Automated Optical Counting of Blood Platelets

By Geoffrey M. Brittin, Shirley A. Dew, and Elvi K. Fewell

We have evaluated the use of an optical particle counter to perform automated platelet counts on whole blood. The erythrocytes were lysed by dilution of whole blood with 2 M urea and the remaining platelets and leukocytes were enumerated by a darkfield microscope optical system that detects light diffracted by them. A suspension of fixed human platelets available commercially was highly satisfactory for standardization. The method gave accurate and reproducible platelet counts, comparable with those of electronic particle counting on venous blood and substantially more reliable platelet counts on thrombocytopenic and finger-puncture blood samples. We believe that errors resulting from the electronic method were caused by technical difficulties of sample handling and not to an intrinsic error in electronic counting. By using the automated optical method we found no significant difference between the platelet counts of capillary and venous blood, although capillary platelet counts had twice the variability of venous counts. The optical technique has important advantages over electronic platelet counting, and its superiority appears to be due to the ability to count platelets in diluted whole blood rather than in plasma. It should prove especially useful in performing the large numbers of platelet counts on thrombocytopenic and finger-puncture blood samples that are increasingly important for management of patients receiving chemotherapy.

Electronic counting of platelets, introduced as a practical clinical method by Bull et al. in 1965, has made it possible for hematology laboratories to perform the large number of platelet counts required by modern chemotherapy. This method involves electronic counting of platelets in platelet-rich plasma after it has been separated from red blood cells by sedimentation or centrifugation. Only the actual enumeration of platelets is automatic, and all other operations are manual. Special precautions are necessary to avoid interference from background and variable platelet carryover in counting thrombocytopenic blood samples. Use of the method to count platelets of capillary blood requires anticoagulation of the blood in glass capillary tubes and subsequent transfer to sedimentation tubes. In addition, the raw plasma platelet count must be corrected for coincidence error and the hematocrit. Since management of patients receiving chemotherapy re-
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quires frequent measurements of low platelet counts on capillary blood specimens, an automated method for counting platelets in whole blood instead of platelet-rich plasma would offer important advantages. We have evaluated the use of an automated optical counter for this purpose.

MATERIALS AND METHODS

Electronic counting of platelets was performed by the method of Bull, Schneiderman, and Brecher,1 which has been shown to give results identical to the phase contrast microscope method of Brecher and Cronkite.5 Duplicate electronic counts were performed on a single 1:3000 dilution of platelet-rich plasma in Isoton (Coulter Diagnostics, Miami Springs, Fla.) with a Coulter counter Model B used exclusively for platelet counting. The background was maintained at 30 counts/100 μl or less. Dilutions were made using a 3-μl Microcap (Drummond Scientific, Broomall, Pa.). For platelet counts below 25,000/cu mm, a dilution of 1:300 was made by adding 20 μl of platelet-rich plasma with a Microcap to 6.00 ml of diluent. This was done in order to minimize the effect of the background count and carryover.5

Automatic optical counting of platelets was performed with the AutoCounter (Technicon Corp., Tarrytown, N.Y.), which consists of a sampler, peristaltic-type pump, plastic tubing manifold, glass mixing coils, darkfield microscope optical system, and a recorder. Anticoagulated blood samples are placed in plastic cups and inserted in the turntable, where they are mixed twice by Teflon-coated mixing paddles before and during sampling. The sample size is approximately 0.6 ml. The instrument automatically dilutes the blood samples approximately 1:1500 with 2 M urea, which lyses the erythrocytes. The remaining leukocytes and platelets are counted by a darkfield microscope optical system, which detects the forward-scattered component of light diffracted by them. The optical counter is of the rate-meter type and operates with a 10-sec time constant. For a blood sample with a platelet count of 350,000/cu mm, approximately 10,000 light-scattering events are counted to determine the maximum concentration of particles flowing through the optical sensing volume of 44,000 cu μ. The small size of this sensing volume gives the instrument almost an ideal linear response. The detailed characteristics of the optical system and the advantages of optical over electronic particle counting and discussed by Mansberg.6 Because both platelets and leukocytes are detected, the white blood cell count in all of our studies has been performed independently with a Coulter counter Model S or F (Coulter Electronics, Hialeah, Fla.) and subtracted in order to obtain the corrected platelet count. The optical counter was operated at 40 samples/hr. In most of our studies a saline wash was included between samples to minimize the effects of specimen interaction. Both venous blood anticoagulated with K3-EDTA and a suspension of fixed human platelets obtained from the Technicon Corp. were used to standardize the optical counter. Platelet counts of both the blood and reference material were performed by electronic counting.1

Blood obtained by finger puncture was diluted immediately 1:200 with specially constructed Unopettes,6 having a 20-μl capillary and containing 3.98 ml of diluent (saline 0.85%, containing K3-EDTA 0.1 g/100 ml). To accommodate such specimens the manifold of the optical counter was modified to give an automatic dilution with 2 M urea of 1:7.5, so the final dilution of the blood was approximately 1:1500. The instrument was calibrated with platelet reference material also diluted 1:200. Because the precision of counting deteriorates as the platelet count decreases, samples of venous and finger-puncture blood with platelet counts less than 50,000 cu mm were diluted 1:40 instead of 1:200 with Unopettes having a 40-μl capillary and containing 1.56 ml of saline-EDTA diluent. Platelet counts of these samples were divided by 5 and then corrected for the leukocyte count. At our institution, most patients with thrombocytopenia have platelet counts every few days on finger-puncture specimens, and the 1:40 dilution is obtained routinely. For patients whose platelet count has not been determined previously,

*Courtesy of Horace W. Gerarde, M.D., Ph.D., Becton-Dickinson Co., Rutherford, N.J.
both the 1:200 and 1:40 dilutions are obtained from a capillary blood sample and the 1:40 dilution is counted if the platelet count is low.

Results

Effect of 2 M Urea: The manifold of the optical counter exposes blood to 2 M urea for approximately 3 min before platelets and leukocytes are enumerated by the optical system. Study of the effluent from the optical system with the phase contrast microscope showed only platelets and leukocytes and no erythrocytes. Increasing the time of exposure of blood to 2 M urea an additional 8 min by introduction of a time-delay coil produced a small but insignificant increase in the platelet count. Serial observations with the phase contrast microscope showed that exposure of blood to 2 M urea for longer than 20 min led to swelling and fragmentation of platelets. It is therefore possible that the slightly higher platelet count observed with introduction of the time-delay coil represented early fragmentation of platelets not detected by visual examination. These studies indicate that exposure of blood to 2 M urea for 3 min lysed all the erythrocytes and that small variations in the time of exposure are not critical.

Specimen Interaction: The interaction of specimens was measured from platelet counts of the instrument, as described elsewhere.7 Carryover by the mixing paddles from one sample cup to the next was 1.1% ± 0.1% (SD), and carryover for the entire system was 3.4% ± 0.8% (SD), expressed as the percentage of the volume of one sample carried over into the next.

Linearity: The linearity of the optical platelet counts was determined by counting saline dilutions of whole blood. The magnitude of each dilution was determined from its red blood cell count, performed with the Model S Coulter counter. Platelet counts were linear between 30,000 and 535,000/ cu mm, which was the highest concentration tested.

Reproducibility: Precision of counting was determined from replicate platelet counts of venous blood. The coefficient of variation (C.V.) of platelet counts on undiluted blood varied from 2.0 to 2.5% and on blood diluted 1:200 it was 2.3%. This precision compared favorably with that of electronic counting of platelets of normal venous blood in our laboratory (C.V. 2.2%).

Calibration: The automated optical counter was calibrated with a suspension of fixed human platelets supplied by the Technicon Corp. This material is proprietary, but is believed to resemble the suspension of fixed human platelets described by Nakatsui et al.8 It contained approximately 300,000 platelets/ cu mm with less than 2000 leukocytes and erythrocytes/cu mm. Examination with the phase-contrast microscope showed that it contained morphologically normal platelets, with only occasional small platelet clumps. The reference material was packaged in glass vials containing 10 ml to be mixed on a Vortex mixer for 1 min. We found that mixing for 0.5 min gave values a few thousand per cubic millimeter less than did mixing for 1.0 min. Mixing for longer times up to 7.0 min produced only small random changes in the platelet count. Variation of platelet counts within a vial and between different vials was studied by both automated and optical electronic platelet counting. Four determinations were performed on each vial by both methods. For this study
The optical counter was calibrated with normal whole blood. The results are shown in Table 1. Automated optical counts of the reference material were more reproducible than electronic counts. The mean platelet counts of different vials varied by as much as 4%, and calibration of the optical method with this material may be expected to vary accordingly. We have found a small advantage of using reference material pooled from several vials, as would be expected from the data of Table 1.

Accuracy: The accuracy was determined by comparison with electronic platelet counts and with phase contrast platelet counts when necessary. In a representative study of 47 venous blood samples the values obtained by the optical method were corrected for the leukocyte counts and for a 1.6% discrepancy between the nominal value and the mean value of the platelet reference material obtained during the study. The mean optical platelet count (230,000/cu mm) was almost identical with the mean electronic count (230,400/cu mm), and the linear regression and correlation \( r = 0.98 \) between the two methods were excellent. In this study the initial electronic platelet counts were more than 50,000/cu mm lower than the optical counts on six specimens. Repeating the determinations on these specimens by both methods showed that the initial electronic platelet counts were erroneous in all six cases. Examination of discrepancies between optical and electronic platelet counts in many other studies also indicated that it was usually the electronic counts that were erroneous. For example, in a study of 72 thrombocytopenic venous blood specimens (range 4200–66,000/cu mm) optical and electronic platelet counts differed by more than 6000/cu mm in 27 specimens. Optical counts were higher than electronic counts in 14 cases and lower in 13. Repeating the determinations by both methods and/or by the phase-contrast method resolved 21 of the discrepancies in favor of the optical method and one in favor of electronic counting. In the remaining five specimens it was not possible to obtain agreement between the three methods.

The data of Table 2 shows that the accuracy of platelet counts of the automated optical method adapted for diluted blood samples also agreed closely with electronic counts. The comparative study on thrombocytopenic samples involved 20 samples. In six of them electronic counting gave values more than
Table 2.—Accuracy of Automated Optical Platelet Counts on Diluted Venous Blood Samples

<table>
<thead>
<tr>
<th></th>
<th>Venous Blood Samples Diluted 1:200</th>
<th>Thrombocytopenic Venous Blood Samples Diluted 1:40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Range of values × 10^9/cu mm</td>
<td>54.0–450.0</td>
<td>3.9–33.4</td>
</tr>
<tr>
<td>Mean values × 10^9/cu mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electronic counting</td>
<td>255.7</td>
<td>13.9</td>
</tr>
<tr>
<td>Optical counting</td>
<td>256.7</td>
<td>14.9</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.99</td>
<td>0.98</td>
</tr>
<tr>
<td>Linear regression</td>
<td>Y = 0.96X + 10.7</td>
<td>Y = 1.03X + 0.6</td>
</tr>
<tr>
<td></td>
<td>S_Y,X = 13.0</td>
<td>S_Y,X = 1.6</td>
</tr>
</tbody>
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6000/cu mm lower than those obtained by the optical method. Three of these samples were eliminated from the study because phase-contrast microscopy showed clumping of platelets. In the other three, phase-contrast platelet counts confirmed the results of the automated optical method. The data of Table 2 therefore include only the 14 values for which the electronic counts can be assumed to be correct.

Use of Automated Optical Method To Count Platelets of Capillary Blood Samples: The automated optical method was used to obtain 52 comparisons of capillary and venous platelet counts of normal laboratory personnel. The finger was cleansed with alcohol, allowed to dry, and lanced with a Monolet (Sherwood Medical Industries, Deland, Fla.). The first drop of blood was wiped away, and 20 μl of each of the next two drops was diluted 1:200 into Unopettes. Duplicate 1:200 dilutions of venous blood were also prepared in Unopettes. The automated optical method was calibrated with the Technicon platelet reference material diluted similarly. The diluted samples were separated by a saline wash to eliminate any effects of carryover, and the turntable was loaded so that all the diluted samples of each person were grouped together to obtain the best internal comparison of capillary and venous platelet counts. The mean platelet count of the first drop of capillary blood was 298,800/cu mm and that of the second drop was 299,800/cu mm. The small difference in the platelet counts of the two drops was not significant (p > 0.6). The mean platelet count of capillary blood (299,200/cu mm) was slightly higher than that of venous blood (297,600/cu mm) and the small difference was not significant (p > 0.4). Reproducibility of the two platelet counts performed on finger-puncture blood of each person was 3.4% (C.V.), compared with 1.8% (C.V.) for the duplicate platelet counts on venous blood.

Comparison of Automated Optical and Electronic Methods for Counting Platelets of Capillary Blood Specimens: The study was performed on 19 normal laboratory personnel. Blood from the finger was diluted 1:200 into two Unopettes for automated optical counting. Blood from each of the next two drops was sampled into capillary tubes containing both EDTA and heparin (Drummond Scientific), transferred into a sedimentation tube, and then processed for electronic counting exactly as described by Hart et al. Reproducibility of platelet counts on capillary blood was calculated from counts on the
duplicate Unopettes and the duplicate sedimentation tubes. For each person the platelet count of venous blood was determined by optical counting of a 1:200 dilution and by electronic counting by the method of Bull et al. There was close agreement of the mean platelet counts of capillary (284,400/cu mm) and venous (281,100/cu mm) blood determined by the automated optical method, and reproducibility of the counts on capillary blood was 4.0% (C.V.). By contrast, electronic platelet counts on the capillary blood samples were irreproducible (19.8% C.V.) and much lower than those of the venous samples (205,100/cu mm for capillary blood vs. 280,900/cu mm for venous blood). Two additional studies in which the first drop of blood from the finger was sampled for electronic counting and the latter drops for automated optical counting confirmed these findings.

**DISCUSSION**

An automated optical method was used to count platelets in whole blood with promising results by Upton, Spaet, and LaMantia in 1967. The present method differs from theirs by using 2 M rather than 2.5 M urea and an optical system with a much smaller sensing volume. In addition, a suspension of fixed human platelets is used for calibration instead of latex particles. The principle of the method involves selective lysis of erythrocytes by dilution of whole blood with 2 M urea, detection of the forward component of light diffracted by the remaining platelets and leukocytes, and enumeration of the light scattering events with a counter of the rate-meter type. Because both platelets and leukocytes are enumerated, the white blood cell count must be performed independently and subtracted to obtain the true platelet count. The principle of this method is quite different from that of electronic platelet counting, which involves separation of platelet-rich plasma from red blood cells by sedimentation or centrifugation and selective enumeration of the voltage pulses produced by platelets as they pass through the aperture and change the electrical resistance between two electrodes of the Model B Coulter counter.

In our studies, automated optical platelet counts of venous blood were comparable with those of electronic counting. Discrepancies between the two methods were usually caused by errors of the electronic counts, especially with thrombocytopenic samples, or were found to be caused by platelet clumping, which did not permit consistent counts by any method. We believe that errors in the electronic platelet counts were caused by technical difficulties in sample handling and not to an intrinsic error in electronic particle counting. Automated optical platelet counts of capillary blood by the method of Hart et al. were irreproducible and much lower than those of venous blood. The reasons for this consistent loss of platelets in our hands is unexplained, since the method appears to have worked for others. We have not investigated the problem further, since reliable capillary platelet counts may be conveniently obtained on large numbers of samples with the automated optical method or for individual samples by the phase-contrast microscope method.

The output of the optical counter may be changed by moving a poten-
tiometer and is not an absolute value. A suitable reference material (standard) is therefore required for calibration, which must be performed for each batch of determinations. Fresh whole blood on which platelet and leukocyte counts are determined in the user’s laboratory may be used, but difficulties of availability and stability make this approach unattractive. Convenient calibration therefore requires a reliable platelet reference material. Nakatsui et al. have recently described a suspension of human platelets fixed in glutaraldehyde and stable for at least 8 mo. We have obtained excellent results with the platelet reference material supplied by Technicon, which also contains fixed human platelets minimally contaminated with erythrocytes and leukocytes. Our experience suggests that this material will be satisfactory for calibration and quality control of platelet counts by the automated optical and possibly other methods and that it should be evaluated for this purpose by independent laboratories.

The automated optical method has important advantages over electronic platelet counting. The ability to use whole blood instead of platelet-rich plasma allows extensive automation of the procedure, including automatic mixing, dilution, and counting. The ability to use diluted whole blood permits immediate dilution of finger-puncture blood specimens with Unopettes, thereby obviating the necessity to anticoagulate and sediment micro blood samples. Thrombocytopenic blood samples may be conveniently diluted fivefold less than those with higher counts, so the signal-to-noise ratio remains favorable. This capability also minimizes the error caused by carryover in the flow system, which is a linear function of the ratio of any two successive counts. However, since platelet counts encountered in disease may differ by as much as 3 logs, it is necessary to re-assay thrombocytopenic samples that are immediately preceded by samples with elevated platelet counts. There are several problems with the automated optical method that detract only slightly from its advantages: (1) The requirement for standardization of the method for each batch of samples is more cumbersome than calibration of the electronic particle counter, which need be performed only infrequently. The availability of a suitable platelet reference material minimizes this difficulty. (2) The leukocyte count must be performed independently and subtracted from the total of leukocytes and platelets enumerated. This correction is insignificant when platelet and leukocyte counts are normal, but becomes important in dealing with thrombocytopenic samples, especially those with elevated leukocyte counts. The correction for the leukocyte count is no more difficult to perform and is usually less critical than the correction for hematocrit in electronic platelet counting. (3) Although it is necessary only to read peak heights on a strip chart to obtain the total platelet and leukocyte count, technologists occasionally read the peak heights erroneously, associate them with inappropriate samples, or forget to apply corrections for dilutions. In addition, more time is required to determine the peak height than to read the output of a Coulter counter. However, the output of the optical counter is probably preferable to that of the Coulter counter, which gives no permanent record of the raw data and requires corrections for coincidence error and hematocrit. (4) Since the error caused by carryover is a linear function of the ratio of any two successive cell counts, the measured
carryover of 3% is sufficient to cause serious overestimation of platelet counts of thrombocytopenic samples if they are immediately preceded by samples with elevated platelet counts or greatly elevated leukocyte counts. This problem is minimized by the ability to dilute thrombocytopenic samples less than others, but it is still advisable to re assay thrombocytopenic samples that are immediately preceded by samples with high counts. (5) Platelet cold agglutinins that cause spuriously low platelet counts by electronic counting have recently been described by Watkins and Shulman. Such agglutination could not be reversed by warming the agglutinates to 37°C. We have encountered several patients with similar platelet clumping and observed that the automated optical method gave spuriously low platelet counts just as did electronic counting. Apparently exposure of blood to 2 M urea in the optical method does not reverse agglutination of platelets.

The automated optical method should permit an exact comparison of the platelet counts of venous and capillary blood. The precision of the method is much greater than that of the phase-contrast microscope method, and capillary and venous blood samples may be counted with the identical technique, which is not possible with the electronic counting method. Our findings indicate that the variability of capillary platelet counts is about twice that of venous counts but that there is no significant difference between capillary and venous platelet counts. These studies confirm the earlier finding of Brecher et al. with the phase-contrast microscope method11 that capillary platelet counts are more variable than venous counts, but refute their conclusion that capillary platelet counts are on the average 28% lower than venous counts. Because of the relatively low precision of the phase contrast method, the study of Brecher et al. required platelet counts on eight successive drops of finger-puncture blood. It is likely that there was a loss of platelets from the latter drops caused by adhesion of platelets in the wound. Such loss is the basis of the in vivo platelet function test devised by Borchgrevink, and the automated optical method applied to finger-puncture blood samples may be expected to increase the reliability of this test.

We believe the advantages over electronic platelet counting and the availability of a satisfactory platelet reference material makes the automated optical technique the present method of choice for counting of blood platelets. It should prove especially useful in performing the large numbers of platelet counts on thrombocytopenic and finger-puncture blood samples that are increasingly important for management of patients receiving chemotherapy.

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REFERENCES

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GEOFFREY M. BRITTIN, SHIRLEY A. DEW and ELVI K. FEWELL