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Platelet Microparticles are Heterogeneous and Highly Dependent on the Activation Mechanism: Studies Using a New Digital Flow Cytometer

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Background: Platelet-derived microparticles (MPs) are believed to play an important role in coagulation and inflammatory disorders. Unfortunately, MP size renders them difficult to study and analyze by conventional flow cytometry.

Methods: We analyzed and characterized platelet-derived MPs, using antibodies against the major surface glycoproteins (GP), the platelet activation antigen P-selectin (CD62P), and a marker of procoagulant activity (phosphatidylserine exposure). MPs were generated by exposure of platelets to thrombin receptor activating peptide (TRAP) or ionophore. Both agonists induced significant microvesiculation of platelets, and the resulting MPs were analyzed by a new digital flow cytometer: Becton-Dickinson FACSAria™.

Results: Membrane GPs were equally well represented in MPs generated by either reagent. In contrast, P-selectin was more intensely expressed in TRAP-MPs, while phosphatidylserine (PS) expression was markedly increased in ionophore-MPs. Two distinct populations of TRAP-MPs (one PS-positive and another PS-negative) were apparent. The latter characteristic facilitated sorting of MPs according to their PS exposure.

Conclusions: The data presented herein show a significant improvement in the methodology applied until now to the characterization of MPs. The ability to characterize and sort MP subpopulations may help to resolve their contributions to normal and pathological functions. © 2007 International Society for Analytical Cytology

Key terms: platelet; microparticle; sorting; flow cytometry; phosphatidylserine

Platelet-derived microparticles (MPs) are small portions of membrane shed from activated platelets following activation (1). These MPs support procoagulant activity and contribute to formation of the hemostatic plug (2). MP analysis constitutes an important new approach for the investigation of pathologies in various diseases, and their characterization is of interest as they have potentially important roles in hemostasis and thrombosis, inflammation, and other aspects of vascular biology (3).

A working definition of MPs is based on the criteria of size and expression of antigens specific to the cell type of origin (3–5). In the case of platelets, exposure to different agonists also results in an activated state characterized by specific changes in membrane phospholipid distribution (exposure of aminophospholipids such as phosphatidylserine in the external membrane leaflet), accompanied by shedding of MPs from the membrane (5–8). Therefore, in many laboratories, the expression of phosphatidylserine (PS) on the surface of MPs (usually detected in flow cytometry by the binding of labeled Annexin V) has been used as an additional criterion for the definition of a MP (4).

Numerous studies in the last few years have shown that MPs are present in blood from healthy individuals and patients with a variety of coagulation and inflammatory disorders (3,9–13). Platelets are currently recognized to be the dominant source of circulating MPs in vivo in the blood of healthy subjects (14). Ex vivo, MP formation by platelets can be induced by activation, using agonists such as thrombin, collagen, or calcium ionophore (15). In the present work, we studied platelet-derived MPs generated ex vivo by two different agonists, thrombin receptor activating peptide (TRAP) and calcium ionophore. One goal of these studies was to determine the feasibility of sorting MPs by flow cytometry. Separating subsets of platelet-derived MPs can be considered a prelude to our ultimate goal of characterizing the procoagulant and other functional profiles of specific populations of MPs in whole blood samples.

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The analysis of MPs has several limitations, most importantly their size (<1 μm is used by many authors as the range of interest (4)). Although conventional flow cytometry has been applied to their study, several authors have agreed on the technical difficulties of current analog technologies (16). Our present study introduces the recently available first generation BD FACSAria™ digital flow cytometer for the analysis of cell-derived MPs. The numerous technological advances embodied in the standard configuration of this new cell sorter provide a high performance sorting and analysis capability for MPs and many other cell types. The FACSAria was used for flow cytometry characterization of the MPs, and specific sorting strategies were developed and applied for the separation of MPs according to the expression of PS.

MATERIALS AND METHODS

Reagents

TRAP [SFLLRNPNDKYEPF] and ionophore A-23187 were purchased from Sigma Chemical (St Louis, MO). Fluorescent microbeads ranging from 0.3 to 1.0 μm in size (FluoSpheres™ fluorescent microspheres) were obtained from Molecular Probes (Eugene, OR). Platelet glycoprotein (GP) IIIa (a subunit of the complex GP IIb–IIIa) was detected with a monoclonal antibody to human CD61 (clone RUU-PL 7F12, BD Biosciences, San José, CA) conjugated to fluorescein 5-isothiocyanate (FITC) or peridinin–chlorophyll-protein complex (PerCp), while GP Ibα was detected using a monoclonal anti-CD42b (clone HIP1, BD Biosciences) conjugated to allophycocyanin (APC). An isotype-matched IgG_1 (clone, BD Biosciences) conjugated to APC, phycoerithrin (PE), and FITC was used as a negative control in all studies. Procoagulant activity associated with anionic aminophospholipid exposure in the outer leaflet of the MP membrane was inferred by binding of Annexin V (8) (Pharmingen, San Diego, CA) conjugated to PE or lactadherin conjugated to FITC (kindly provided by Gary E. Gilbert, Department of Medicine-VA Boston Healthcare System, Boston, MA) (17,18). As a control for Annexin V binding, a parallel determination was performed in which the Ca^{++} chelator EDTA was added to the buffer for each sample.

Preparation of Platelet-Derived MPs

Human blood samples were obtained from healthy donors who had not taken any drug potentially affecting platelet function for the previous 10 days. Blood was collected into sodium citrate anticoagulant at a 3.2% (0.105 M) final concentration. Platelet-rich plasma was obtained by centrifugation of whole blood at 200*g* for 10 min at 22°C. Platelets were isolated from platelet-rich plasma by centrifugation at 800*g* for 20 min, and washed twice with equal volumes of citrate–citric acid–dextrose (93 mmol/L sodium citrate, 7 mmol/L citric acid, and 140 mmol/L dextrose), at pH 6.5, containing 5 mmol/L adenosine and 3 mmol/L theophylline. The final pellet was resuspended in a buffer containing Hepes (10 mmol/L), NaCl (137 mmol/L), KCl (5.38 mmol/L), and $CaCl_2$ (5 mmol/L), at pH 7.4 and kept at 37°C for 20 min. Aliquots of platelet suspensions were independently activated by TRAP (10 μM) or the calcium ionophore A23187 (3 μM) for 10 min at 37°C. Samples were then centrifuged at 12,000*g* for 10 min at 20°C to remove platelets, and the MP-rich supernatant was harvested for flow cytometry studies. MPs were enumerated by combining gating and labeling strategies with BD TruCOUNT™ tubes (BD Biosciences) following the manufacturer's instructions.

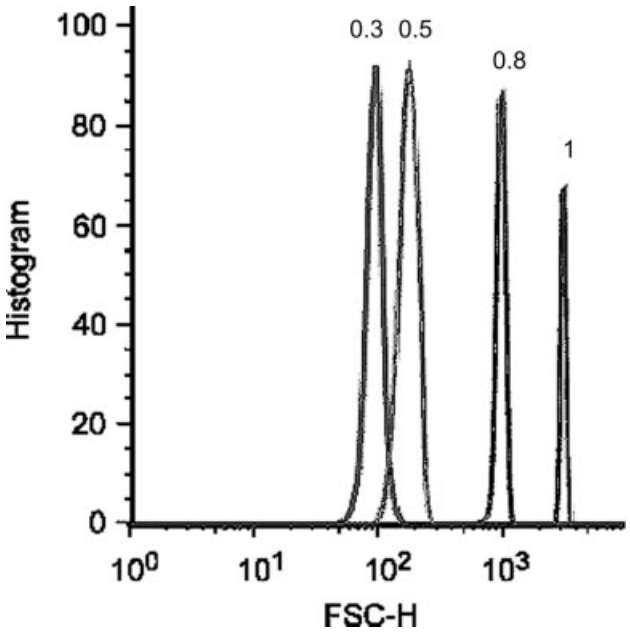

FIG. 1. Discrimination of fluorescent beads according to their size (FSC) by FACSAria. The histogram displays the correct distribution of 0.1-, 0.3-, 0.8-, and 1.0-μm fluorescent microbeads regarding their forward side scatter characteristics.

Flow Cytometry Studies

Immunolabeling of MPs with antibodies and Annexin V or lactadherin was performed using tricolor analysis. After collection, aliquots of MPs (180 μL) were added to polypropylene tubes preloaded with Hepes (10 mM), NaCl (137 mM), KCl (5.38 mmol/L), $CaCl_2$ (5 mM) at pH 7.4. MPs were first incubated with saturating concentrations (predetermined by titration) of anti GP IIIa (CD61 conjugated to FITC or PerCp) or GP Ibα (CD42b-APC) and incubated in the dark, without stirring, for 15 min at room temperature. Next, the addition of saturating concentrations of the remaining APC-, FITC-, and PE-conjugated antibodies was followed by another incubation for 15 min as described earlier. Samples were kept in the dark, awaiting flow cytometry analysis within the next hour.

Data were acquired using the Becton Dickinson FACSAria digital flow cytometer (Becton-Dickinson, Mountain View, CA) that incorporates three air-cooled lasers at 488-, 633-, and 407-nm wavelengths. Fluorescence and scatter signals were calibrated with Calibrite beads (Becton Dickinson, BD Biosciences, San José, CA). MPs were gated according to their size, using fluorescent beads of 1.0 μm

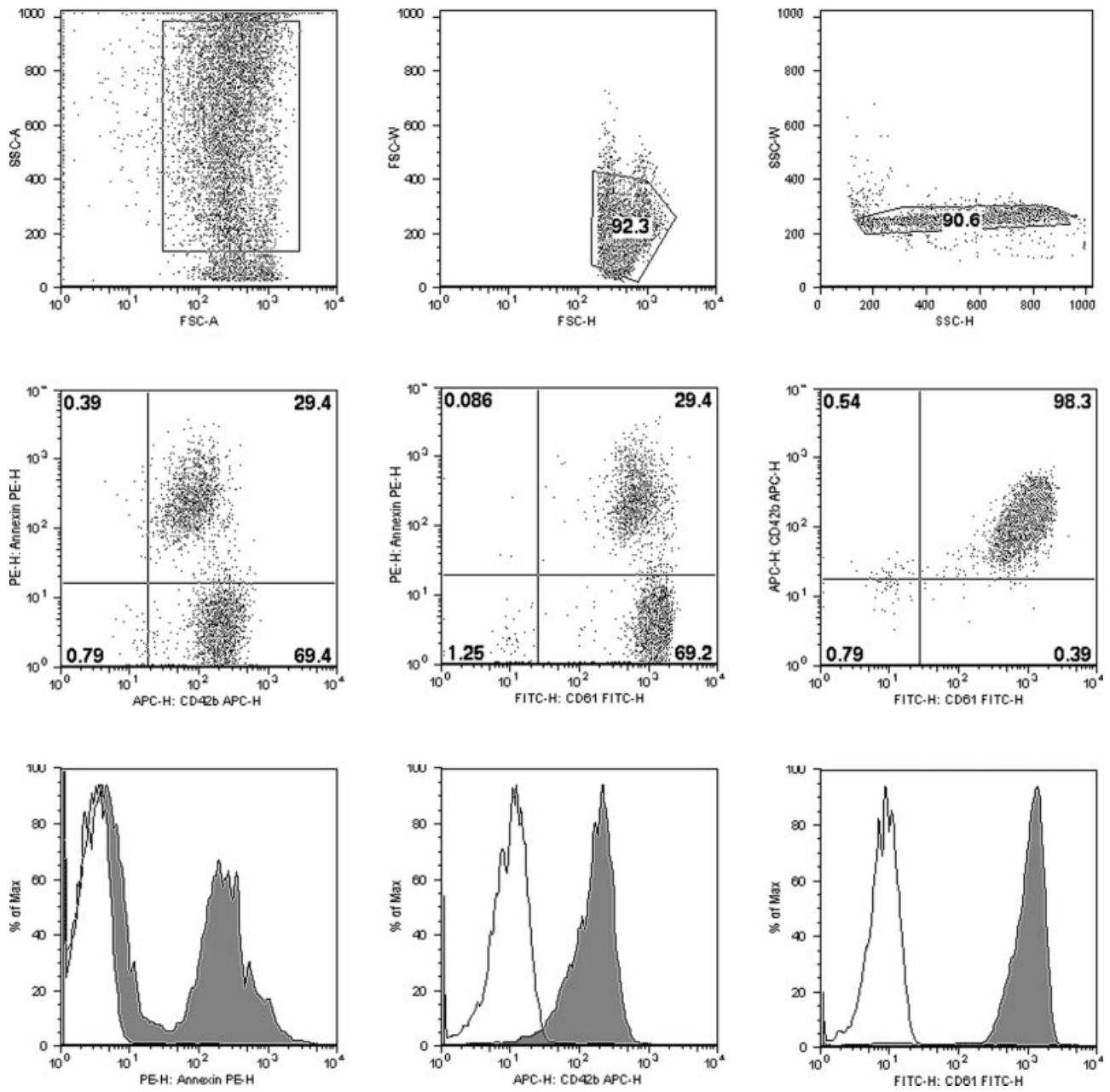

FIG. 2. Acquisition and analysis of TRAP-derived platelet MPs with FacsAria digital flow cytometer. MPs were defined as events <1.0 μm in size, with characteristic forward vs. side scatter, and positivity for CD61 and CD42b. Analysis was performed using dot plots and histograms that were composed of fluorescence data obtained in the logarithmic mode from 10,000 events analyzed in each sample. TRAP-generated MPs were positive for both platelet markers, as shown in the right hand middle section panel. Two populations, one Annexin V-positive and the other Annexin V-negative were observed (left hand dot-blot panel, middle row). Histograms on the bottom row show the clear positivity (full gray) obtained for CD42b (left), CD61 (right), and the two Annexin V populations identified (middle) in comparison to their respective controls (white) run in paralell in each analysis.

that served to establish the upper and the outer limit of the gate. The combination of direct signal acquisition, microbubble eliminating fluidics, and high signal-to-noise fiber optics in the FACSAria allows direct observation of particles <1.0 μm in size. MPs were gated by their characteristic forward vs. side scatter, and positivity for anti CD61 or CD42b.

The fluorescent Annexin V-positive MP population was found in the entire forward/side scatter population, including doublets, fragments, and larger contaminating particles. To center the MP population in a FSC-A/SSC-A dot plot, a histogram of all events was created in the Annexin V channel. The Annexin V-positive events were gated, and the results were displayed by back gating into a forward (FSC-A) and side scatter area (SSC-A) dot plot. This allowed final adjustment of the FSC-A/SSC-A PMT voltages and gates. The positive events in the FSC-A/SSC-A were gated and displayed in a hierarchical scheme to a dot plot with FSC-height (FSC-H) vs. FSC-width

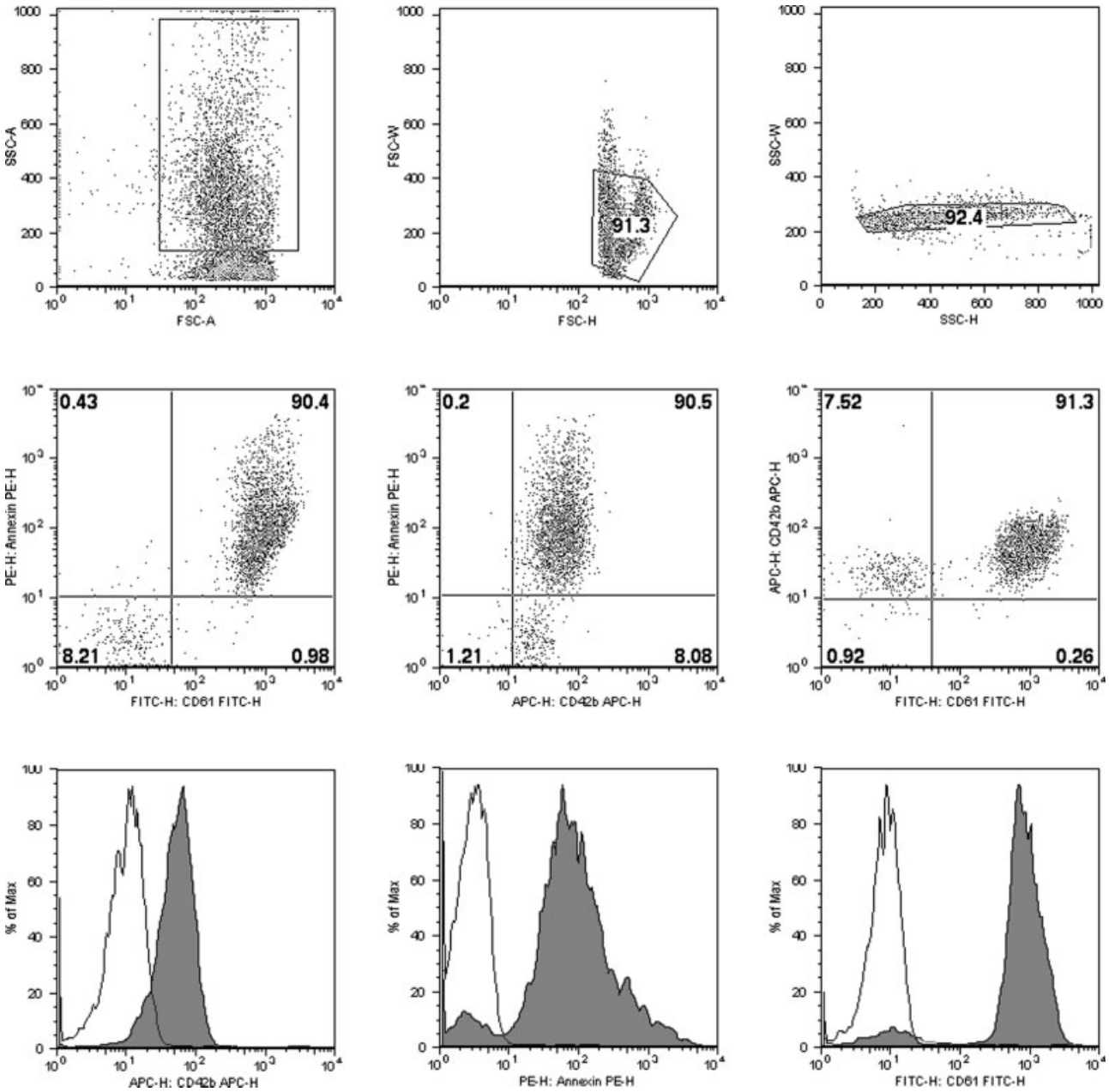

FIG. 3. Acquisition and analysis of ionophore-generated MPs from washed platelets. MPs were gated according to the strategy previously described and analyzed regarding both the expression of specific platelet markers (CD61 and CD42b), and expression of surface PS. After 10,000 events were acquired, analysis using dot plots and histograms demonstrated that the majority of ionophore-derived MPs were positive for GPIIIa (CD61), GPIbα (CD42b), and phosphatidylserine (PS) (middle panels). The positivity for each determination (gray) is again compared with their respective negative controls (white) in the bottom histograms.

(FSC-W). This plot eliminates doublets by FSC geometry by drawing a gate around the dominant population and eliminating outliers. These gated events were then displayed in a final dot plot of SSC-H vs. SSC-W that further eliminates doublets through side scatter geometry. After gating the major population, the result was displayed in the appropriate channel (FITC, PE, or APC), where only the singlet MP population was evaluated. As a final step, increasing the SSC-A threshold eliminated residual buffer noise below the 0.22-μm level.

As an approach to a sorting strategy for MPs, PS was detected by using lactadherin conjugated to FITC. This novel protein has demonstrated higher affinity than Annexin V for PS, and has the potential advantage of noncalcium-dependent binding to PS (18). MP suspensions were incubated with CD61-PerCp and lactadherin-FITC to be sorted according to PS exposure. Two populations of CD61-positive events were collected, one positive and the other negative for lactadherin-FITC binding.

Table 1
Summary of Characteristics of TRAP- and Ionophore-Generated Microparticles (MPs) Labeled with Monoclonal Antibodies to GP IIIa, GP Ibα, P-selectin (CD62P), and Annexin V

	$CD61^+/AV^+$	$CD61^+/AV^-$	$CD42b^+/AV^+$	$CD42b^+/AV^-$	$CD42b^+/CD62P^+$	$CD42b^+/CD62P^-$	$CD61^+/CD42b$
Trap MPs	47.68 ± 9	52.31 ± 10.1	47.37 ± 12.4	52.62 ± 11.3	85.2 ± 3.5	15.6 ± 4.9	96.4 ± 1.2
Iono MPs	87.65 ± 2.2	12.34 ± 5.3	87.83 ± 10.3	12.16± 3.9	35.83 ± 7.1	64.16 ± 7.2	91.4 ± 2.7

Data are expressed as the percentage of fluorescence-positive MPs (mean ± SEM; $n = 6$). The majority of both TRAP- and ionophore-derived MPs were positive for CD61 and CD42b staining (>90%). Variable expression of surface phosphatidylserine (PS) was detected in TRAP- or ionophore-derived MPs, as assessed by Annexin V-positive events. Treatment of washed platelets with TRAP resulted in two populations, one that was Annexin V-positive (47%) and the other Annexin V-negative (52%). In contrast, all MPs generated using ionophore were positive for Annexin V binding. Interestingly, positivity for P-selectin expression was greater in MPs produced by TRAP (85%) vs. ionophore (35%).

Data analysis was performed using dot plots and histograms that were composed from fluorescence data obtained in the logarithmic mode from 10,000 events analyzed in each sample. Using the Flow Jo™ conversion software (Becton Dickinson) running on a Macintosh Power computer (Apple Computer), data were expressed as the percentage of fluorescence-positive MPs. In order to do so, quadrant analytical markers were set in the corresponding fluorescence channel to define the corresponding negative controls. These markers were used as a threshold to determine the proportion of MPs exhibiting immunofluorescence above this level in subsequent sample analyses.

RESULTS

In preliminary experiments, we set out to determine the ability of the FACSAria to accurately discriminate between beads <1.0 μm on the basis of size. As illustrated in Figure 1, a clear FSC discrimination between fluorescent beads of 0.3, 0.5, 0.8, and 1.0 μm in size is evident.

Ex vivo, both TRAP and ionophore stimulation of washed platelets resulted in important microvesiculation. Numerically, approximately twice as many MPs were generated by TRAP-stimulated platelets compared to ionophore-stimulated platelets ($0.42 \times 10^3/\mu L$ with TRAP compared to $0.22 \times 10^3/\mu L$ with ionophore). MPs prepared by exposure of platelet suspensions to either TRAP or ionophore showed intense positivity (>90%) for the abundant platelet GPs, GP IIIa, and Ibα, as shown by the detection of platelet markers CD61 and CD42b, respectively (Figs. 2 and 3). Results obtained in these experiments are summarized in Table 1. However, the expression of P-selectin (CD62P) varied depending on the activating agent, insofar as the majority (85%) of MPs generated by TRAP expressed P-selectin, compared to only 36% of ionophore-derived MPs (Table 1). Other differences were noted between MPs, according to the agonist used to generate them. For example, virtually all MPs (>95%) derived from ionophore treatment of platelets were positive for Annexin V binding, indicative of PS exposure (Fig. 3). However, TRAP-derived MPs consisted of two distinct populations, one being Annexin V-positive (48%) and the other Annexin V-negative (52%); notably, both populations were doubly positive for CD61 and CD42b in similar proportions (47%) (Fig. 2).

When MPs isolated from TRAP-activated platelets containing two populations of MPs (defined by Annexin V binding) were exposed to 3 μM A-23187—but not 100 μg/mL collagen (not shown)—for 10 min, we observed a rapid shift to a single population of Annexin V-positive MPs (compare Figs. 2 and 4 for before and after ionophore stimulation of TRAP-generated MPs).

Sorting of MPs

Prior to sorting of MPs, we performed a comparison of PS detection on MPs, using fluorescently labeled Annexin V and lactadherin as probes. These studies demonstrated that lactadherin binds with a higher affinity (about two-fold more) than Annexin V and seems to be more sensitive for the detection of PS changes (data not shown) (18). Therefore, MPs generated by TRAP were double-labeled with CD61-PerCp and lactadherin-FITC, and sorted according to their positivity for CD61 and lactadherin. Sorting was terminated after 10,000 positive events were collected. The sorted samples were then acquired again, and positivity for CD61 and lactadherin were reassessed, as shown in Figure 5. Notably, all CD61-positive events—whether positive or negative for lactadherin—were displayed in the initial panel acquisition gate that was established for all particles <1 μm in size.

DISCUSSION

We have demonstrated that the BD FACSAria offers an improved resolution and higher ability to discriminate and characterize cell-derived MPs. This instrument utilizes a digital acquisition of signals, and appears to be a vast improvement over analog systems. The number of signal channels for each photomultiplier tube (PTM) has increased from the industry standard of 1,024 to 2.64×10^5. The laser core focus spot on most instruments, such as FACSCalibur™, is 3:1, or $25 \times 75\mu m^2$, whereas the beam geometry for the FACSAria is 9:1, roughly 9×81 μm^2. The reduction in the focus spot translates into increased signal discretion from the PTMs as a particle is interrogated. The combination of direct signal acquisition, fixed cuvette position, microbubble eliminating fluidics, and the high signal-to-noise fiber optics allows direct observation of particles <1.0 μm in size. In support of this statement, Figure 1 clearly establishes the ability of FACSAria to discriminate between fluorescent beads sized between 0.3 and 1.0 μm. The digital fluorescence signal in the BD FACSAria allowed us to clearly distinguish between subpopulations of MPs (Figs. 2 and 3, respectively), an

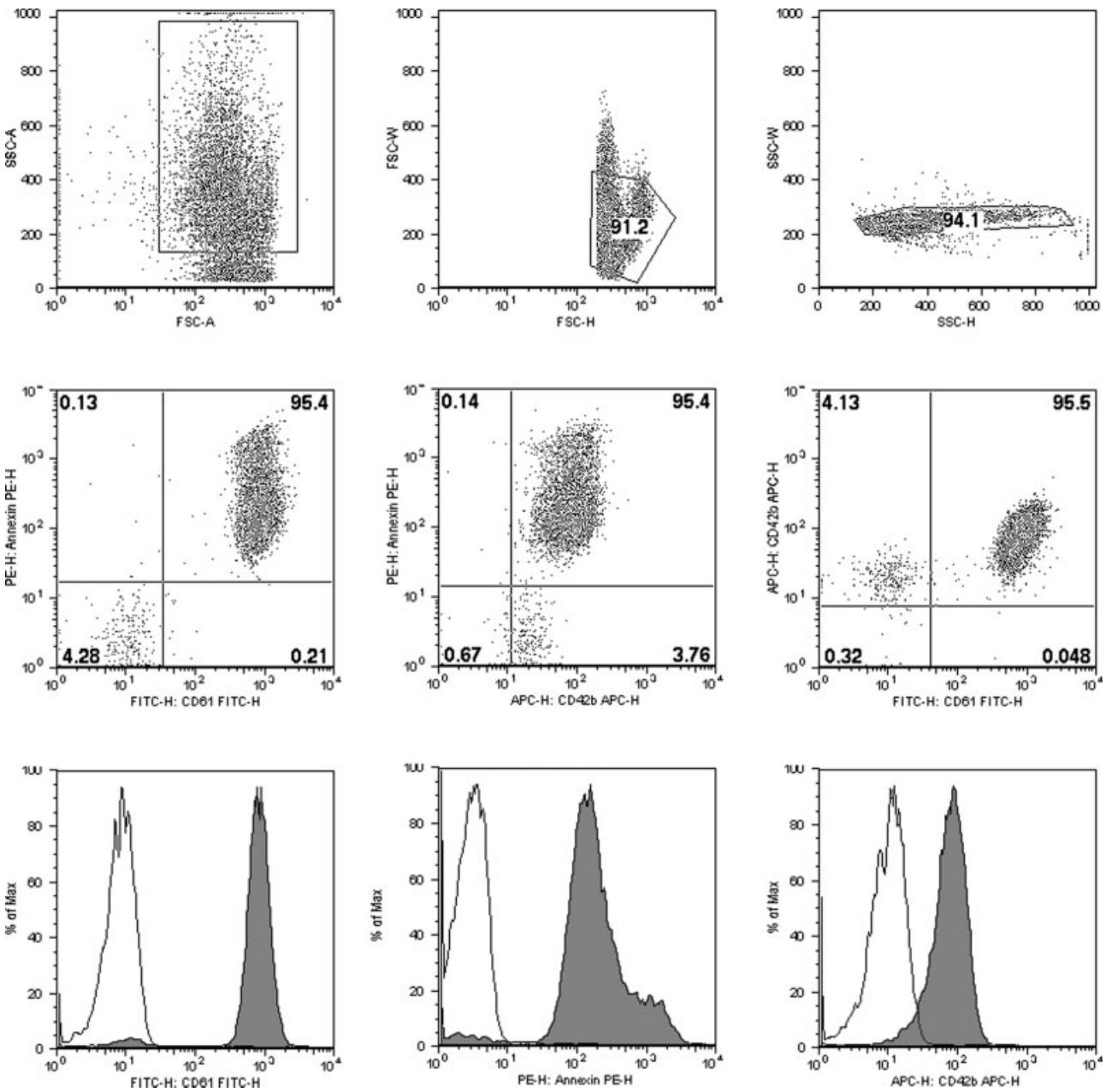

FIG. 4. Acquisition of MPs isolated by centrifugation from TRAP-stimulated platelets, and subsequently stimulated with the calcium ionophore A-23187 (3 μM) for 10 min. Analysis showed that the sequential activation of platelets with TRAP followed by ionophore resulted in a homogenous population of MPs that were triply positive for CD61, CD42b, and PS.

achievement that is not attainable using current analog flow cytometers. Furthermore, the FACSAria demonstrated the ability to cleanly sort MP subsets. TRAP-derived MPs were sorted according to GP IIIa and PS expression, and separated into two different populations according to PS expression (Fig. 5). In our sorting strategy, we elected to use lactadherin, a milk-derived protein that binds PS. Previous studies have shown that compared to Annexin V, lactadherin has an increased affinity for anionic phospholipids, and binds preferentially to highly curved membranes (18). In addition, lactadherin is a more sensitive marker of small changes in PS expression, and offers the advantage of facilitating sorting in a calcium-free environment (such as citrate- or EDTA-anticoagulated clinical samples) (17,18). To our knowledge, ours is the first reported demonstration of lactadherin as a probe for PS expression in MPs, and of MP sorting using a cell sorter approach.

Many groups (including our own) have defined circulating MPs in blood according to three criteria, namely: (1) size <1 μm; (2) specific binding of well-characterized monoclonal antibodies to cell-specific antigens; and (3) the fact that ultracentrifugation will deplete the measured events

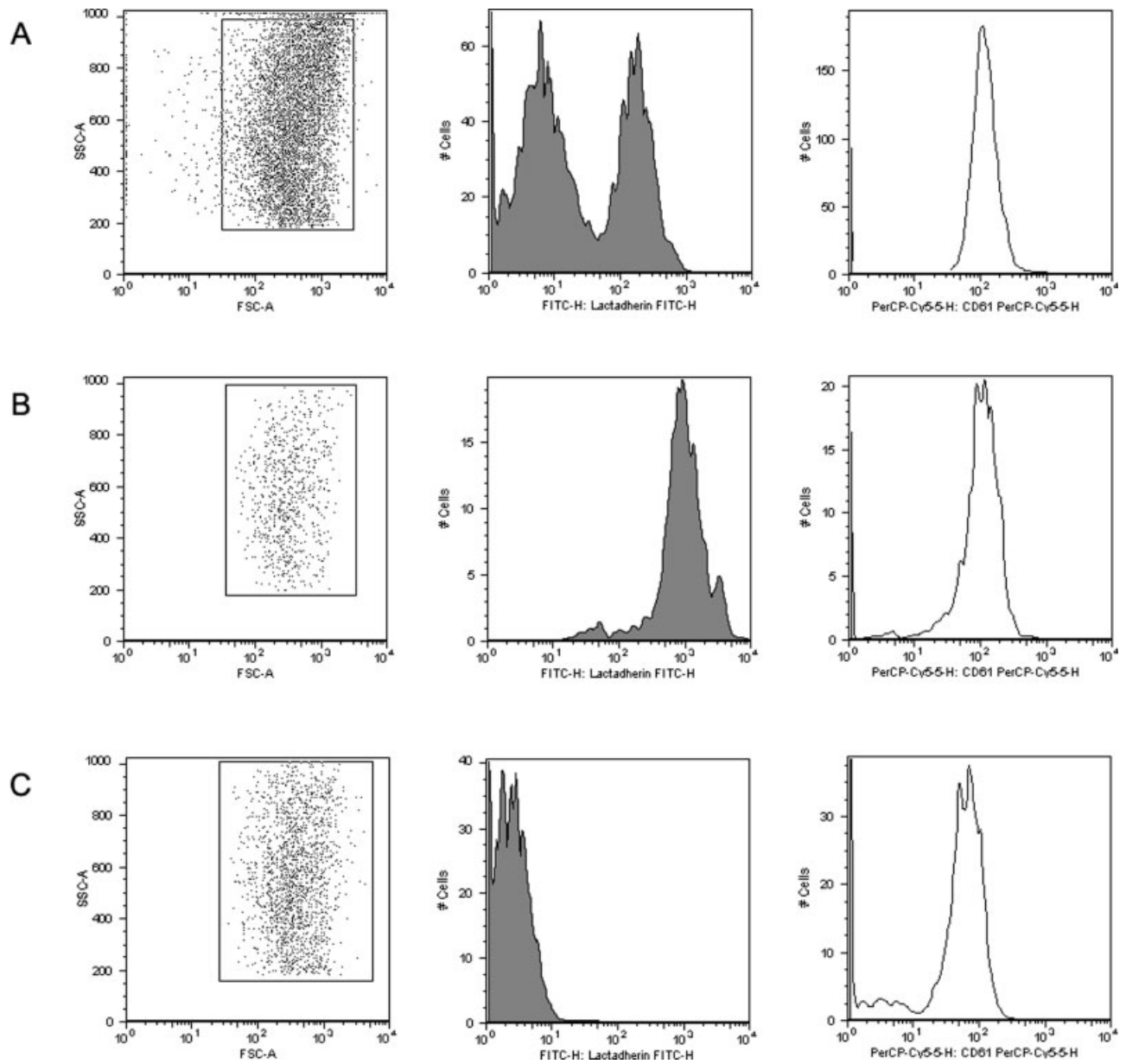

FIG. 5. Sorting of TRAP-generated MPs according to PS exposure. Expression of PS on MPs was detected using a novel noncalcium-dependent binding protein, lactadherin. MPs were gated as described earlier and sorted according their positivity for anti-CD 61 and their positivity or negativity to lactadherin (middle panel, Row **A**). Post sorting acquisition and analysis showed the ability of FacsAria to separate MPs into two populations, i.e. one that was positive for anti-CD61 and lactadherin (row **B**), and one that was positive for anti-CD61 but negative for lactadherin (row **C**).

from platelet-free plasma (4,12). Our findings confirmed previous reports (19) showing that platelet-derived MPs contain GP IIb-IIIa and GP Ibα in easily detectable amounts on the membrane, allowing these antigens to be used as surface markers for platelet-derived MPs. Previous studies using more conventional FACS technology have suggested that MPs could be relatively deficient in GP Ib (20) while being enriched in GP IIb-IIIa (21). Our present results demonstrate that both antigens are well represented, and may be used for flow cytometry detection in platelet-derived MPs produced by either TRAP or ionophore. It is frequently assumed that levels of P-selectin expressed on the platelet surface will depend on the potency of the activating agent (22,23). According to this paradigm, higher levels of P-selectin in ionophore-derived MPs might be predicted. On the contrary, our results suggest that levels of P-selectin were increased in MPs generated by TRAP (Table 1) (24).

In addition to the usual properties used to define MPs (listed above), most laboratories have also included PS exposure (detected by binding of labeled Annexin V) as an essential additional criterion (25,26). However, our results support the concept that MPs are actually quite heterogeneous with respect to PS exposure, depending on the agonist used to generate them. Specifically, although MPs derived

from TRAP activation were virtually 100% positive for GPIbα and GP IIIa, the analysis of PS expression detected two different populations, one Annexin V-positive and the other Annexin V-negative (Fig. 2). When comparing Annexin V-positive MPs with Annexin V-negative MPs, similar percentages of GPIb- and GP IIIa-positive events were detected (Table 1). The ability of platelets to express PS seems to be dependent on both the agonist and the concentration used (8). Similarly, our results support the concept that the PS exposure—like P-selectin expression—on MPs may be dependent on the specific agonist used to generate them. The heterogeneity of platelet-derived MPs prepared by various activating agents ex vivo may also be reflected by heterogeneity of PS expression in vivo. Therefore, criteria used for detection of platelet-derived MPs in blood by flow cytometry should include PS-negative events that otherwise fit the profile of MPs bearing antigens known to be platelet-specific. The heterogeneity observed in the expression of anionic phospholipids may lead to new insights regarding the function and the biological significance of MPs in health and disease states. For example, there are few data on the half-life of circulating MPs in vivo, but it may be speculated that the circulation time of PS-negative MPs is relatively prolonged, because of their failure to be recognized by senescence/apoptosis PS receptors in macrophages. This and other characteristics of MP subsets may help to understand the apparently paradoxical effects of platelet-derived MPs in disease states. For example, these MPs are postulated to play a protective role against hemorrhage in thrombocytopenic patients with autoantibody-mediated immune thrombocytopenic purpura (1–3,13), yet be responsible for frequent and severe thrombotic events in the immune-mediated condition of heparin-induced thrombocytopenia (9). Furthermore, the potential value of distinguishing MPs according to PS expression may not be limited to platelet-derived MPs. Analogous studies have demonstrated that endothelial cell-derived MPs that circulate in vivo may be positive or negative for PS expression, depending on whether they were generated by activation or apoptosis, respectively (27).

Interestingly, our observations also suggest that with appropriate second stimulation, "nonprocoagulant" MPs (i.e., those not exposing PS) retain the pathways required to undergo loss of membrane phospholipid asymmetry (Fig. 4). We are currently investigating the mechanism by which ionophore induces the loss of phospholipid asymmetry in TRAP-generated MPs. This and a wealth of other data (3,13) suggest that far from being inert end-stage byproducts of cell activation or apoptosis, MPs may play a variety of crucial active roles in health and in disease states.

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