −70°C until use. All plasma samples from a single patient were tested in the same experiment to avoid day-to-day variation of the flow cytometer between samples from 1 patient. Healthy volunteer samples, collected in the same period and stored identically, were used for comparison.

Thrombin generation by microparticles
The thrombin generation assay as described by Kessels et al was used to assess the in vitro thrombin-generating capacity of microparticles. To prepare normal plasma, we collected citrate-anticoagulated blood from 40 healthy volunteers who had not taken any medication during the previous 10 days. Plasma was prepared by centrifugation for 15 minutes at 1550 g at room temperature. The plasma samples were pooled and treated with reptilase (40 µL per 2 mL plasma) for 10 minutes at 37°C and then for 10 minutes on melting ice. Subsequently, fibrin and microparticles were removed by centrifugation for 1 hour at 17 500 g (20°C), and plasma was stored in 1-ml aliquots at −70°C until use. Microparticles were prepared as described in flow cytometric analyses. At t = 0, thrombin generation was started by the addition of 30 µL CaCl2 (17 mmol/L) to 120 µL of the prewarmed (37°C) normal plasma, to which 20 µL of buffer A (50 mmol/L Tris-HCl, 100 mmol/L NaCl, pH 7.35) and 10 µL of the washed microparticle suspension had been added. At fixed intervals after t = 0, 3 µL portions were removed from this mixture and added to prewarmed (37°C) buffer A containing 4 mmol/L of the chromogenic substrate S2238 and 20 mmol/L EDTA (to block further thrombin generation). After 180 seconds, the conversion of S2238 was stopped by the addition of 90 µL citric acid (1.0 mol/L), and the generated p-Nitroaniline was determined on a spectrophotometer at λ = 405 nm. For inhibition experiments, mAbs (anti–tissue factor factor VII, or anti–factor XII; 0.1 mg/mg) were added to both plasma (20 µL) and microparticles (10 µL), which were preincubated separately for 30 minutes at room temperature before the microparticles were added in the thrombin generation assay.

Statistical methods
Data were analyzed with SPSS for Windows, release 8. Differences were considered statistically significant at P < .05. For direct comparison of the number of microparticles in blood samples, the Wilcoxon matched-pairs signed-rank test was used.

Results

Number and cellular origin of circulating microparticles in patients with meningococcal sepsis
Microparticles were isolated from plasma samples, labeled, and analyzed by flow cytometry as described in “Materials and Methods.” Figure 1 shows a representative picture of microparticles stained with annexin V, which binds to negatively charged phospholipids and can be used to stain microparticles, and anti-CD66b, which labels granulocytes. To correctly identify
annexin V–positive microparticles, we determined a threshold in a microparticle sample that was prepared without any additions to correct for intrinsic autofluorescence (Figure 1A). This threshold is also depicted in panels B, E, and F. To identify microparticles that bound cell-specific mAbs, we also incubated microparticles with identical concentrations of control antibodies to set a threshold. This threshold is shown in Figure 1C and is also used in panels D, E, and F. Labeling with anti-CD66b revealed that part of the microparticles originated from granulocytes (Figure 1D) and that the microparticles also bound annexin V (Figure 1E, upper right). Figure 1F shows the virtual absence of granulocyte-derived microparticles in a representative dot plot of microparticles from a healthy volunteer.

Table 1 summarizes the numbers of circulating microparticles, identified by staining with annexin V–PE and anti-CD mAb-FITC, in patients at study entry (n = 7) and healthy volunteers (n = 5). For these experiments, a panel of mAbs was used directed against T \( \delta \) cells (CD4), T \( \delta \) cells (CD8), monocytes (CD14), B cells (CD20), platelets (CD61), endothelial cells (CD62E), granulocytes (CD66b), and erythrocytes (glycophorin A). Compared with healthy volunteers, the patients had significantly increased numbers of circulating platelet (CD61)-derived and granulocyte (CD66b)-derived microparticles at study entry. Monocyte (CD14)-, B cell (CD20)-, and endothelial cell (CD62E)-derived microparticles were also increased, although the difference was not statistically significant. Notably, the nonsurviving patient A, who died on admission after a disease course of less than 18 hours, had the highest plasma levels of microparticles derived from T \( \delta \) cells, monocytes, B cells, endothelial cells, platelets, granulocytes, and erythrocytes. The other nonsurvivor showed no marked differences in microparticle numbers when compared with patients who survived meningococcal sepsis.

**Time course of circulating microparticles**

The time course of microparticle numbers in the patients is given in Figure 2. In all 5 survivors, the number of granulocyte-derived...
microparticles decreased during the first 10 hours after admission (Figure 2, upper left). During the first 10 hours, the number of monocyte-derived microparticles (CD14; Figure 2, middle left) increased slightly in 4 of the survivors, although this increase was not significant ($P = .117$ at 24 hours and $P = .462$ at 36 hours). In contrast, microparticles originating from platelets, erythrocytes, or endothelial cells (Figure 2; upper right, lower right, and lower left, respectively) showed no apparent changes during the observation period.

### Relation of circulating microparticles to coagulation in vivo and in vitro

To investigate whether the microparticles detected in the patients promoted coagulation, we first measured the activation of the coagulation system in vivo by assessing the concentration of the prothrombin fragment $F_{1+2}$ in plasma during the course of the disease, which reflects the concentration of thrombin formed in vivo.20 Table 2 shows that the concentration of $F_{1+2}$ on admission was higher in the patients than in volunteers ($P = .004$). This difference was still present at 24 hours ($P = .016$), but not thereafter. The concentration of $F_{1+2}$ in survivors decreased slowly after admission, becoming significantly lower than baseline values at 36 hours ($P = .016$), indicating progressively less activation of coagulation in vivo. To substantiate a link between coagulation activation and microparticles, we studied the thrombin-generating capacity of isolated microparticles from patients and healthy volunteers in vitro. Virtually all microparticle preparations of the patients generated more thrombin than those of healthy controls (data are summarized in Table 2).

Microparticles released from monocytes in vitro express tissue factor,21,22 so we investigated whether such particles also circulate in vivo. Non-survivor A, who suffered from severe DIC as evidenced by a low platelet number ($7 \times 10^{9}/\mu L$), a prolonged prothrombin time (> 60 seconds; normal range, $< 14.5$ seconds), a decreased fibrinogen level (< 0.1 g/L; normal range, 1.7-3.7 g/L), the presence of fibrin degradation products, and a prolonged activated partial thromboplastin time (> 120 seconds; normal range, < 36 seconds), had extremely high numbers of monocyte-derived microparticles (CD14 positive). These microparticles double stained for tissue factor (Figure 3A). In contrast, microparticles of non-survivor B, with a less fulminant disease course (platelet number $258 \times 10^{9}/\mu L$, prothrombin time 17.1 seconds, fibrinogen 2.5 g/L, presence of fibrinogen degradation products, and activated partial thromboplastin time of 42.9 seconds), hardly stained for CD14 or tissue factor (Figure 3B).

As indicated in Figure 4A, the addition of microparticles from non-survivor A to normal plasma resulted in a strong generation of thrombin, whereas those of non-survivor B induced only modest thrombin generation. The generation of thrombin in normal plasma by the microparticles from non-survivor A was extremely delayed when both microparticles and the normal plasma were preincubated with mAbs against tissue factor or factor VII (Figure 4B), indicating that the microparticle-associated tissue factor is active and stimulates the extrinsic pathway of the coagulation system. In contrast, preincubation with OT-2, a mAb that inhibits factor XIA and blocks kaolin-induced thrombin generation in normal plasma (data not shown), had no effect.

### Discussion

This study shows that patients with meningococcal sepsis have elevated numbers of microparticles originating from various cell populations in their circulation. These microparticles evoked a stronger generation of thrombin in normal plasma than those from volunteers, suggesting that the elevated numbers of microparticles in the circulation or their cellular origin may be related to the increased activation of the coagulation system in vivo. It could be argued that the increased procoagulant activity of the patients’ microparticle fractions is mainly due to the increased number of microparticles and not to their cellular origin or properties. Because we wished to estimate the total procoagulant activity of the fractions, the microparticles were not diluted to a standard concentration in the thrombin generation assay. In addition, dilution cannot be done easily because the cellular composition of microparticles varied among patients, by the course of the disease, and most likely also by the type of disease. We also demonstrated tissue factor on microparticles, especially in a patient with an extremely fulminant course of DIC, which was functional in the thrombin generation assay as established with specific activity-blocking antibodies to tissue factor, factor VII, and factor XII.

Tissue factor is a transmembrane protein, the extracellular domain of which functions as a receptor for factor VII.23 Binding of factor VII to tissue factor is a first step in a series of events in which soluble coagulation proteins become assembled on a phospholipid surface. Evidence that tissue factor is important for coagulation and inflammation in vivo comes from a number of animal studies. Infusion of recombinant activated factor VII into normal chimpanzees raised the plasma levels of activation peptides of factor IX, factor X, and prothrombin. This was blocked by the administration of an anti–tissue factor mAb.24 Infusion of endotoxin reduced the

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### Table 2. Thrombin generation in plasma of patients with meningococcal sepsis in vivo and by microparticles in vitro

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>$F_{1+2}$ (nmol/L)</th>
<th>$N_{1}/N_{1}$</th>
<th>$P$</th>
<th>TGT (OD 405 nm)</th>
<th>$N_{1}/N_{1}$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.5 (4.1-10.0)</td>
<td>7/7</td>
<td>.004</td>
<td>0.105 (0.088-0.149)</td>
<td>7/7</td>
<td>.003</td>
</tr>
<tr>
<td>4</td>
<td>4.1 (3.4-20.0)</td>
<td>5/5</td>
<td>.009</td>
<td>0.080 (0.070-0.132)</td>
<td>5/5</td>
<td>.009</td>
</tr>
<tr>
<td>10</td>
<td>5.5 (2.1-8.6)</td>
<td>4/5</td>
<td>.028</td>
<td>0.084 (0.068-0.106)</td>
<td>5/5</td>
<td>.009</td>
</tr>
<tr>
<td>16</td>
<td>3.8 (2.2-12.7)</td>
<td>4/5</td>
<td>.016</td>
<td>0.100 (0.090-0.116)</td>
<td>5/5</td>
<td>.009</td>
</tr>
<tr>
<td>24</td>
<td>4.2 (2.4-4.9)</td>
<td>4/5</td>
<td>.016</td>
<td>0.070 (0.055-0.079)</td>
<td>3/5</td>
<td>.117</td>
</tr>
<tr>
<td>36</td>
<td>3.2 (1.4-8.3)</td>
<td>4/5</td>
<td>.140</td>
<td>0.078 (0.067-0.142)</td>
<td>4/4</td>
<td>.014</td>
</tr>
<tr>
<td>Volunteers</td>
<td>2.1 (1.1-2.9)</td>
<td>—</td>
<td>—</td>
<td>0.057 (0.044-0.063)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Time after admission (t = 0).
†$N_1$ indicates the number of patient plasma samples that contained a higher concentration of $F_{1+2}$ than the highest concentration found in healthy volunteers; $N_1$ indicates the total number of patient samples tested.
‡$P$ indicates thrombin generation test.
§$N_1$ indicates the number of patient microparticle fractions that generated more thrombin than the highest of the volunteer samples tested.
#Median (range).
A number of platelets in rabbits and decreased the concentrations of fibrinogen, antithrombin, and factor VIII, whereas it prolonged the activated partial thromboplastin time. These changes were counteracted by tissue factor pathway inhibitor. Infusion of *Escherichia coli* into baboons caused sepsis with severe DIC, which could be prevented by concurrent infusion of tissue factor pathway inhibitor. Although these studies clearly indicate the importance of tissue factor for the development of DIC and sepsis, the location of functionally active tissue factor expression is not well known. Our results suggest that tissue factor exposed by microparticles, particularly those released by monocytes, may be important in this respect. However, another component required for the assemblage of the coagulation factor complexes on the phospholipid surface is phosphatidylserine, which is not exposed on normal cells but on, for example, activated platelets. In the present study, microparticles stained positive for annexin V, indicating the presence of phosphatidylserine on their surfaces. Presumably, this also explains in part the thrombin-generating capacity of circulating microparticles. The relative contributions of phosphatidylserine and tissue factor to coagulation activation remain to be established. Possibly, the presence of tissue factor may enhance the coagulation activation associated with phosphatidylserine.

Monocytes are the only cells found in peripheral blood currently known to be capable of expressing tissue factor. Isolated monocytes stimulated by endotoxin express tissue factor. Under flow conditions, endotoxin-stimulated monocytes stimulate fibrin deposition and thrombus formation. Anti–tissue factor mAb inhibits both of these processes. Monocytes have been shown to express tissue factor in patients suffering from invasive tumors, leukemia, sepsis, myocardial infarction, and diabetes, and in patients requiring extracorporeal circulation. In addition to expressing tissue factor, monocytes can release tissue factor–exposing microvessicles in vitro upon stimulation with endotoxin. Recently, Mallat et al recently reported the presence of membrane vesicles of monocytic and lymphocytic origin that retained tissue factor activity in atherosclerotic plaques. Our present results extend these findings and demonstrate, for the first time, that procoagulant microparticles of monocyte, granulocyte, and endothelial cell origin can be detected in the circulation. It is interesting that of the 7 patients studied, only the patient with severe DIC had an extremely elevated (7-fold increase) number of endothelial cell–derived microparticles (244 × 10⁶/L) compared with controls. Although we cannot exclude the possibility that these microparticles also express tissue factor, 85% of the tissue factor–positive microparticles were CD14 positive. We therefore presume that the contribution of tissue factor to the overall procoagulant activity is especially due to the increased number of monocyte-derived microparticles.

The samples analyzed in the present study had been collected between 1989 and 1993. This may raise concerns about the validity of the present findings (i.e., the microparticle profile). Therefore, microparticles were also isolated immediately after blood collection from 2 patients with sepsis and multiple organ failure and 2 healthy controls. Both patients clearly showed granulocyte-derived microparticles, which were absent or present at low numbers in the controls. The numbers of monocyte- and endothelial cell–derived microparticles were not increased in these patients compared with the controls (data not shown). On the basis of these preliminary data, we cannot make definitive conclusions about the presence or absence of either monocyte- or endothelial cell–derived microparticles in other diseases, but we hypothesize that such microparticles may be especially prevalent in patients with severe DIC. Combes et al recently showed the presence of endothelial cell–derived microparticles in blood from healthy individuals and their increased presence in blood obtained from patients with lupus anticoagulant. Thus, the presence of endothelial cell–derived microparticles is evidently not unique for patients with meningococcal sepsis. Combes et al, however, did not report the presence of tissue factor on these microparticles.

All patients studied had elevated numbers of microparticles. It is tempting to speculate that interference with the release of microparticles may be a target for therapeutic intervention. Recently, an mAb against the glycoprotein IIb-IIIa complex on platelets, which inhibits platelet–platelet interaction or aggregation, has been used successfully in patients undergoing stent implantation. In vitro, this mAb prevents the release of microparticles from platelets. Our findings imply that this mAb may inhibit clotting in vivo as well. Indeed, infusion of this mAb into baboons with lethal *E. coli* sepsis prevented fibrin deposition and renal insufficiency. Thus, therapeutic interference with microparticle release in general, and possibly of platelets in particular, seems a realistic option.

In conclusion, elevated levels of microparticles were observed in patients with meningococcal sepsis. These microparticles enhanced coagulation by providing a suitable phospholipid surface and, at least in part, by exposing tissue factor. We suggest that such microparticles are involved in the pathogenesis of DIC during meningococcal sepsis and may constitute a novel target for therapeutic intervention in this disease, and possibly also in other clinical conditions with enhanced coagulation activation.
References

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