Original Article

Flow Cytometric Analysis of Microparticle Phenotype and Their Role in Thrombin Generation

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Background: Microparticles may be generated from a number of cell types and are known to play a role in haemostasis by a variety of mechanisms. We investigated the role of platelet, red cell, and leucocyte-derived microparticles in the measurement of thrombin generation.

Methods: Four parameters of thrombin generation (the endogenous thrombin potential (ETP), lag time, time to peak, peak height) and microparticle content was determined in 35 plasma samples from normal individuals pre and post filtration to remove microparticles. Immunofluorescent flow cytometry was used to identify and enumerate platelet, leucocyte, monocyte and red cell derived microparticles in plasma samples based on the expression of CD42b, CD45, CD15, and Glycophorin A respectively. Expression of phosphatidylserine and tissue factor by microparticles was determined by Annexin V and anti CD142 binding. The pre and post filtration results were compared.

Results: There was a significant decrease in ETP and Peak Height, and an increase in the time to peak post filtration ($P < 0.001$). A significant decrease in the number of CD42+, CD45+, CD15+, CD142+, and Annexin V+ microparticles was also observed. The change in CD42b+ microparticles correlated highly with the change in Annexin V+ microparticles ($r = 0.68$). Whilst the change in ETP correlated best with the change in CD15+ microparticles ($r = 0.45$) and the change in time to peak correlated with the change in Annexin V binding ($r = 0.52$) ($P < 0.01$).

Conclusion: The presence of micropartcles in plasma significantly affects thrombin generation. © 2010 Clinical Cytometry Society

Key terms: plasma filtration; calibrated automated thrombogram® (CAT) thrombin generation assay; CD15; CD45

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Microparticles are vesicles derived from the budding of cell membranes of multiple cell types including: platelets, endothelial cells, monocytes, granulocytes, and erythrocytes. Typically they are <1 μm in diameter (1). Platelet derived microparticles are by far the most abundant in the circulation, constituting between 70 and 90% of the total number of microparticles (2). Recent studies have shown that some of these microparticles are produced directly by megakaryocytes (1).

The presence of phosphatidylserine on the surface of microparticles provides binding sites for a number of activated clotting factors including those associated with both the tenase and prothrombinase complexes (3,4). Expression of phosphatidylserine by microparticles has been shown to be necessary for generation of thrombin (5). There is also a large body of evidence that microparticles express tissue factor, a potent activator of the coagulation cascade. The exact cellular origin of tissue factor expressing microparticles is the subject of some

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debate, while it is clear that monocytes can produce microparticles enriched with tissue factor (6), it was shown that 90% of tissue factor expressing microparticles in the circulation of healthy volunteers originated in equal shares from microparticles derived from platelets and monocytes (6).

The involvement of microparticles in haemostasis may also not be limited to thrombus formation, as microparticles have also been shown to express negative regulators of thrombosis such as thrombomodulin, TFPI (7), and Protein C (8). It is therefore likely that the development of a thrombus is dependent on the relative signal strengths of the procoagulant and anticoagulant factors.

The physiological roles of microparticles are varied, but since they are known to be implicated in thrombosis, this intuitively suggests that they probably have an effect on a patients capacity to generate thrombin. However, the most appropriate method to measure thrombin generation is uncertain. The endogenous thrombin potential (ETP) has been developed as one method with the advantage that it measures the composite effect of the multiple factors that determine coagulation capacity (9). In this assay coagulation is initiated ex vivo with the addition of tissue factor to patient plasma and thrombin generation is continuously registered by measuring cleavage of a fluorogenic substrate. A thrombin generation curve is produced from which parameters such as peak thrombin generation and the area under the thrombin generation curve (the ETP) are derived. Such measures have been used to predict, for example, risk of venous thromboembolism (10). The aim of this investigation was therefore to determine the impact that microparticles have on thrombin generation as measured by the Calibrated Automated Thrombogram® (CAT) thrombin generation assay. We routinely double centrifuge citrated blood samples for use in the CAT assay. However we investigated by flow cytometry whether such samples contained residual microparticles. We found that they did and decided to investigate whether further filtration of the double centrifuged sample removed the residual microparticles and what effect this may have on endogenous thrombin potential. We report our findings.

MATERIALS AND METHODS

Blood Samples for Normal Ranges

To establish whether age or sex affected thrombin generation, blood samples (80) were collected from either normal healthy staff working in the Department of Haematology or from healthy transplant donors at The Royal London Hospital. Ethics approval for the study had been obtained and all individuals gave informed consent. Using both parametric and non parametric statistics we showed that age and sex do not affect the thrombin generation parameters investigated (Table 1).

Table 1
Age and Sex Related Normal Ranges for ETP, Lag Time, Peak Height, and Time to Peak in 80 Normal Plasma Samples

		All		20–29		30–39		40–49		50–60	
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
ETP	Mean	2,047	2,175	1967	2,164	1,987	2,226	2,081	2,088	2,152	2,221
	SD	409	407	398	407	363	396	419	457	484	256
	Median	2,007	1,996	2,014	1,996	1,869	2,229	2,048	2,026	2,186	2,251
	Interquartile	1,818–2,251	1,874–2,553	1,932–2,164	1,874–2,553	1,739–2,118	1,924–2,405	1,858–2,236	1,781–2,271	1,839–2,672	1,996–2,402
	Range										
Lag Time	Mean	3.0	2.9	2.9	2.6	3.0	3.0	3.1	3.1	3.0	3.0
	SD	0.6	0.6	0.7	0.5	0.4	0.7	0.5	0.7	0.7	0.3
	Median	3.0	2.7	3.0	2.6	3.0	2.7	3.1	3.0	2.8	3.2
	Interquartile	2.7–3.3	2.5–33	2.5–3.3	2.2–3.2	2.7–3.3	2.5–3.3	2.7–3.3	2.7–3.3	2.7–3.3	2.7–3.3
	Range										
Peak Height	Mean	347	365	349	359	338	368	358	362	341	370
	SD	58	40	61	48	46	40	76	48	48	22
	Median	349	364	365	351	337	366	353	360	314	368
	Interquartile	304–381	339–380	356–373	335–372	311–351	343–375	303–398	326–399	305–360	356–380
	Range										
Time to Peak	Mean	6.0	6.0	5.8	5.7	5.9	6.0	6.1	6.2	6.2	6.1
	SD	0.9	0.8	1.0	0.7	0.9	0.8	1.0	1.1	0.9	0.5
	Median	6.0	6.0	5.8	5.7	6.2	6.0	6.0	6.0	6.0	6.0
	Interquartile	5.3–6.7	5.3–6.7	5.0–6.7	5.3–6.0	5.4–6.6	5.4–6.0	5.8–6.7	5.8–7.0	5.5–6.5	6.0–6.7
	Range										

Blood Samples for Microparticle Analysis

Blood samples (35) were selected from specimens sent for routine clotting screens to the Coagulation Laboratory at The Royal London Hospital. All patients samples selected had normal clotting screens as determined by the INR, APTT, and Thrombin time, therefore excluding any patients known to be undergoing anticoagulant therapy. Age and sex was not recorded as we have shown that these parameters do not affect thrombin generation as determined in 80 normal individuals (see above).

Reagents

FITC-conjugated Glycophorin A CD235a (clone JC159) was from Dako (UK). FITC-conjugated CD15 (clone HI98) and PE-conjugated anti TF (CD142; clone HFT-1) were from Pharmingen (BD UK), CD2PECy7 (clone LFA-2), CD14APC (clone MOP9), CD19PerCP (clone 4G7), and CD64 FITC (clone 10.1), were from BD (UK). FITC Annexin V and PE CD42b (clone SZ2) were from Beckman Coulter. Pacific Blue (PB) conjugated CD45 (clone H130) was from Invitrogen. PE IgG1 isotype controls were from Dako, Beckman Coulter, and Pharmingen. Internal negative control cells were used for CD45PB. Tyrodes Solution containing 1mM Hepes (TS) was from Sigma (UK) and was filtered through a 0.22-μm syringe filter from Millipore (UK). Polystyrene latex beads (1.1 μm, 0.6 μm, and 0.1 μm) were from Sigma (UK). Accucount Beads were from Spherotech (USA). Cell Tracker Beads were from BD (UK). FluCa Kit, PPP Reagent and Thrombin Calibrator were from Diagnostica Stago (FR).

Plasma Sample Preparation and Filtration

Blood samples for thrombin generation were collected into 0.109 M tri-sodium citrate vacutainers. Samples were centrifuged at 1,500*g* for 10 min. Following this time the top two thirds of the plasma were removed and aliquoted into LP4 tubes. These aliquots were then centrifuged for a further 10 min at 1,500*g*. The top two thirds of the double centrifuged plasma were then removed, half was placed in a corning tube and frozen at −70°C and the other half was filtered.

Plasma was filtered using a Ceveron microparticle filtration unit (MFU) 500. Centrifuged plasma was loaded into the 96 well 0.2-μm filtration plates, no more than 200 μl of sample was placed into each well. The number of wells used was dictated by the size of the plasma sample being filtered. The filter plate was then covered using plate sealer foil. The instrument was then activated, producing a vacuum of ∼70 kPa. The vacuum sucked the plasma through the filter plate into a preloaded 96-well receiver plate. Plasma samples were then aspirated from the receiver plate using a Pasteur pipette placed in corning tubes and frozen at −70°C.

Thrombin Generation Assay

Thrombin generation was performed on a Fluoroscan Ascent FL (Thermo Electron Corporation) using the Calibrated Automated Thrombogram® system (Thrombinoscope bv) and reagents produced by Diagnostica Stago. Thrombin generation was initiated by the addition of 20 μl of both PPP reagent and FluCa reagent to 80 μl patients plasma, giving final concentrations of 5 pM tissue factor and 4 μM phospholipid. Thrombin calibrator wells contained 20 μl of thrombin calibrator and FluCa reagents and 80 μl of patient plasma. Both calibrator and thrombin generation wells were measured in triplicate. Fluorescence was measured at 390/460 nm every 30 s for 40 min (11).

Flow Cytometry

The flow cytometric analysis of microparticles in plasma samples was performed on a Canto II flow cytometer (BD) using Diva Software version 6.1. The instrument was calibrated daily with Cell Tracker Beads (BD). The instrument had three lasers and a standard optical filter setup with a window extension of 7.0. The forward scatter and side scatter thresholds were set low. Compensation was performed using Comp Beads (BD) and photomultiplier voltages suitable for cellular analysis.

Limit of Sensitivity for Detection of Microparticles

To determine whether particles less than 1 μm could be detected on the Canto II., whole aged blood (5 μl) from a normal individual was diluted in filtered TS (1 ml) then 1 μl each of beads size 1.1 μm, 0.6 μm, and 0.1 μm was added either singly or in combination from a stock solution diluted 1:1,000 in filtered TS, Accucount 10-μm beads (25 μl) were also added. Platelets were identified in a plot of side scatter (linear scale; *y*-axis) and forward scatter (linear scale; *x*-axis). The position of the bead populations were identified first singly then in combination (Fig. 1).

Immunolabelling

To phenotype microparticles in plasma samples (50 μl) was labeled for 15 min at 4°C with, FITC CD15 (5 μl), PE-CD42b (5 μl) and Pacific Blue (PB) CD45 (5 μl), or FITC Annexin V (5 μl) and PE-CD142 (5 μl), or PE IgG2a or PE-IgG1 isotype controls (5 μl). Beads 1.1 μm (1 μl) from a manufacturers stock solution diluted 1:1,000 in filtered TS were added to each sample together with Accucount beads (25 μl). Samples were diluted to 1 ml with filtered TS and analysed immediately by flow cytometry as described previously (11–13).

Flow Cytometric Analysis

Platelets and microparticles were first identified based on size determined by the 1.1-μm beads in a plot of side scatter (linear scale; *y*-axis) and forward scatter (linear scale; *x*-axis. Gated microparticles were then displayed on a plot of PE fluorescence (logarithmic scale; *y*-axis) and FITC fluorescence (logarithmic scale; *x*-axis) or on a plot of PB fluorescence (logarithmic scale; *y*-axis) and FITC fluorescence (logarithmic scale; *x*-axis). The

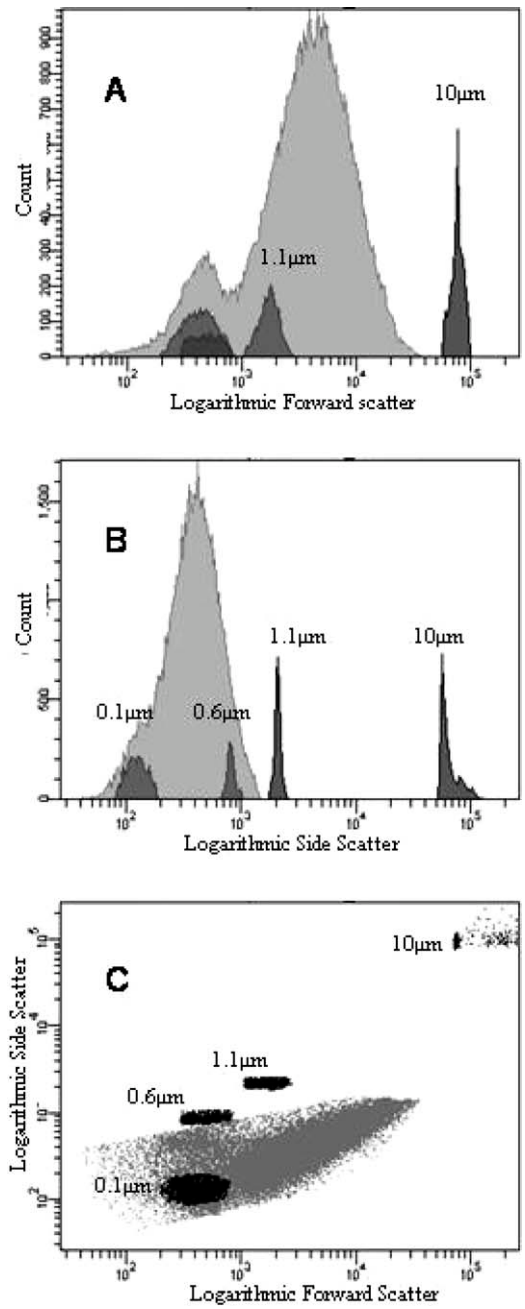

FIG. 1. **A**: Histogram of logarithmic forward scatter (*x*-axis) versus count (*y*-axis). The 10-μm and 1.1-μm beads are clearly seen but the 0.6-μm and 0.1-μm beads are not. The platelet distribution may be seen to the right of the 1.1-μm beads and the microparticle distribution to the left. **B**: Histogram of logarithmic side scatter (*x*-axis) versus count (*y*-axis). The 10-μm, 1.1-μm, 0.6-μm, and 0.1-μm beads are all clearly seen. **C**: Plot of logarithmic forward scatter (*x*-axis) versus logarithmic side scatter (*y*-axis). The 10-μm, 1.1-μm, 0.6-μm, and 0.1-μm beads are all clearly seen.

number of positive events for CD15, CD42b, CD45, Annexin V, and CD142 was calculated after accumulation of 1,000 Accucount beads. (Fig. 2). The number per μl of each microparticle type was calculated.

Analysis of CD45 on Microparticles

To investigate the expression of CD45 on leucocyte derived microparticles four platelet poor plasma samples (50 μl) were labeled with either CD15 FITC or CD64 FITC (5 μl) and CD2 PECy7 (5 μl), CD14 APC (5 μl), CD19 PerCP (5 μl), and CD45 PB (5 μl). Samples were processed as above and analyzed as above but gating to dot plots of FITC, PerCP, APC, PECy7 (logarithmic scale *x*-axis) versus PB (logarithmic scale *y*-axis). The percentage of single positive and dual positive events were recorded.

Statistical Analysis

Data for analysis pre and post filtration were assessed using the Paired *t*-test and the Wilcoxon Matched Pairs Test. The mean, SD, Median and Interquartile Ranges of the positive events are given for the different test groups. Correlations were made using the Spearman Rank Order Correlation. Statistical significance was considered at $P < 0.01$.

RESULTS

Age- and Sex-Related Normal Ranges for ETP

The results of the ETP parameters for 40 males and 40 females are shown in Table 1. There were no significant differences in any of the parameters between males and females. The samples were also grouped into four cohorts according to age. There were no significant differences between the four age groups for either the males or females.

Thrombin Generation Pre- and Postfiltration

The Median and Interquartile Range values for the thrombin generation parameters pre and post filtration are shown in Table 2. The change in the ETP and Peak Height showed a significant decrease post filtration ($P < 0.001$) and Time to Peak gave a significant increase post filtration ($P < 0.001$). There was not a significant difference in the lag time pre- and postfiltration although the lag time tended to increase post filtration.

Limit of Sensitivity for Detection of Microparticles

The analysis of different sized bead diluted in whole aged blood showed that 10, 1, 0.6, and 0.1 μm beads could be distinguished. The use of logarithmic forward scatter versus count did not allow differentiation of the 0.6 and 0.1 μm beads Figure 1A. However all bead populations were clearly differentiated on a plot of logarithmic side scatter versus count Figure 1B. A plot of logarithmic side scatter and logarithmic forward scatter is shown in Figure 1C.

Microparticle Phenotype

When analyzed as a percentage of Annexin V positive events in non filtered plasma, 75% were $CD42b^+$, 6.9% were Glycophorin A^+, 8.9% were $CD45^+$ and 10.6% were $CD15^+$ positive. Only 2.9% of microparticles were Annexin V^+ and $CD142^+$.

There was a significant decrease in the number of $CD42b^+$, $CD15^+$, Glycophorin A^+, Annexin V^+, and Annexin$V^+$$CD142^+$ microparticle post filtration ($P <$

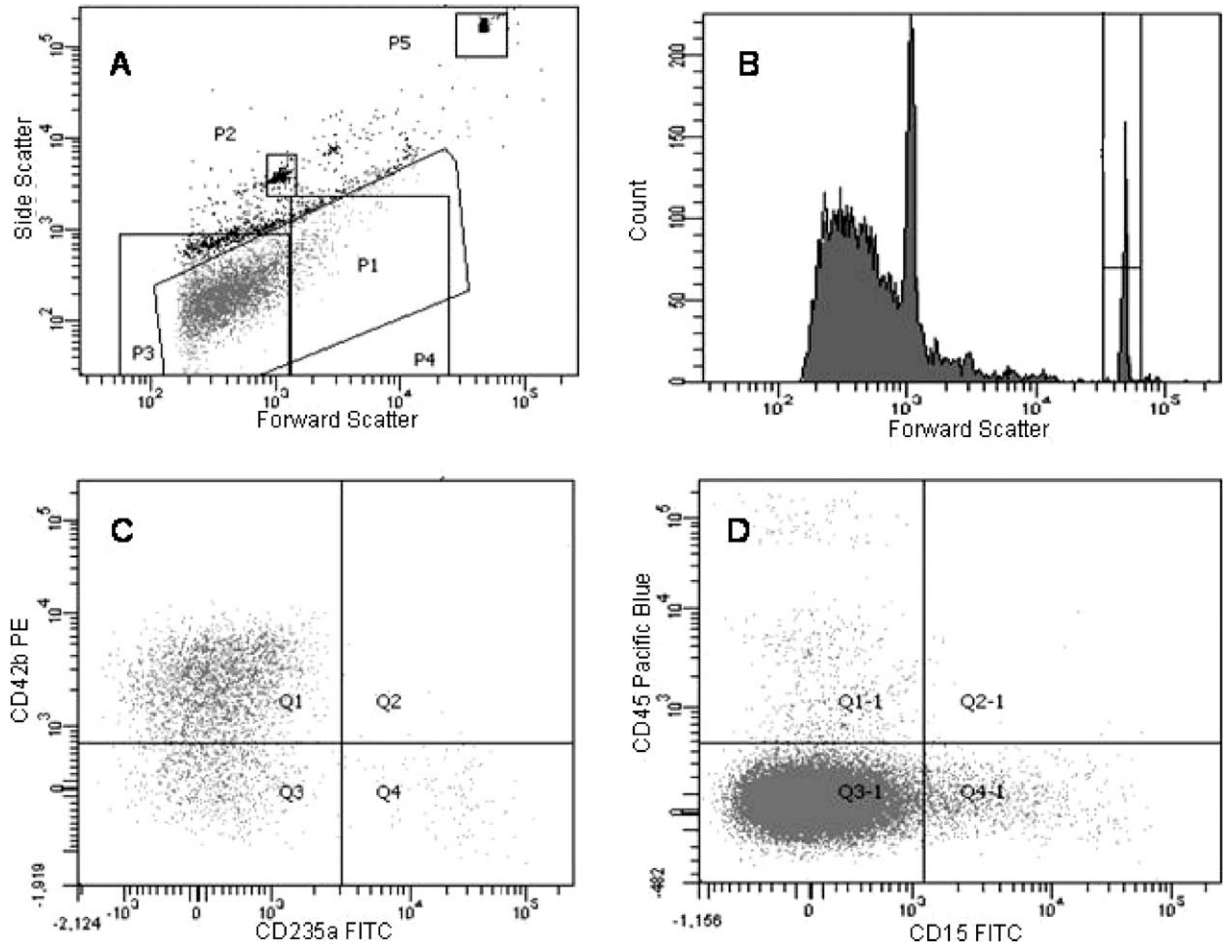

FIG. 2. **A**: Dot plot forward scatter logarithmic scale (*x*-axis) versus side scatter logarithmic scale (*y*-axis). P1 is drawn to encompass microparticles and platelets. P2 is drawn around 1.1-μm beads. P3 and P4 are drawn to define particles greater and less than 1.1 μm. P5 is drawn around 10 μm Accucount beads. **B**: An histogram of forward scatter logaristhnic scale (*x*-axis) and count (*y*-axis) showing the distribution of microparticles in relation to the 1.1-μm and 10-μm beads. **C**: Dot plot gated on P3 showing the CD235a FITC$^+$ (*x*-axis) and CD42b PE $^+$(*y*-axis) micoparticles. **D**: Dot plot gated on P3 showing the CD15 FITC$^+$ (*x*-axis) and CD45 PB $^+$(*y*-axis) micoparticles.

0.001). There was also a reduction in the number of CD45$^+$ microparticles in many samples (20/35) ($P = 0.03$). Table 3.

The number of microparticles expressing both CD15 and CD45 was lower than expected. To investigate this four platelet poor plasmas were labeled with antibodies to leucocyte antigens using CD2 PECy7, CD14 APC, CD15 FITC, CD19 PerCP, and CD64 FITC together with CD45 PB. The mean, SD and SE are shown in Table 4. CD15$^+$ microparticles were the most abundant (12%) with CD2 microparticles the least abundant (0.3%). CD45$^+$ microparticles represented 6% of the population. None of the leucocyte microparticle subtypes showed 100% coexpression with CD45.

Correlations

The ETP correlated with the number of CD15+ microparticles both before and after filtration of the plasma samples ($r = 0.59$ and $r = 0.58$ respectively, $P < 0.001$). Also the change in ETP correlated with the change in CD15+ microparticles ($r = 0.48$, $P < 0.01$).

The number of AnnexinV$^+$ microparticles correlated with the number of CD42b$^+$ microparticles prior to plasma filtration ($r = 0.65$, $P < 0.001$) and the change in CD42b+ and Annexin V positive microparticles post filtration also correlated ($r = 0.68$, $P < 0.001$). Whilst the change in time to peak correlated with the change

Table 2
The ETP, Lag Time, Peak Height, and Time to Peak in Plasma Samples Pre- and Postfiltration

	Median (mean) interquartile range	
Thrombin generation	Prefiltration	Postfiltration
ETP	2,022 (2,035)	1,985 (1,935)
(nM Thrombin)	1,813–2,284	1,417–2,166
Lag time	3.83 (4.00)	4.00 (4.09)
(min)	3.33–4.25	3.33–4.50
Peak height	347 (344)	334 (327)
(nM thrombin)	308–383	295–375
Time to	6.67 (7.00)	6.67 (7.20)
peak (min)	6.19–7.20	6.25–7.50

Table 3
The Number of Different Microparticles per μl of Plasma Pre- and Postfiltration

Microparticle positivity	Median (mean); Interquartile range; number per μl	
	Prefiltration	Postfiltration
Annexin V	2,817 (6755) 811–8,488	6 (33) 0–13
Annexin V + CD142	81 (103) 44–119	6 (9) 0–13
CD42b	2,101 (5,336) 795–4,904	13 (28) 4–26
CD15	298 (503) 83–882	48 (322) 1–608
CD45	250 (319) 162–442	175 (216) 83–336
Glycophorin A	193 (307) 89–283	8 (24) 2–48

in Annexin V binding ($r = 0.52$, $P < 0.01$) and with the change in CD42b ($r = 0.44$, $P = 0.01$) similarly the change in peak height correlated with the change in CD42b ($r = 0.46$, $P < 0.01$ and the change in Annexin V ($r = 0.40$, $P = 0.02$).

DISCUSSION

In this study we have investigated the effect of the presence of microparticles in 35 plasma samples used in the Calibrated Automated Thrombogram® (CAT) thrombin generation assay. We routinely double centrifuge citrated blood samples for use in the CAT assay. However, when we investigated by flow cytometry whether such samples contained residual microparticles we found that they did. Here we have investigated the effect of microparticles in plasma on the thrombin generation assay. Double centrifuged plasma samples were filtered using a Ceveron microparticle filtration unit (MFU) 500. The ETP of each plasma sample was measured pre- and postfiltration. There were significant effects on the thrombin generation parameters, the ETP, and peak height showed a decrease post filtration ($P < 0.001$) suggesting that procoagulant activity had been lost by the removal if microparticles. While the time to peak showed a significant increase postfiltration ($P < 0.001$) suggesting that the removal of microparticles led to a delay in thrombin generation. There was not a significant difference in the lag time pre- and postfiltration although the median value did increase post filtration. Our findings are consistent with previous studies which showed that removal of microparticles from fresh frozen plasma significantly reduced clot formation (14). The importance of the role of platelet microparticles in procoagulant activity was illustrated by Sinauridze et al. (15) who demonstrated that the platelet microparticle surface is ~50–100 fold more procoagulant than activated platelets. This may explain why the removal of residual microparticles from the plasma samples had such a marked effect on the thrombin generation assay.

To investigate which phenotype of the microparticles affected the thrombin generation assay we enumerated the number and type of microparticles pre and post filtration. Filtration removed 99.8% of Annexin V^+ microparticles and this change in Annexin V^+ microparticles correlated with the change in $CD42b^+$ platelet microparticles. CD42b+ platelet microparticles represented the most abundant type (75%) of microparticles in the plasma samples studied which is consistent with previous reports (2). However, the ETP correlated most closely to the number of CD15+ microparticles both pre- and postfiltration rather than with the number of CD42b+ microparticles. The change in ETP also correlated best with the change in CD15+ microparticles. This would suggest that monocyte/neutrophil-derived microparticles have a greater effect on the thrombin generation assay than platelet-derived microparticles. Monocyte microparticles express tissue factor, a potent activator of the coagulation cascade (6), and this may explain the greater influence of CD15+ microparticles on ETP. However in our study only a low percentage (2.9%) of Annexin V+ microparticles expressed tissue factor and there was no correlation with this phenotype and any of the endogenous thrombin potential parameters. Also platelet-derived microparticles have been shown to express tissue factor (6). Our findings are consistent with those of Stampfuss et al. (16) who showed that microparticles derived from apoptotic U937 cells were highly procoagulant, compared to intact cells, but that this was not associated with increased tissue factor expression or Annexin V binding. They suggest that the membrane environment rather than tissue factor expression plays an important role in procoagulant activity of microparticles. Other studies have shown that the

Table 4
CD45 Expression on Leucocyte-Derived Microparticles

	Antigen expression				
	CD2	CD14	CD15	CD19	CD64
Mean	0.25	3.50	11.73	1.20	3.40
SD	0.06	1.87	2.85	0.50	1.96
SE	0.03	0.90	1.42	0.26	0.98
	CD2CD45	CD14CD45	CD15CD45	CD19CD45	CD64CD45
Mean	0.013	1.25	2.70	0.23	1.80
SD	0.01	1.07	1.64	0.13	0.69
SE	0.01	0.53	0.82	0.06	0.34

Results are the percentage positive events within the microparticle gate.

concentration of tissue factor on the surface of microparticles in the circulation of healthy individuals, whose blood remains unstimulated with cytokines, is below the threshold needed to initiate in vivo thrombin generation (16,17).

Interestingly the change in time to peak correlated with both the change in Annexin V^+ and $CD42b^+$ microparticles post filtration. The time to peak showed a significant increase post filtration. The correlation with the number of Annexin V^+ and $CD42b^+$ microparticles suggests that the removal of phosphatidylserine led to a delay in thrombin generation. Further evidence showing the importance of phophatidylserine in thrombin generation is provided by the correlation of change in peak height (significant decrease post filtration) with the change in the number of $CD42b^+$ events and to a lesser extent Annexin V^+ events post filtration, which shows that removal of phosphatidylserine correlates with a decrease in the maximum rate of thrombin production. These findings are supported by previous studies that showed that the expression of phosphatidylserine by microparticles was required to allow the concentration of clotting factors to reach the levels necessary to achieve optimum kinetics for the generation of thrombin (5).

Studies on the coexpression of CD45 and other leucocyte antigens on microparticles revealed that CD45 was not expressed on 100% of leucocyte-derived microparticles. The reason for this is unclear and requires further investigation.

In conclusion we have shown that the presence of microparticles within plasma samples does influence the thrombin generation assay. The number of $CD15^+$ myeloid microparticles correlates better with the ETP than that of $CD42b^+$ platelet microparticles and phosphatidylserine exposure plays a role in the timing of the thrombin generation assay.

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