QUANTITATION OF CELL-DERIVED MICROPARTICLES IN PLASMA USING FLOW RATE BASED CALIBRATION

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Abstract. Activation of vascular endothelium and blood cells can result in the formation of microparticles (MPs), which are membrane vesicles with a diameter < 1 μm which can play a pathogenetic role in a variety of infectious and other diseases. In this study, we validated a modified quantitative method called "flow rate based calibration", to measure circulating MPs in plasma of healthy subjects and malaria patients using FACSCalibur flow cytometry. MPs counts obtained from "flow rate based calibration" correlated closely with the standard method (R^2=0.9, p=0.001). The median (range) number of MPs in healthy subjects was 163/μl (81-375/μl). We demonstrated a flow rate based calibration for the quantitation of MPs in *P. falciparum* malaria-infected patients. The median (range) number of MPs was 2,051/μl (222-6,432/μl), n=28 in patients with falciparum malaria. The number of MPs in plasma from patients with severe falciparum malaria was significantly higher than in uncomplicated falciparum malaria (2,567/μl (366-6,432/μl), n=18 versus [1,947/μl (222-4,107/μl), n=10, p<0.01]. Cellular origin of MPs in malaria patients were mainly derived from red blood cells (35%), platelets (10%), and endothelial cells (5%). There was no significant correlation between the total number of MPs and parasitemia. Flow rate based calibration is a simple, reliable, reproducible method and more affordable to quantitate MPs.

INTRODUCTION

MPs are phospholipid vesicles derived from red blood cells, platelets, endothelial cells, and white blood cells, still containing certain membrane receptors as well as other proteins inherited from their parental cells. They form a heterogeneous population but are defined by their size of less than 1 μm in diameter and the presence of phosphatidylserine (PS) on the vesicle surface (Combes *et al,* 1999; VanWijk *et al,* 2003). A variety of pathological conditions generate MPs, such as cardiovascular disease (VanWijk *et al,* 2003), idiopathic thrombocytopenic purpura (Jy *et al,* 1992), and thalassemia (Pattanapanyasat *et al,* 2004, 2007). MPs also have pathophysiological relevance through their interaction with neighboring or remote cells. Research on this topic

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requires an accurate, reliable, and affordable method to quantitate MPs in clinical samples. At present flow cytometry is most commonly used for MPs analysis. The conventional method uses a precisely known concentration of beads in Trucount™ tubes. If the sample with the unknown number of particles is added, the relative number of these is assessed in comparison to the Trucount™ tube standard, so that the absolute number of particles can be calculated (Pattanapanyasat *et al,* 2004). However, this method is limited by high cost, since every sample has to be added to a Trucount™ tube. Malaria is an important public health problem in the tropics, with an annual mortality of at least one million people (http://www.cdc.gov). MPs may have a role in the pathogenesis of malaria. Using malaria as an example we evaluated a new method to quantitate MPs using flow rate based calibration with a FACSCalibur flow cytometer. With this method the exact volume per unit of time that flows through the flow cytometer detector is assessed with Trucount™ tubes. The concentration of particles in the unknown sample can then be assessed by counting the number of particles over the same time period corresponding to the known volume.

MATERIALS AND METHODS

Flow rate based calibration

A new method to quantify MPs uses flow rate based calibration in a flow cytometer (FACSCalibur, Becton Dickinson Biosciences, San Jose, CA). The method is based on the principle that a volume of sample passes through a detector of a flow cytometer over a set period of time (usually 120 seconds). This can be assessed very accurately by calibrating the machine with Trucount™ beads at the same flow rate (same rate of sample acquisition). "Trucount™" beads (Cat no. 340334; Becton Dickinson Biosciences, San Jose, CA) are tubes containing a precisely defined concentration of particles with a known diameter. By running a Trucount™ beads sample at a certain flow rate (acquisition rate) over a defined period of time (120 seconds), the exact volume that passes through the detector can be easily derived from the number of counts divided by the concentration of Trucount™ beads, as specified by the manufacturer. This volume is then the reference volume to assess MP concentrations in unknown samples. If the settings regarding flow rate remain the same, the MP count over the same time period (120 seconds) corresponds to the same acquired sample volume, so that the concentration of MPs in the sample can thus be calculated. For accuracy this sample volume calibration is assessed at the beginning and the end of the assay. However, a calibration factor needs to be applied if a sample with a different viscosity is measured, since this will slightly alter the acquired volume. The calibration factor can be calculated by adding the buffer or the mixture of buffer and plasma into Trucount™ beads and assessing the acquired sample volume over the same time period (120 seconds). The calibration factor is the ratio of the sample volume acquired in the mixture of binding buffer plus plasma compared to buffer alone. Calibration was done using samples, from 10 healthy subjects, of citrated blood (1 volume of trisodium citrate : 9 volume of whole blood). Plasma MPs were obtained by a two-step centrifugation: first at 1,500*g* for 15 minutes, followed by centrifugation of the supernatant at 13,000*g* for 2 minutes (Combes *et al*, 1999,2004). Thirty microliters of MPs enriched plasma of each sample was then added to separate Trucount™ tubes, after which one milliliter of binding buffer was added and mixed thoroughly. The sample volume (μl) acquired in 120 seconds was calculated from the number of beads counted in 120 seconds divided by the concentration of Trucount™ beads specified by the manufacturer.

Quantitation of microparticles

Three milliliters of citrated-blood were obtained each from 11 healthy subjects and 28 patients who were diagnosed with malaria on admission at the Hospital for Tropical Diseases, Bangkok, Thailand. Of the malaria patients, 18 had severe falciparum malaria (SM) and 10 had uncomplicated falciparum malaria (UM). Severity was defined by the criteria described by the WHO (2000). This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University.

Plasma MPs were obtained by a two-step centrifugation as described above (Combes *et al*, 1999, 2004). Annexin V-FITC (Cat No. 556454; Becton Dickinson Biosciences, San Jose, CA), a specific marker for PS, was used to distinguish MPs from nonspecific particles. The cellular origin of MPs was identified using an antibody to a specific surface marker of the cells; phycoerythrin (PE)-conjugated anti-glycophorin A (Becton Dickinson Biosciences, San Jose, CA, Cat. No. R7078) was used for RBCs, PE-conjugated anti-CD51 (Becton Dickinson Biosciences, San Jose, Cat no. 340928) for endothelial cells, and PE-conjugated anti-CD41a (Becton Dickinson Biosciences, San Jose, CA, Cat. No. 340928) for platelets. Briefly, 30 μl of plasma sample were mixed with 2 μl of FITC-conjugated annexin V, 2 μl of PE-conjugated anti-glycophorin A (Becton Dickinson Biosciences, San Jose, CA), and 26 μl of binding buffer (Becton Dickinson Biosciences, San Jose, CA). This mixture was incubated for 15 minutes at room temperature in the dark. Then, one milliliter of diluted binding buffer solution (1:10 v/v in distilled water) was added and the number of MPs analyzed by FACSCalibur flow cytometer. The binding buffer-bead mixture was mixed gently for 10 seconds by vertexing at low frequency immediately prior to flow cytometric acquisition.

The MPs upper size limit was defined by using 0.8-μm beads in a logarithmic FSC-logarithmic SSC dot plot and a gate was drawn around the population gate R1 (Fig 1A). MPs stained with FITC-conjugated annexin V were detected (Fig 1B). Only events included within this box were further analyzed for positive staining with annexin V and a specific cell surface marker (Fig 1C). The bead event data were acquired and analyzed with the use of CellQuest™ software (Becton Dickinson Bioscience, San Jose, CA). The acquisition time was 120 seconds. Calibration of the volume using Trucount™ tubes was done at the beginning and the end of each batch of samples. The mean value of the two calibrations was used in subsequent calculations. In order to assess the reproducibility of the method, ten replicate samples were measured. To compare the number of MPs quantitated by flow rate based calibration with the reference method, ten samples were assessed in parallel using both methods.

Statistical analysis

Statistical analyses were carried out using the SPSS 11.0 statistical program (SPSS Corporation, Chicago, IL). The precision of the method was determined by the coefficients of variation (CVs). Statistical correlation was analysed using linear correlation and Bland-Altman plots. Non-normally distributed parameters were compared by the Mann-Whitney *U* test. The correlation between the number of MPs and parasitemia were calculated using the Spearman's rank correlation method. A p-value ≤ 0.01 was considered as statistical significance.

RESULTS

Flow rate based calibration

The number of beads in the Trucount™ tubes assessed at the beginning (prebead) and the end (postbead) for each batch of samples showed consistent counts over the harvesting period (120 seconds). The calibration factor to correct for differences in viscosity be-

tween the latex bead suspension and the plasma samples as described above was 1.043. (Table 1).

Quantitation of MPs using flow rate based calibration

Sample volume (μl) acquired in 120 seconds (A). Pivotal in this method is the assessment of the exact sample volume (A) acquired during the measurement acquisition period of 120 seconds. The volume (A) was calculated using the following formula (Storie *et al*, 2003a):

$$(A) = \frac{\text{Events (beads) counted in 120 seconds x Dilution}}{\text{Bead concentration in tube (provided by manufacturer)}}$$

In our experiment the bead concentration in the Trucount™ tubes was 49,255 beads/μl; we diluted to an end volume of 1,000 μl. The acquired volume in 120 seconds is thus:

$$\text{“Prebead”} = \frac{3{,}694 \times 1{,}000}{49{,}255} = 74.99\ \mu l$$

$$\text{“Postbead”} = \frac{3{,}706 \times 1{,}000}{49{,}255} = 75.24\ \mu l$$

The sample volume (μl) acquired in 120 seconds (*A*) was defined as the average of these measurements:

$$= \frac{\text{Prebead + Postbead}}{2} = \frac{74.99+75.24}{2} = 75.11 \mu l$$

Calculation of concentration of MPs in a blood sample (B) (Storie *et al*, 2003a). MPs were localized within the R1 region, and were distinguished from debris by their positive staining for annexin V responses in the histogram plot (Fig 1B).

The concentration of MPs/μl (B) in the diluted sample (uncorrected for viscosity) can be calculated as: *B* = MPs counted in 120 seconds = volume of sample acquired in 120 seconds (*A*)= 1,460/75.11 = 19.44 /μl

From this, the concentration of MPs in the undiluted sample (uncorrected for viscosity) can be calculated, using the dilution factor (C) (Storie *et al*, 2003a)

$$\text{Dilution factor (C)} = \frac{\text{Sample volume (μl)}}{\text{Buffer volume (μl)+sample volume (μl)+MAb volume (μl)}}$$

$$= \frac{30}{1{,}016+30+4} = 0.028$$

Thus the MPs concentration in the plasma blood sample (D) (uncorrected for viscosity) is: $D = (B)/ (C) = 19.44 / 0.028 = 680/\mu l$

As described in the methods section, the change in viscosity of the plasma/buffer mixture compared to the buffer alone used in the calibration of the machine should be included. Thus the corrected plasma concentration (E) (/μl) is:

E = MPs/μl *(D)* x calibration factor

= 680 x 1.043

= 709 MPs/μl

This can be simplified using the following equation (Storie *et al*, 2003a):

$$\left(\frac{\text{Number of MP counts in the R1 region x bead concentration in the Trucount™ tubes}}{\text{Average number of counted beads in 120 seconds}^*} \right) \times K$$

* = (number of prebead + number of postbead) / 2
With K= (dilution factor x calibration factor)/diluent volume = 2.9×10^{-5} in our experiment

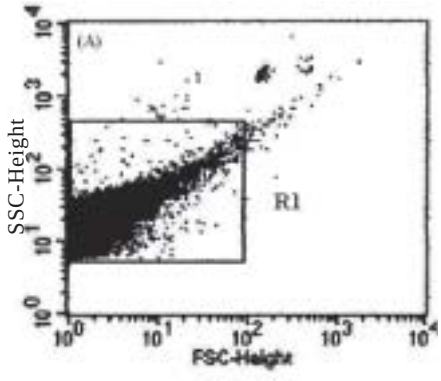
In order to assess the reproducibility of this method, the mean CVs of MPs derived from analyzing 10 replicate samples was 2.6%. The MPs count from flow rate based calibration were highly correlated with those from the reference method using absolute counting by the bead-based method (R^2= 0.7, p= 0.001). In order to determine whether the two assays agreed sufficiently to be used interchangeably, the difference between each data pair of agreements was plotted as described by

Table 1
Calculation of the calibration factor.

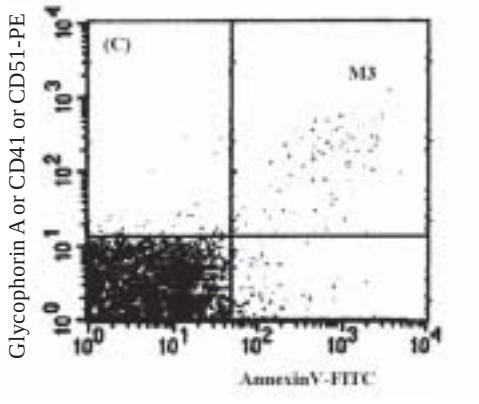
Sample	Binding buffer (Blank)		Binding buffer+Plasma MPs (Sample)		Ratio
	Bead count	Blank[a]	Bead count	Sample[a]	Counts (Blank:Sample)
1	3,505	70.651	3,230	64.563	1.094
2	3,537	71.296	3,301	66.539	1.071
3	3,653	73.634	3,629	73.150	1.006
4	3,785	76.295	3,553	71.618	1.065
5	3,838	77.363	3,539	71.336	1.084
6	3,740	75.388	3,655	73.674	1.023
7	3,905	78.714	3,631	73.190	1.075
8	3,678	74.138	3,700	74.581	0.994
9	3,467	69.885	3,513	70.812	0.986
10	3,757	75.730	3,663	73.835	1.025
Mean	3,686.5	74.309	3,541	71.330	1.043[b]

[a]Volume (μl) acquired in 120 seconds

[b]Calibration factor is the ratio of the sample volume acquired in the mixture of binding buffer and plasma compared to the buffer alone (blank) over the same time period (120 seconds).



Marker	Events
M1	19,430
M2	1,460



Quadrant	Event
M3	975

Fig 1–Flow cytometric quantitation of plasma MPs in malaria patients. (A) MPs gated by size on an FSC/SSC cytogram and named as R1. Only events included within gate R1 were further analyzed for fluorescence associated with annexin V. (B) Histogram analysis showed the events with negative for annexin V (M1) and events with positive for annexin V (M2). (C) Quadrant analysis showed the events that positive staining with annexin V and a specific cell surface marker (glycophorin A or CD41 or CD51) (M3).

Bland and Altman (1986). Analysis of the Bland-Altman plot showed agreement between the numbers of MPs obtained from flow rate based on those obtained from absolute counts using the bead-based method. The mean (95%CI) difference between the two methods was 168 (74-262), which is within the range of 1.96SD (88-425).

The median (range) plasma MPs in healthy subjects was 163/μl (81-375/μl). MPs were also quantitated in the plasma of malaria-infected patients. The median (range) plasma concentration of circulating MPs was 2,051/μl (222-6,432/μl) in patients with falciparum malaria, *n*=28). In the plasma of patients with severe falciparum malaria, the MPs concentrations were significantly higher than in uncomplicated falciparum malaria [2,567/μl (366-6,432/μl), *n* = 18, p < 0.01 versus 1,947/μl (222-4,107/μl), *n* = 10, p <0.01]. The median (range) concentration of MPs in the blood samples from healthy donors [464/μl (360-741/μl), *n* = 18] and from malaria infected patients [875/μl (288-5,681/μl), *n* = 9] were compared with the reference method (absolute counting by the bead based method) in healthy donors [354/μl (177-573/μl)) and malaria infected patients [656/μl (279-7,645/μl)]. There was no significant difference (p=0.02 and p=0.66, respectively). The cellular origin of MPs in malaria infection was further analysed. The mean (95%CI) percentage of MPs derived from RBCs (RMPs) in patients infected with *P. falciparum* was 32% (29-41), 14% (11-20) in platelets (PMPs), and 6% (4-10) in endothelial cells (EMPs). There was no significant difference among the percentages of the different types of MPs for uncomplicated and complicated falciparum malaria. There was no correlation between parasitemia and the total number of MPs, RMPs, PMPs, or EMPs in *P. falciparum* infected patients.

DISCUSSION

Several reports have been published on the presence MPs in the blood with of patients with a variety of disorders, mainly non-infectious diseases (Jy *et al,* 1992; Combes *et al,* 1999, 2004; VanWijk *et al,* 2003; Pattanapanyasat *et al,* 2004, 2007). Although many aspects of the pathophysiological role of MPs are still unclear, a picture develops in which MPs may play an important role in inflammation, coagulation, and vascular homeostasis (Distler *et al,* 2005; Coltel *et al,* 2006). The absolute counting by a bead based method using flow cytometry has commonly been used as the reference method for quantitating MPs in various diseases because of its accuracy, precision and reproducibility. A flow rate based calibration has previously been described for the quantitation of $CD4^+$ T cells. The method is user-friendly, reliable and has low inter-laboratory variation (Storie *et al,* 2003a,b; Walker *et al,* 2006). In this study, we demonstrate that flow rate based calibration is a valid method for MPs quantitation. The major advantages of the here-described method are lower-cost, easiness to perform, accuracy and reproducibility. This makes it a good method to use for quantitation of MPs in serial samples. The technique requires the use of Trucount™ tubes only for initial calibration and in order to adjust for differences in viscosity between the latex bead suspensions in buffer and the study samples. However, this method does not need the addition of Trucount™ tubes for each sample. This can reduce the cost of analysis considerably. It should be noted that the flow rate based calibration method requires a flow cytometer machine with very consistent pumping rates. The favorable small inter-laboratory variation is maintained by running the latex bead counts as an internal control at the beginning and the end of each batch of samples measured. Thus the method does need technical expertise and maintenance. In other applications, measuring $CD4^+$ and $CD8^+$ populations, this technique has been shown to compare favorably

to other volumetric single-platform techniques (Storie *et al,* 2003a,b).

MPs are found in plasma of healthy subjects and malaria patients. MPs in malaria infection are derived from different cell types. Phenotypic analysis of the cellular origins of MPs from malaria patients have demonstrated that most MPs are derived red blood cells [mean (95%CI): 35 (26-41)], platelets [mean (95%CI): 10 (8-20)], and endothelial cells [mean (95%CI): 5 (2-9)]. WBCs can be a possible source for these unidentified MPs. The WBCs common antigen (CD45) is usually used to identify white blood cell derived microparticles (WMPs); CD66b, CD14, CD8, and CD20 are used to detect MPs originating from monocytes, granulocytes, helper T cells, cytotoxic T cells, and B-lymphocytes, respectively. In the current study, PMPs were detected by using monoclonal antibody to glycoprotein (GP) IIb-IIIa (CD41a). It is possible that some PMPs do not sufficiently express this antigen and that other PMPs surface antigens like the previously reported GPIIa (CD41), GPIX (CD42a), GPIb (CD42b), or GPIIa (CD61) (Simak and Gelderman 2006), would reveal that some of the unidentified MPs could be platelet derived. Similarly, EMPs may be underdetected. Alternative surface antigens used for the detection of EMPs include integrin (CD51) (Combes *et al,* 1999), CD146, E-selectin (CD62E) (Berckmans *et al,* 2001), vascular endothelial (VE)-cadherin (CD144) (Shet *et al,* 2003), or PECAM-1 (CD31). In this study, there was no significant correlation between the total MPs and parasitemia. However, the relationship between the level of MPs and clinical parameters needs to be evaluated in a larger study.

ACKNOWLEDGEMENTS

We thank the staff of the Hospital for Tropical Diseases, Mahidol University, Bangkok, Thailand for their help and support. We would like to thank all the patients who donated blood samples for this study. This work was supported by The Royal Golden Jubilee PhD program, Thailand Research Fund, and the Mahidol-Oxford Tropical Medicine Research, The Wellcome Trust of Great Britain.

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