Clin. Lab. Haem.
1995, **17**, 163–172

Enumeration of platelets by multiparameter flow cytometry using platelet-specific antibodies and fluorescent reference particles

R. DICKERHOFF *Department of Haematology and Oncology, Kinderklinik St. Augustin, D-53754 St. Augustin, Germany*

A. VON RUECKER *Department of Clinical Biochemistry, University of Bonn, D-53105 Bonn, Germany*

Summary The correct enumeration of platelets is still an elusive matter. This is mainly due to the fact that commercial instruments which are used for platelet counting cannot discriminate platelets from other cellular particles and precipitates that cause similar signals. Visual (chamber counting) methods are still frequently used in routine laboratories to verify low automated platelet counts ($< 50 \times 10/l$) despite obvious technical and statistical drawbacks. The following report shows how platelet counts can be measured by multiparameter flow cytometry with the help of reference particles (fluorescent latex beads) and platelet-specific antibodies i.e. *anti*-GPIIb/IIIa(CD41a), *anti*-GP Ib-α (CD42b) and *anti*-GP IIIa (CD61). The linearity of this method was highly satisfactory and the observed imprecision was within acceptable limits. At a platelet concentration of $10 \times 10^9/l$ the coefficient of variation (CV, $n = 10$) ranged from 5.3% (PCV = 0.456) to 5.6% (PCV = 0.148). Accuracy was evaluated by comparing results to the ICSH-selected method for platelet counting. The correlation of both methods was significant ($P < 0.005$) and Passing-Bablok's linear regression analysis showed no systematic differences between the two methods. Comparisons of this new platelet counting technique were also performed with routine visual methods, automated blood analysers (Technicon H-1, Sysmex E-5000) and a different flow cytometric method using only forward and side light scatter properties of platelets for their discrimination. The linear correlation of all methods was significant ($P < 0.01$) at platelet concentrations above $50 \times 10^9/l$. At lower platelet concentrations, our new platelet counting technique correlated significantly only with the visual and the forward/side scatter methods. These findings stress the necessity to confirm low platelet counts by automated blood analysers and suggest that using multiparameter flow cytometry with platelet-specific antibodies may be a proficient way to do so. The possibility of using this technique as a reference method is discussed.

Keywords platelet counts, platelet-specific antibodies, CD41a, CD42b, CD61, reference particles, flow cytometry

Introduction

Clinical reports have pointed out that prophylactic platelet transfusions in patients with reduced or absent platelet production are indicated if morning platelet counts are below $5 \times 10^9/l$ or below $10 \times 10^9/l$ in the presence of haemorrhagic manifestations or fever (Kottke-Marchant 1994; Gmür *et al.* 1991; National Institutes of Health, 1987). On the other hand, it has been demonstrated that the accuracy of platelet counts with all currently available methods is most suspect when levels are below $50 \times 10^9/l$ (Rowan 1991). The following report outlines this discrepancy and suggests a method for confirming present-day platelet counting in questionable cases.

Fairly accurate platelet counts are achieved with visual, chamber counting methods if a standardized protocol is followed, but this is rather time-consuming and not realistic in routine practice. The imprecision of routine chamber

Accepted for publication 15 March 1995
Correspondence: A. von Ruecker

counting due to technical errors, subjective mistakes by technicians and counting statistics is variable: coefficients of variation (CVs) can be from 3% to 11% at a platelet count of 250×10^9/l to well over 20% at platelet levels below 50×10^9/l (England 1990; Brecher, Schneiderman & Cronkite 1953). Automated blood analysers frequently used in laboratories today have limitations in platelet counting. Most blood analysers are flow-through cytometers that register optical or electrical signals from cells passing through a sensing zone. The amplitudes/intensities of these signals are proportional to the particle's size and are categorized accordingly as platelets, erythrocytes or leucocytes. Erythrocytes and leucocytes can be quantified quite efficiently in whole blood with the use of size thresholds (ICSH 1994). However, the use of this technique for distinguishing and counting platelets has several drawbacks. Especially at low platelet counts, microcytic erythrocytes, schizocytes, debris, precipitation of chemicals, air bubbles and electronic noise may cause signals similar to platelets and lead to false high counts. Platelet aggregates and giant platelets on the other hand may fall outside the platelet upper size threshold and not be recognized properly (Bode 1993; Rowan 1991; England & van Assendelft 1986).

Instead of using size thresholds to identify platelets, we applied immunological techniques. Platelet-specific antibodies conjugated to the chromophore fluorescein (FITC) were bound to platelets, thus enabling the discrimination of platelets and platelet-derived particles from erythrocytes, leucocytes and other cells or particles by setting the fluorescence (530 nm) channel of our multiparameter flow cytometer to detect FITC positive particles. Immunologically intact platelets were then distinguished from platelet-derived microparticles and larger platelet aggregates by their characteristic forward and side light scatter properties (Abrams & Shattil 1991).

Most multiparameter flow cytometers are not capable of measuring sample volume with sufficient accuracy unless additional tools are used. In our study we examined two different approaches to avoid instrument modifications. First, we determined the ratio of FITC-stained platelets to erythrocytes from our list mode data. By multiplying this ratio with the erythrocyte count that was obtained from an accurately calibrated and controlled alternative analyser, the platelet count was calculated. Secondly, we added a defined amount of FITC-fluorescent latex particles (FITC beads) to an accurately known volume of blood. We then used a fluorescence threshold to count FITC-stained platelets and FITC beads selectively and could determine the platelet count by multiplying the FITC platelet/FITC bead ratio with the known FITC bead concentration.

Patients, materials and methods

Patients, blood samples

Thirty thrombocytopenic patients (platelet counts below 50×10^9/l) were studied including two patients with acute idiopathic thrombocytopenia, 12 patients with thrombocytopenia of various origins and 18 receiving cytotoxic therapy. The control/normal group consisted of hospital personnel with no signs of illness who came for routine check-ups ($n = 45$) and five blood samples were from patients with high platelet counts ($>400 \times 10^9$/l) of unknown cause. Blood samples were collected in K_2EDTA tubes during routine monitoring or check-ups and, in the case of thrombocytopenic patients, after thrombocytopenia had been confirmed on previous occasions. No blood samples in excess of those collected for routine procedures were necessary. All samples were processed within 1 h after blood had been drawn.

Materials

Fluorescent monoclonal antibodies were mouse anti-human from Becton Dickinson (Heidelberg; CD61-FITC) and Immunotech (Hamburg; CD41a-FITC, CD42b-FITC). Molecular Probes Inc. (Eugene, OR, USA) provided the FITC conjugated latex particles (beads) that had distinctly different forward and side light scatter characteristics than platelets. Calibrite™ beads for calibration purposes were supplied by Becton Dickinson (Heidelberg). All pipettes (Eppendorf-Netheler-Hinz GMbH, Hamburg) were checked for accuracy and precision with help of a micro-scale (A&C Instruments, Frankfurt), CVs over 1% ($n = 10$) were not accepted. Reagents and chemicals were from E. Merck (Darmstadt) unless indicated otherwise.

Methods

(a) Platelet counts by automated blood analysers. A full blood count was performed in duplicate on all patient and control samples using a Technicon H-1 (Bayer Diagnostics, Munich) and a E-5000 (TOA-Sysmex, Digitana, Hamburg). Platelet counts of each patient and instrument were averaged. If two platelet counts of the same sample and by the same instrument differed by more than 7% (mostly due to blood clotting), the sample was not included in the study.

(b) Platelet counts by Neubauer counting chamber. The ammonium oxalate method was applied essentially as described by Dacie and Lewis (Dacie & Lewis 1991). In short, a 1% aqueous ammonium oxalate solution was prepared and filtered through a micropore filter (0.22 μm).

100 μl of blood was added to 1.9 ml solution. After mixing the suspension for 10 min, a Neubauer counting chamber (5 × 16 fields) was filled and placed in a moist Petri dish. After 20 min a phase-contrast or (in routine procedures) light microscope was used to count at least 200 platelets. Counts were always performed in duplicate. To determine platelet count accuracy, two experienced haematologists working separately used the somewhat more meticulous selected method suggested by the International Committee for Standardization in Haematology (ICSH 1988). If sample platelet counts established by these haematologists differed by more than 5%, the sample was not used in the study.

(c) Platelet counts by flow cytometry. Two aliquots (50 μl) of a 1 in 5 dilution of EDTA-anticoagulated whole blood with PBS (phosphate buffered saline) were mixed with saturating concentrations of monoclonal FITC conjugated platelet-specific antibodies (see below, 10 μl of commercially available stock solutions) and incubated for 30 min at room temperature in the dark. Following incubation, one sample was diluted with plain PBS (1 ml) and the other was diluted with PBS containing an explicit amount of FITC conjugated latex beads (1% to 5% of the erythrocyte count; 5–10 μm in diameter). FITC bead counts in stock solutions were calibrated with the help of a ZM particle counter (Coulter Inc., Krefeld) in accordance with the German DIN reference protocol used for determining erythrocyte counts (Deutsches Institut für Normung 1994). In short, this protocol prescribes a series of at least 10 measurements at five different particle dilutions to enable correction for coincidence and to guarantee linearity and accuracy. The between-day imprecision (CV) of the measured FITC bead concentrations was less then 2% ($n = 10$) in all cases.

The platelet-specific antibodies used were *anti*-CD41a, *anti*-CD42b and *anti*-CD61. CD42b or glycoprotein (GP) Ib is part of the von Willebrand and thrombin receptor complex and considered to be an activation-independent membrane marker. CD41a (GP IIb/IIIa) and CD61 (GP IIIa) form the fibrinogen receptor and are activation-dependent, i.e. they increase on surface membranes if platelets are traumatized or stimulated (Abrams & Shattil 1991; Bode 1993). After initial testing, we chose to use a mixture (5 μl + 5 μl) of either *anti*-CD42b/*anti*-CD41a or *anti*-CD42b/*anti*-CD61 FITC conjugated antibodies for our study, because we often had observed that the binding of *anti*-CD42b fluorescent antibodies was reduced after platelets were traumatized or stimulated, thus requiring FITC threshold readjustments. This decrease in CD42b on platelets has also been reported by others and is probably due to agonist-induced redistribution of CD42b from the platelet's surface membrane to the membranes of the open canalicular system (Hourdille *et al.* 1990). Both antibody mixtures were equally suited for platelet counting; for financial reasons we chose the *anti*-CD42b/*anti*-CD41a mixture.

A FACScan (Becton Dickinson, Heidelberg) flow cytometer was used for measuring the stained platelets with logarithmic amplification on all parameters. Calibrite™ beads and the Calibrite software (Becton Dickinson) were always used prior to measurements to align the instrument. Next, green and red amplifier gains were fine-adjusted with the help of a pre-selected batch of Calibrite™ beads so that the beads' green and red fluorescence peak channels were comparable in all experiments. The stained platelet sample without FITC beads was then run and events were recorded in forward/side scatter (FSC/SSC) and florescence-1/side scatter (FL1/SSC) mode with forward scatter threshold settings on level 90 (of 1024 channels) (see Figure 1). If the particle flow rate was too high, coincident counting of FITC-labelled platelets and erythrocytes occurred which caused false-positive immunofluorescence on erythrocytes (see Figure 1B). Samples were diluted until no coincident counting could be detected. This was usually the case at flow rates of approx. 2000 particles (blood cells) × sec^{-1}. Similar flow rates to avoid coincidence in FACScan flow cytometers have been reported by others when examining immunofluorescence on platelets in whole blood (Scharf *et al.* 1992).

After optimal flow rates were established, samples with and without beads were diluted similarly and 10–50,000 particle events were registered without further instrument adjustment (Figure 2). The threshold was then changed to FL1 and set at level 500–540 (of 1024 channels) to exclude all FITC-negative particles: 5–10,000 fluorescence events were registered (Figure 3). Sedimentation of particles may affect the results if registration times exceed 3 min. To assure constant readings the particle inlet must be adjusted above the sedimentation zone in the sample tubes (e.g. by using a tight-fitting plastic/rubber hose as an extension between the machine nozzle and the sample tube).

Platelet counts were determined from samples with and without FITC beads using Lysis II software (Becton Dickinson) as follows:

1. Forward and side scatter method (see Figure 1A). Platelets were discriminated from debris and erythrocytes visually with the help of histograms. The ratio of platelets to erythrocytes was determined and multiplied by the erythrocyte count obtained from an accurately calibrated and controlled blood analyser (here a Technicon H-1);

2. FITC immunofluorescence method (Figures 2 and 3). Particles with positive FITC immunofluorescence (platelets,

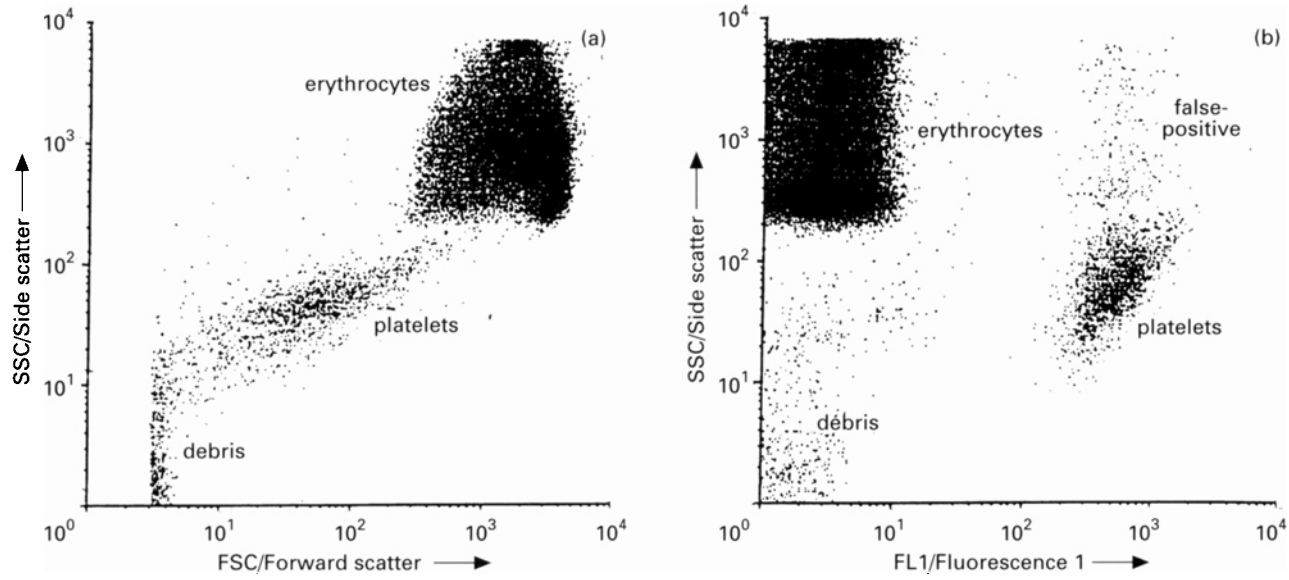

Figure 1. Whole blood analysis of platelets by flow cytometry. (a) Dot plot of platelets in whole blood. (b) False-positive FITC immunofluorescence associated with erythrocytes due to coincident counting of platelets labelled with *anti*-CD42b and *anti*-CD41a FITC conjugated antibodies at high flow rates (10,000 particles $\times s^{-1}$).

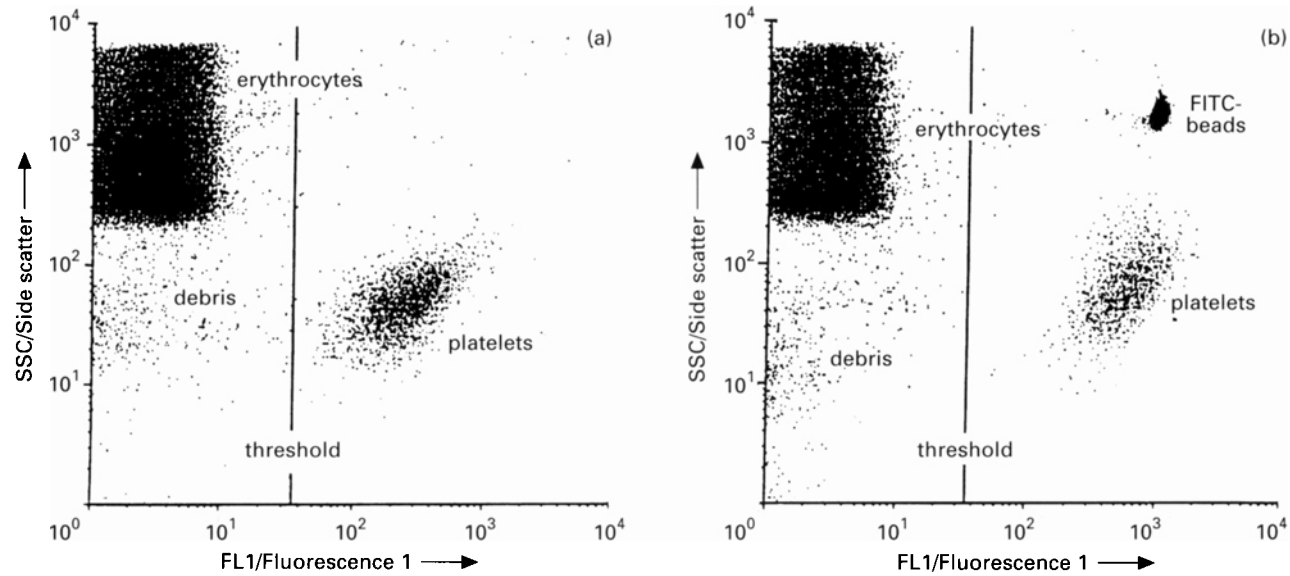

Figure 2. Whole blood analysis of platelets by flow cytometry in the absence (a) and presence (b) of FITC beads. Platelets were labelled with FITC-conjugated antibodies against CD41a and CD42b. 50,000 events were registered. Vertical lines indicate fluorescence-1 (FL1) threshold settings used in Figure 3.

platelet derived microparticles, larger platelet aggregates, FITC beads) were separated from FITC-negative particles by threshold analysis (Figure 3B) or region settings (Figure 2) and re-plotted in forward/side scatter mode (Figure 3A). Platelets were then discriminated from platelet derived microparticles and platelet aggregates by visual criteria (cluster formation). The ratio of platelets to erythrocytes (Figure 2A) or platelets to FITC beads (Figure 3A) was then formed and the platelet count calculated by means of the known erythrocyte or FITC bead count.

It is important to check the correctness of the FITC bead concentration by calculating the erythrocyte count from the erythrocyte to FITC bead ratio (Figure 2B). If there is a large discrepancy ($\geqslant 5\%$) between the bead-derived erythrocyte count and the erythrocyte count from the calibrated blood analyser, this is often due to blood clotting or

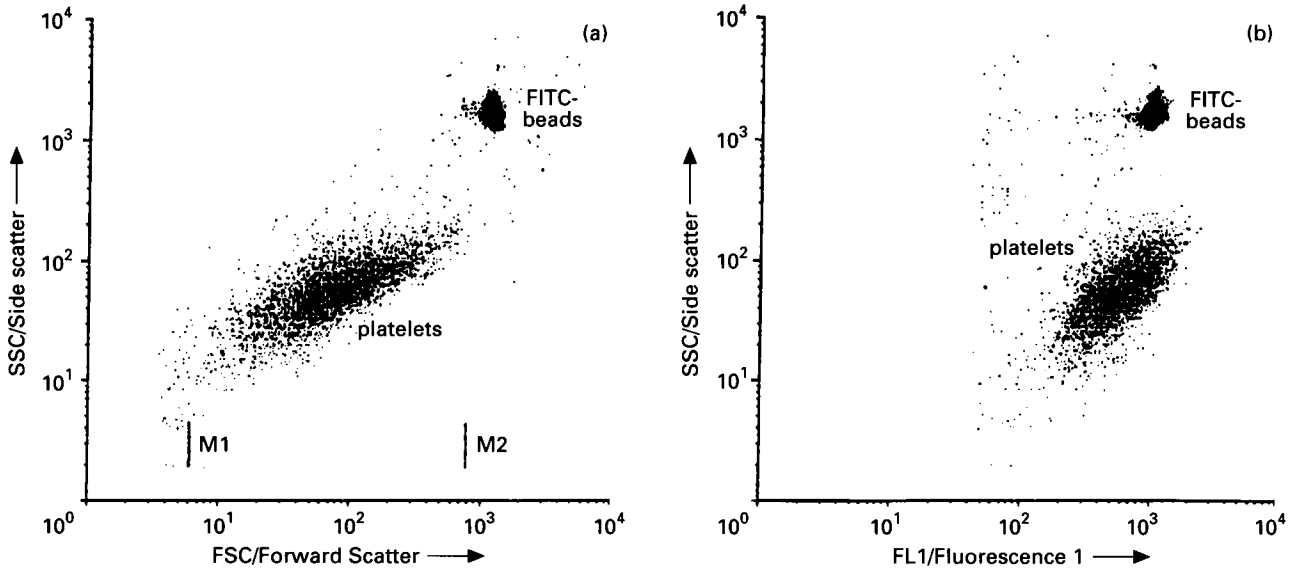

Figure 3. Platelet analysis in the presence of FITC beads. Platelets labelled with FITC-conjugated antibodies were analysed after the FL1 threshold was set as indicated in Figure 2. (a) Sample in forward/side scatter mode with marker settings to exclude platelet-derived microparticles (M1) and aggregates (M2); (b) Same sample in FL1/side scatter mode.

adherence of old FITC bead preparations to artificial surfaces (Thom 1985). Microscopic inspection of the sample and careful testing of glassware and plastics prior to use usually helps. Normal leucocyte counts registered along with the erythrocytes can be ignored.

(d) Platelet-depleted/platelet-enriched whole blood preparations. For linearity measurements fresh whole blood containing ACD (acid citrate dextrose) from one healthy donor was acquired from the local blood transfusion service. After centrifugation (500 × g, 10 min) in appropriate glassware, plasma and the buffy coat were removed. Red cells were then washed with an excess of physiological saline (9 g/l NaCl) until the remaining platelets were below 2×10^9/l ($n = 5$). The plasma fraction was also centrifuged at 2000 × g (30 min) to remove all cellular particles and added in different amounts to the washed erythrocytes to achieve PCVs of approx. 0.15, 0.30 and 0.45. A defined amount of fresh platelet concentrate from blood-group identical donors from the local blood transfusion service was then given to parts of each blood preparation to obtain platelet counts of approx. 1000×10^9/l ($n = 5$). Platelet counts were then varied by mixing platelet-depleted and platelet-enriched blood preparations with the same PCV. All platelet counts were performed using the ICSH-selected chamber counting method as described above.

Statistical analysis

The statistical tests used to compare the results obtained from different platelet counting methods were Spearman's correlation coefficient and Passing-Bablok's non-parametrical linear regression analysis (Passing & Bablok 1983). The Wilcoxon matched-pairs, signed-rank test and Student's *t* test were used for individual comparisons. Differences were considered significant at the 5% level.

Results

Linearity of the platelet count measured with the help of platelet-specific antibodies and FITC beads (Figure 4)

Visual assessment of the plots shown in Figure 4 indicate no substantial deviations of linearity at platelet concentrations in the range of 10–1000 × 10^9/l. In the range below 10×10^9/l, the FITC bead method has a slight tendency towards higher-than-expected values. When platelet counts were determined with the help of the erythrocyte/platelet ratio instead of with the bead/platelet ratio (see Methods), no differences in linearity were observed at platelet concentrations above 20×10^9/l. Below this level the platelet count was 7% to 18% higher using the erythrocyte/platelet ratio. This was probably due to the fact that, at low platelet concentrations, FITC bead concentrations were only 1% to 5% as large as erythrocyte concentrations (see Methods) thereby allowing more platelets to be counted which improved platelet counting statistics and made discrimination between platelets, platelet-derived microparticles and platelet aggregates easier (also see Table 1). Linearity was not affected by the different PCVs (0.15, 0.30 and 0.45) of the tested blood preparations.

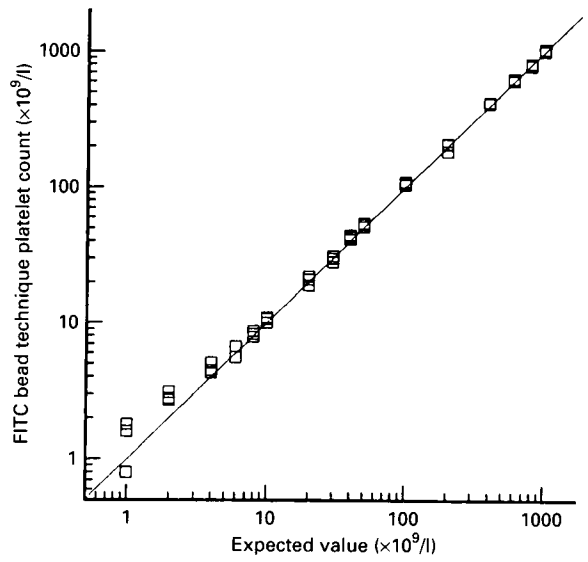

Figure 4. Linearity of platelet counts determined with platelet-specific antibodies (*anti*-CD42b, *anti*-CD41a) and FITC beads. A platelet-depleted blood preparation (platelet count approx. 1×10^9/l) was mixed with a platelet-enriched blood preparation (platelet count approx. 1000×10^9/l) to form different platelet concentrations at a constant PCV of 0.45 as described in Methods. The graph shows the values obtained as opposed to the expected values. Each symbol represents one measurement done in duplicate; three measurements were done at each level.

Precision (Table 1)

Precision was evaluated at low (10×10^9/l) and normal (250×10^9/l) platelet counts in the presence of different erythrocyte concentrations (PCVs). Within-run precision ($n = 10$) was satisfactory at low platelet concentrations for the methods using FITC immunofluorescence and precision was not influenced noticeably by different PCVs. In contrast, the platelet count in the Neubauer chamber at low concentrations was associated with a high imprecision and was distinctly influenced by the PCV. At normal platelet concentrations all methods performed satisfactorily and the platelet count was not significantly changed by the PCV.

Between-run precision of the flow cytometric methods was studied by running specified samples (with low and normal platelet concentrations) in 10 successive batches of 10 patient samples over a period of 12 hours. No significant changes of the platelet counts and CVs were observed.

Accuracy, comparison of methods

Lacking a true reference method, we used the ICSH-selected chamber counting method to study accuracy of platelet

Table 1. Within-run precision of different platelet counting methods. Ten replicate analyses were performed on single samples with each method. The influence of different PCVs on the coefficient of variation is shown (see text).

Method	Visual chamber counting*	FITC platelet/erythrocyte ratio	FITC platelet/FITC bead ratio
1. PCV = 0.148			
platelet count ($\times 10^9$/l)	10.4†	11.3	10.2
expected: 10×10^9/l			
CV (%)	14.7	8.9	5.6
2. PCV = 0.456			
platelet count ($\times 10^9$/l)	12.3†	11.8	10.4
expected: 10×10^9/l			
CV (%)	17.4	9.1	5.3
3. PCV = 0.145			
platelet count ($\times 10^9$/l)	255.7	252.3	254.7
expected: 250×10^9/l			
CV(%)	7.3	3.3	2.9
4. PCV = 0.448			
platelet count			
($\times 10^9$/l)	259.4	254.9	251.6
expected: 250×10^9/l			
CV (%)	8.9	3.7	3.1

* 200 platelet count in routine laboratory; † $P < 0.05$

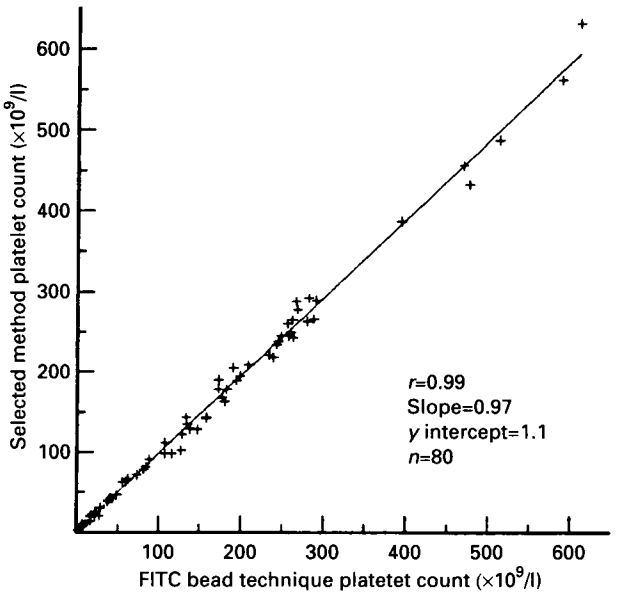

Figure 5. The accuracy of platelet counting by the FITC bead technique is demonstrated by comparison to the ICSH-selected method. Thirty thrombocytopenic patient samples (platelet counts $< 50 \times 10^9$/l) and 50 samples from randomly chosen patients were analysed. The linear regression is shown.

counts as described in Methods. The samples of 80 patients were used including 30 samples from patients with low platelet counts ($< 50 \times 10^9$/l). A very significant correlation ($P < 0.005$) between the selected method and FITC bead technique was observed (Figure 5). The Passing-Bablok linear regression analysis showed that the y-intercept is not significantly different to 0 and the slope is not significantly different to 1. Similar results were obtained if the data of the 50 patients with platelet counts above 50×10^9/l and the 30 patients with platelet counts below 50×10^9/l were analysed separately. The FITC bead technique was next compared to different platelet counting methods and instruments:

Method A: the chamber counting method in our routine laboratory using light microscopy;
Method B: the technique described in Methods that uses only the forward and side light scatter properties of platelets for platelet discrimination (see Figure 1A);
Method C: the Technicon H-1, a multichannel whole blood analyser using laser light;
Method D: the Sysmex E-5000, an automated whole blood analyser using aperture-impedance and hydrodynamic focusing.

A good and significant ($P < 0.01$) correlation of all these methods with the FITC bead method was observed at platelet concentrations above 50×10^9/l ($n = 50$):

Method A: $r = 0.99$
slope = 1.00, y-intercept = $+6.7 \times 10^9$/l
Method B: $r = 0.99$
slope = 1.04, y-intercept = $+4.1 \times 10^9$/l
Method C: $r = 0.97$
slope = 0.98, y-intercept = $+1.8 \times 10^9$/l
Method D: $r = 0.98$,
slope = 1.05, y-intercept = $+2.1 \times 10^9$/l

The slopes were not significantly different to 1 and the y-intercepts were not significantly different to 0 in methods B, C and D. The y-intercept of Method A is significantly too high ($P < 0.05$; confidence range from 0.66 to 4.1×10^9/l) which indicates that there was a constant systematic difference between the routine chamber counting and FITC bead technique at platelet concentrations above 50×10^9/l.

At platelet concentrations below 50×10^9/l ($n = 30$) correlation data is shown in Figure 6. There was no significant correlation between the FITC bead technique and both automated blood analysers, whereas a significant correlation was found with the routine chamber counting method ($P < 0.03$) and the forward/side scatter technique ($P < 0.01$). Only the slope of the routine chamber counting method was not significantly different to 1. All other methods showed a significant difference in slope to the FITC bead technique which reveals that there is a proportional systematic difference between the results of the FITC bead technique and methods B, C and D at low platelet counts. All y-intercepts were classified significantly different to 0 by Passing-Bablok's linear regression analysis.

Discussion

There is no disagreement about the fact that platelet counts are of clinical utility even if platelet function is becoming more important (Bode 1993; Kottke-Marchant 1994). Whether or not platelets can be counted adequately by existing methods is contentious. Our study shows that platelet counting with all the methods tested is satisfactory at platelet concentrations above 50×10^9/l. At lower levels, it seems fair to say that present-day platelet counting for diagnostic reasons may be adequate but that counting for the purpose of therapeutic decisions is not. Others have come to similar conclusions (Rowan 1991).

Most commercially available blood analysers still use size thresholds to discriminate platelets from other particles. This can cause various problems owing to microcytic erythrocytes and debris as already pointed out. Our results with thrombocytopenic patients illustrate this point. At platelet levels below 50×10^9/l there was no significant correlation between platelet counts from the tested auto-

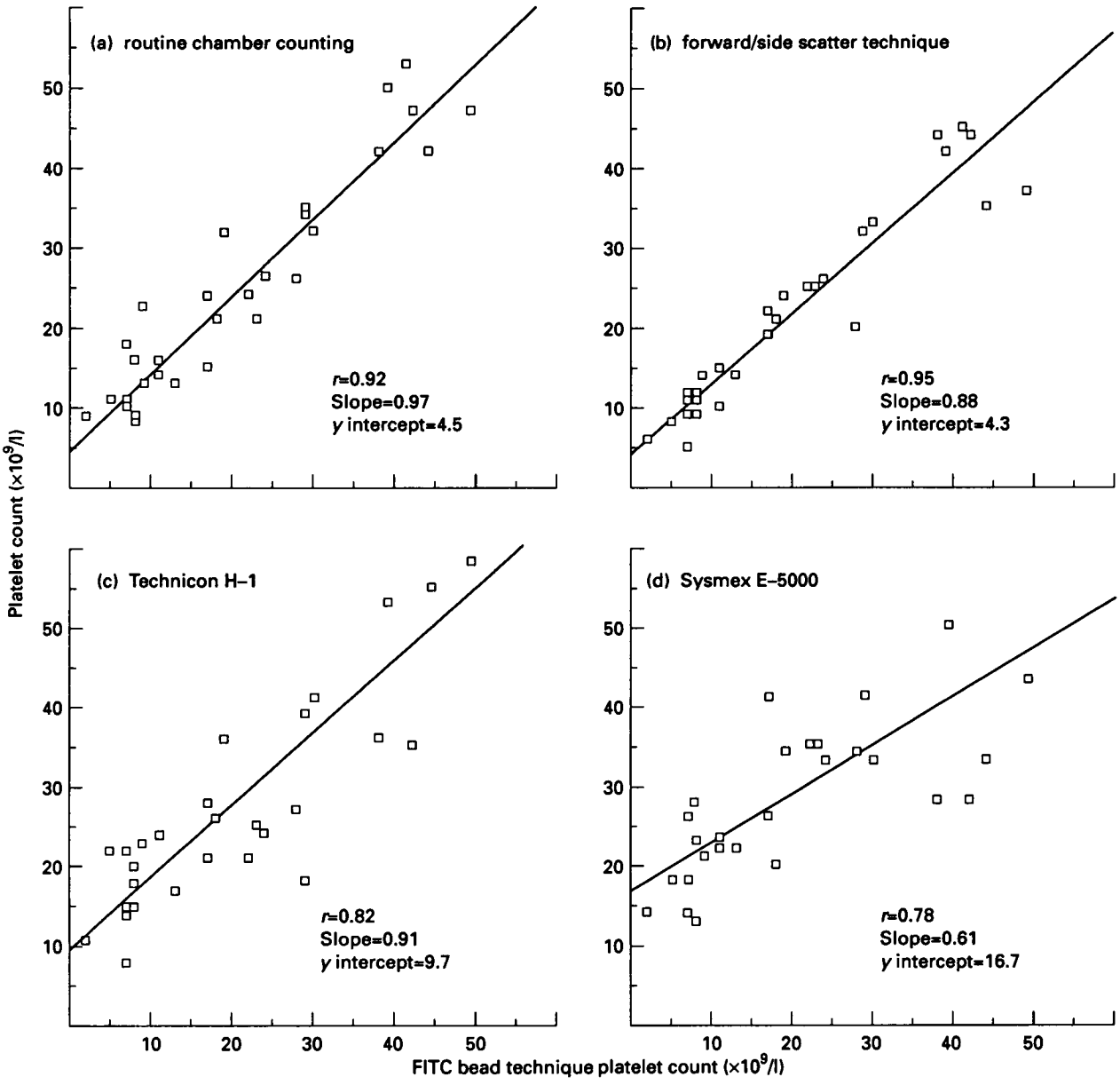

Figure 6. Comparison of different platelet counting methods and instruments with the FITC bead technique at low platelet concentrations ($< 50 \times 10^9$/l). Thirty thrombocytopenic patients' samples were analysed (see text).

mated blood analysers and those determined with the help of our FITC bead technique which proved to be equivalent to the ICSH-selected method. Manual platelet counts by chamber counting techniques in our routine laboratory correlated surprisingly well with the FITC bead technique (and the ICSH-selected method) and may be due to the fact that the technicians' honour was at stake. Manual platelet enumerations in routine laboratory settings are not a technician's delight and must follow a scrupulous protocol to ensure acceptable reproducibility and sensitivity. It seems unwise to recommend platelet transfusions at platelet levels below 20×10^9/l, a practice still followed at many institutions, unless the time-consuming ICSH-selected method or an equivalent platelet counting method is applied and has been accredited (National Institutes of Health 1987).

Technological advances in instrumentation have not remained at a standstill during the past years, and it was clear to us as we started our study that using a multiparameter flow cytometer with fluorescent platelet-specific antibodies must be more accurate in discriminating platelets from other particles and disturbing signals than conventional techniques. On the other hand, we did not know what difficulties to expect.

One unexpected finding was that a significant

coincidence of erythrocytes and platelets may occur at particle flow rates above $2000 \times s^{-1}$ in flow cytometers, in spite of a hydrodynamic focusing system and the use of laser-optics that decrease dead time. This was quite irritating initially because the instrument's pulse-analysing system did not register this fact or show any warning. Other authors have had similar experiences (Scharf *et al.* 1992). Probably coincident counting was due to the slow analog-to-digital converting system that is still used in our flow cytometer and was not caused by a poor flow cell.

Another important issue was that sedimentation of particles (especially erythrocytes and latex beads) in the sample tubes may influence the platelet to erythrocyte or platelet to bead ratio. This can be avoided by adjusting the instrument's particle inlet above the sedimentation zone and below the dilution zone (e.g. by using a tight-fitting plastic extension on the machine nozzle). In our study, this guaranteed constant particle ratios even during long particle registration periods that were necessary at platelet concentrations below 10×10^9/l with the FITC bead technique.

The careful enumeration and testing of FITC conjugated latex beads is a further important issue. Before setting FITC fluorescence thresholds (Figure 2), it is essential to see if the erythrocyte count can be calculated correctly from the FITC bead to erythrocyte ratio and the FITC bead concentration (see Methods). This applies especially to old and used bead stock solutions. The use of cell lysing solutions to eradicate erythrocytes should be avoided. It caused the imprecision of the platelet count measured by the FITC bead technique to increase substantially. The reason for this phenomenon is still unclear, but may be due to increased adherence of beads to various surfaces (Thom 1985). Sometimes we also observed a drastic increase in platelet microparticles, which suggests platelet breakdown by lysing techniques.

In our opinion, the FITC bead technique is able to verify platelet counts at concentrations below 50×10^9/l and may be a suitable reference method for platelet enumeration if applied meticulously. Installing the technique in a routine clinical laboratory setting is wearisome, but it offers definite advantages over the routine chamber counting method in terms of accuracy and precision. The overall operator time is about the same as for the chamber counting method and decreases with the number of assays. The major disadvantage of the FITC bead technique is that, besides requiring a flow cytometer with the appropriate instrument software and skilled personnel, expensive platelet-specific monoclonal antibodies are needed. On the other hand, expensive platelet transfusions may be avoided.

The FITC bead technique distinguishes platelets from other cellular particles and allows discrimination of platelets from platelet-derived microparticles and platelet aggregates. This was not the case if only forward and side scatter characteristics of platelets were used to distinguish platelets (Figure 1A). The alternative use of the ratio of FITC labelled platelets and erythrocytes to verify platelet counts proved to be sufficient if moderately low platelet concentrations ($20–100 \times 10^9$/l) were analysed. At platelet concentrations below 20×10^9/l, Passing-Bablok's linear regression analysis showed that there is a significant difference between the FITC bead technique and the erythrocyte/FITC platelet ratio method. A blood sample of a thrombocytopenic patient with 5×10^9/l platelets and 3×10^{12}/l erythrocytes will only generate approx. 165 FITC-positive platelet events if 50,000 particles (our instrument's limit) are counted. This means that not only the statistical error of the erythrocyte/FITC platelet ratio method is high at low platelet counts, but also that the identification of platelet-derived microparticles and platelet aggregates can be difficult at such low counts. In the future, unlimited files for collecting sufficient data to meet a predetermined accuracy may show that the erythrocyte/FITC platelet ratio method is sufficient even at platelet levels below 20×10^9/l.

A further alternative to using FITC beads to calculate the absolute platelet count is to use a multiparameter flow cytometer that can measure sample volume with sufficient accuracy. At the present time we know of only one multiparameter flow cytometer that measures sample volume and is being marketed (i.e. from Ortho Diagnostic Systems). A comparison between such a system and the FITC bead technique for platelet counting should be carried out. Finally, we feel that manufacturers of blood analysers should put more emphasis on constructing and marketing affordable machines that use more advanced platelet counting technologies and more efficient computational systems (i.e. analog-to-digital converters) to cope with everyday clinical needs.

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