Morphology and Enumeration of Human Blood Platelets

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MORPHOLOGY AND NUMBER of formed blood elements are not the final criteria, but are useful guides in the evaluation of normal and disturbed function of the hemopoietic system. However, in the case of the blood platelet the correlation between morphology and function remains insecure in spite of the extensive studies of Fonio (1). Furthermore the difficulty of counting platelets, together with the wide range of reported normal values (2), have led some observers to doubt that generally acceptable standards can be set for normal platelet levels (3).

The purpose of the paper is to show that the value of morphologic studies is limited by the prompt reaction of platelets to numerous factors in the environment, but that enumeration of platelets can, nevertheless, be accomplished with the same accuracy as that of white or red blood cell counts when a suitable diluent is used.

MATERIALS AND METHODS

Platelet Morphology. Blood was collected from a cubital vein into a chemically clean, dry syringe, care being taken to avoid admixture of air bubbles. The blood was then rapidly added to test tubes containing the following anticoagulant solutions: 14 per cent magnesium sulfate, 3.2 per cent sodium citrate, 1.6 per cent potassium oxalate, 1 to 4 per cent ammonium oxalate, 0.8 per cent potassium oxalate with 1.2 per cent ammonium oxalate, 1 to 2 per cent sodium oxalate, 0.85 per cent sodium chloride containing enough heparin to provide 100 gamma of heparin for each cubic centimeter of blood added. Dilutions of 10:1, 1:1, and 1:10 and 1:100 of blood to anticoagulant solution were made. From the 1:1 dilutions platelet-rich plasma was removed after one-half-hour period of sedimentation or after slow centrifugation, with the exception of the ammonium oxalate solutions which were hemolytic. The anti-
coagulants used did not affect the pH of the blood significantly in 1:1 dilutions. As measured in the test tube by the glass electrode the pH varied from pH 7.05 to 7.64, and was usually between 7.30 and 7.60.

A small drop of blood or plasma was transferred to a slide and allowed to spread between slide and coverslip. The coverslip was rimmed with vaseline and the preparation studied with a 97× dark contrast phase oil immersion objective. The 1:100 dilutions of blood to anticoagulant were also examined in a counting chamber with a 43× dark phase contrast objective. The slides were examined from 30 minutes to 24 hours after their preparation. Siliconed (GE dri film no. 9986) (4) slides and coverslips were used in some experiments, but unless otherwise stated our results refer to preparations with wettable glass surfaces, and to examination within 30 to 60 minutes after preparation of the slides from freshly drawn blood, or from plasma separated within one-half hour of blood collection.

**Platelet Counting.** Venous blood was collected in a 10-ml. siliconed syringe, through a 20-gauge sharp needle. Only blood collected following a clean venepuncture was used. If bubbling occurred the blood was discarded and new needles and syringes used. One ml. of blood was drawn into the syringe and the needle removed. A few drops of blood were placed on a siliconed watch glass to facilitate filling of the siliconed red blood cell hemocytometer pipettes. Alternatively a 19- or 20-gauge needle was inserted into the cubital vein, 1 to 2 cc. of blood was allowed to flow directly into a siliconed test tube, 10 x 75 mm., and the pipettes were filled from the test tube. The blood was drawn to the 1.0 mark and then diluted with one per cent ammonium oxalate. All of the platelets in 5 blocks of small square (as for RBC) are counted after 15 minutes of settling, using a phase microscope, dark contrast 43× objective, long working distant phase condenser, and a counting chamber with an optically flat bottom. Both sides of the chamber are counted, and the total number counted is multiplied by 2500 to give the number of platelets per cu. mm.

An ordinary coverslip must be used in place of the thick standard hemocytometer coverslip. Figure 1a shows the appearance of platelets in the counting

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3 The ammonium oxalate solution is preferably stored in the refrigerator between 10° and 5°C. to prevent growth of molds and bacteria, and filtered before use.

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Fig.1a: COUNTING CHAMBER filled with blood diluted 1:100 with 1 per cent ammonium oxalate. 43× dark contrast phase objective, final magnification 350x. b-k: Coverslip preparation from blood diluted 1:1 with anticoagulant solutions. 97× dark contrast phase objective, final magnification 1300X. Photographs taken within 30 to 60 minutes of blood collection, except d, h, i, k. All from plasma except b. b: Ammonium oxalate 1 per cent. c, e: Potassium oxalate 1.6 per cent. d: Potassium oxalate 1.6 per cent, 24 hours. f: Potassium oxalate 1.6 per cent with 0.2 per cent formalin. g: Magnesium sulfate 14 per cent. h: Sodium citrate 3.2 per cent, 24 hours. i: Magnesium sulfate 14 per cent, 3 hours. j: Sodium chloride 0.85 per cent with 0.1 mg. heparin per cc. blood. k: Same as j, 24 hours. l: Native blood, 2 hours, 1300X. m: Blood (20 parts) with 14 per cent magnesium sulfate (1 part), 2 hours, 1300X.
chamber. Pseudopodia or ‘tails’ are present in most of the platelets but are obscured in the photograph by halation.

Platelet counts were also performed on oxalated venous blood (Wintrobe’s oxalate: 6 mg. ammonium oxalate, 4 mg. potassium oxalate for 5 cc. of blood).

RESULTS

Platelet Morphology. Using a 1:1 dilution of blood and various anticoagulant solutions, most of which were isotonic with regard to the red cells, the platelet morphology varied markedly with the anticoagulant used.

With all of the ammonium or potassium oxalate solutions the body of the platelet appeared dark, with indistinct granules. Processes formed rapidly and were thick as compared with those seen in magnesium sulfate or sodium citrate, and generally fewer in number. Many platelets had a single, long process (fig. 1b and c). These processes were not present when the preparation was made rapidly and examined immediately. They developed in the test tube and in the coverslip preparation within 30 to 60 minutes, and little further change was noticeable during the following 24 hours (fig. 1d). A varying number of platelets became adherent to the glass slide or coverslip. This occurred more frequently with potassium oxalate than with ammonium oxalate mixtures (fig. 1e). Adhesion of platelets was frequently seen in the one per cent sodium oxalate solution, but less commonly in 2 per cent sodium oxalate. Addition of 0.2 per cent formalin to the oxalate solutions inhibited the formation of processes and largely prevented adhesion of platelets to the glass (fig. 1f).

With sodium citrate or magnesium sulfate (fig. 1g) the body of the platelet had a highly refractile outline and individual granules were not seen. Processes were at first absent, later delicate and short, especially with sodium citrate. Adhesion to the glass surface occurred with varying frequency. Adherent platelets had a highly refractile central body and a round or polygonal outline, occasionally with short, thorn-like processes (fig. 1g). Little change was noted after 24 hours with sodium citrate (fig. 1h). With magnesium sulfate many platelets changed into a ring form with marginal granules after a few hours (fig. 1i).

With sodium chloride containing heparin platelets commonly adhered to the glass, usually in clumps (fig. 1j). Granules were prominent. Processes did not form. After several hours platelets changed into ring forms (fig. 1k). If blood was diluted 1:100 with sodium chloride, a refractile center and processes of the swollen platelet body were usually present.

With the exception of sodium chloride, the 1:10 and 1:100 dilutions showed essentially the same appearance of platelets described for the 1:1 dilutions. When the 1:100 dilutions were examined in the counting chamber with a 43x objective, platelets generally appeared as highly refractile, purplish round
bodies. The thick processes produced in potassium and ammonium oxalate solutions could usually be seen with careful focusing.

When only one part of the anticoagulant solution was added to 10 parts of blood, platelets generally adhered to each other and to the glass slide very rapidly, usually without formation of pseudopodia. However, this did not hold true for the 14 per cent magnesium sulfate solution. There were generally as many adhesive forms when one part anticoagulant was mixed with one part of blood as when 10 parts of blood were added. Ring forms developed rarely in the 10:1 dilution, but after a few hours were seen in great numbers in the 1:1 mixture.

The appearance of platelets was identical in whole blood and in plasma removed after undisturbed sedimentation of platelets.

The morphology of platelets occasionally differed in the same preparation from field to field. It is generally possible to judge the thickness of the preparation in a given field from the appearance of the red cells which tend to hemolyze in thin portions of the slide, even though the diluting fluid is isotonic (5). In such thin areas the platelets were usually flattened out and adherent to the glass, often in marked contrast to the prevalence of freely floating forms in thick areas of the preparation.

Silicone coating of slide and coverslip affected platelet morphology to some extent by minimizing adhesions to the slide or coverslip. However, the silicone coating did not reduce adhesion of platelets to the siliconed surfaces when saline solution with heparin was used as the diluent.

The development of fibrin needles in the coverslip preparation was no indication of whether or not gross clotting would occur in the blood or plasma kept in the test tube. With one part of 14 per cent magnesium sulfate to 20 parts of blood extensive fibrin formation was often seen under the coverslip within 2 hours (fig. 1m) even though the blood in the test tube remained fluid for 24 to 48 hours or longer and fresh preparation made at that time from the test tube showed no fibrin when first examined. On the other hand untreated blood, which clotted readily in the test tube, usually showed only minimal or no visible fibrin formation in coverslip preparation (fig. 1l). When blood was collected without an anticoagulant and the plasma separated in lucite tubes at 5°C., fibrin needles formed readily in coverslip preparation at room temperature. Silicone coating of the slides and coverslips did not prevent fibrin formation. This is in agreement with the findings of Fonio (1) and Mann (6).

Human Platelet Counts. One per cent ammonium oxalate was used in preference to other diluents because the red cells are hemolyzed thus making the platelets more readily seen and identified. In addition platelets are purplish, highly refractile and distinctly different in appearance from bacteria, yeasts, molds, and dust, thus largely removing the element of personal judgment in enumerating platelets.
With the technique described platelet counts were made on 50 normal young men during June and July, 1949. The range of these 50 counts was 87,000 to 498,000/cu. mm. with a mean of 257,000 cu. mm. The distribution of these counts was skewed to the right. The statistical estimate of the 95 per cent limits of variation of such single counts, using a logarithmic transformation, was 140,000 to 440,000 platelets per cu. mm.

Oxalated blood, siliconed technique, from 49 of the 50 individuals, with counts performed within one hour after withdrawal of blood yielded a range of 111,000 to 510,000/cu. mm., with a mean of 232,000/cu. mm. Blood from the same venepuncture was used for both techniques in 49 of the 50 individuals. The difference in the means obtained with the two procedures was statistically significant for the paired observations.

The range of single platelet counts in a group of individuals includes errors of measurement as well as variations between persons. In order to determine the error of the method itself, platelet counts were performed with the technic described on 10 additional healthy males, 20 to 41 years old, on 5 consecutive days, during July, 1950. The daily procedure was as follows:

One of 10 siliconed pipettes was used, in random order, to dilute each sample. Four counting chambers were filled from each of the pipettes and 5 blocks of 16 small squares counted in each half of the chamber. This design permitted the estimation, by analysis of variance, of the chamber and field errors of the method as well as the variability of the platelet count in each individual from day to day. The results are summarized in table 1. The estimate of the field error in the daily count was 4.5 per cent, of the chamber error 2.1 per cent, and of the day-to-day variation for the individual was 3.2 per cent. (All errors here expressed in per cent are coefficients of variation.) The variations from individual to individual were markedly in excess of these errors, indicating that each individual has his own normal platelet level which is being kept within narrow limits from day to day.

Statistical analysis of the data suggested that the daily variation of 3.2 per cent within individuals was probably due to pipette errors. To test this hypothesis the pipette error was determined in 3 separate experiments. In each case dilutions were made in 8 different pipettes from a single sample. Four chambers were filled from each pipette. Through analysis of variance, the field, chamber, and pipette errors were estimated. The field and chamber errors were of the same order as in the preceding experiment, and the pipette errors were 3.2, 2.0, and 4.4 per cent in the 3 experiments. This is in close agreement with the 3.2 per cent value for the day-to-day variation in an individual.

Our chamber error using thin coverslips was of the same order as that found by Berkson with thick coverslips. The use of the thin coverslip was

\[ t = 6.20; P < 0.001. \]
Therefore not considered a major source of error. However, two further experiments were done to explore this point. In one series, 50 duplicate red cell counts were done on 10 samples taken from one individual on the same day. Two chambers were used, and thick and thin coverslips alternated on each chamber.

Table 1. Platelet counts for 10 males for 5 consecutive days (July 1950) (10 squares/hemocytometer)

<table>
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<tr>
<th>INDIVIDUAL</th>
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<th>5-DAY AV.</th>
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<td>Av.</td>
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<td>123.50</td>
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1 First line: Range of counts of 4 chambers filled from one pipette. Second line: Arithmetic mean. The number of platelets per cu. mm. is obtained by multiplying the counts by a factor of 2500.

In a second experiment a similar number of WBC counts was done on a single blood sample and thin and thick coverslips alternated. Thin coverslips gave slightly higher errors than thick, but the difference was not statistically significant for the number of counts made.

To test the reproducibility of our results further, one cc. of blood was di-
luted with 100 cc. of one per cent ammonium oxalate and kept at 5° C. Multiple
counts of this dilution were made for 3 consecutive days. There was no signifi-
cant change in the platelet count during the 3-day period. Next, dilutions of
fresh venous blood were made in 4 non-siliconed pipettes and compared with
dilutions in 4 siliconed pipettes. The pipettes were rotated in Bryan-Garrey
pipette rotor. Pipettes were taken off over a period over 8 hours. There was
no loss of platelets over the 8-hour period, and no difference between siliconed
and non-siliconed pipettes was found.

As a further check on the reproducibility of the count, two series of 32
duplicate counts were performed by three technicians one of whom had no
previous experience with the method of counting platelets here presented.
There was no statistically significant difference between the counting errors of
the three technicians.

DISCUSSION

Platelet clumps and individual platelets have been visualized in the intact
circulation of mammals by Bizzozero (7) and Algire (8) who found that the
circulating platelet is round or lancet shaped and without processes. These
findings are supported by in vitro experiments (1, 9, 10). We have shown that
the variety of shapes that the platelet may assume in coverslip preparations
depends on the thickness of the preparation, the surface property of the glass,
the anticoagulant used and its concentration. Probably variation of other fac-
tors, such as pH, ionic strength and temperature, would also influence platelet
morphology. It appears not unlikely that at least part of these changes in
platelet morphology are brought about by membrane phenomena and ionic
exchanges that may be akin to the swelling, hemolysis, crenation, and sickling
of red cells. Possibly further studies along these lines may throw some light
on the properties of the platelet surface. The importance of interface phenom-
ena for platelet morphology has been stressed by Ferguson (11).

The addition of anticoagulants in the usual concentrations and amounts
changes platelet morphology so markedly that it becomes unlikely that morph-
ologic changes can be considered representative of platelet behavior during
the clotting process as suggested by Fonio (1).

Moreover, our own data show that fibrin formation in the coverslip
preparation does not necessarily indicate fibrin formation or clotting of the
same sample in the test tube. The great variety of known and unknown factors
which influence platelet behavior and morphology in vitro may necessitate the
return to intravascular study of platelet behavior initiated by Bizzozero (7)
and Eberth and Schimmelbusch (12).

For the enumeration of platelets in the counting chamber the changes of

5 A. S. Aloe Co., 1831 Olive St., St. Louis, Missouri.
platelet morphology are of minor importance. The sealed coverslip preparation examined at room temperature over several hours had shown no evidence of disintegration of platelets after addition of most anticoagulants beyond the morphologic changes described. The platelet body remained highly refractile on the addition of most anticoagulants in large amounts, when viewed with the 43x phase objective in the relatively thick layer of fluid in the counting chamber. This is in agreement with Wright's (13) findings that adhesiveness rather than fragility of platelets accounts for the loss of platelets from blood to which anticoagulants have been added, and that larger amounts of anticoagulants decrease adhesiveness. Simultaneous dilution of the blood appears to reduce platelet adhesiveness further. The ammonium oxalate solution was chosen for platelet counts because it hemolyzes red cells thus clearing the background and facilitating identification of platelets. At the same time adhesions to the glass are minimal in ammonium oxalate solution. The purplish sheen and the relatively thick processes formed by most platelets in ammonium oxalate are helpful in the identification of individual platelets. It should be re-emphasized that the visualization of the pseudopodia requires careful focusing but is not essential for the identification of a platelet. Occasionally calcium oxalate crystals are seen but are easily identified by proper focusing. Feissly (14) has recently published a similar method for platelet counts, using hypotonic saline containing cocaine as diluting fluid. The use of a narcotic in a simple laboratory procedure appears inadvisable and offers no particular advantage for the visualization of the platelets. However, we are not here concerned with the value of minor changes in techniques, but rather with the fundamental problem of the reproducibility of platelet counts.

The mean platelet values obtained in normal males agree closely with those found in venous blood by Tocantins (15) and Kristensen (16). However, the final evaluation of such counts must be based on the reproducibility and the error of the method.

An estimate of the expected total error of a hemocytometer count can be derived from Berkson's equation: (17)

\[ V_t = \sqrt{\frac{0.02^2 \cdot 100^2}{n} + \frac{4.6^2}{n_c} + \frac{4.7^2}{n_p}} \]

where \( V_t \) = standard deviation of the count (in percentage of the mean-coefficient of variation); \( n \) = number of cells counted; \( n_c \) = number of chambers used; \( n_p \) = number of pipettes used; and 0.02 \( \times \) 100, 4.6, and 4.7 represent the field, chamber and pipette errors (in percentage of the mean) determined experimentally by Berkson on a large number of red cell counts.

The chamber and pipette errors measured by Berkson and ourselves include not only physical errors in the chamber and pipette, but also technicians' errors in pipetting and variations in the firmness of attachment of the
coverslip to the chamber. These overall pipette and chamber errors are therefore greater than would be expected from the physical tolerances allowed by the NBS for pipettes and chambers.

The first term under the square root represents the field error due to chance distribution of cells in the chamber. This distribution is of the Poisson type and the resulting error should therefore be equal to $\sqrt{m}$ where $m$ is the number of cells per field. Berkson found the expected field errors (1.03 $\sqrt{m}$ and 1.00 $\sqrt{m}$) for leukocyte counts, but reported a smaller field error of 0.92 $\sqrt{m}$ for his own data on red cells, and 0.93 $\sqrt{m}$ for Buerker’s data which he analyzed in detail. Our field error for platelet counts was 0.93 $\sqrt{m}$. Magnuson, Eagle, and Fleischman (18) found a similar deviation from the expected Poisson distribution (0.89 $\sqrt{m}$) when counting spirochetes. These deviations from the Poisson distribution, though small, were statistically significant in each case. It is necessary to consider these deviations in detail because field errors much smaller than Poisson make any procedure suspect. Berkson, following Student (19), suggests crowding as one of the possible sources of field errors smaller than the Poisson error. Crowding of platelets themselves was unlikely because of their low density. The possibility was considered that the red cells, which are still present though hemolyzed, crowd the platelets. This thesis had to be discarded when 40 counts on plasma with 250,000 platelets per cu. mm., and 40 counts on plasma with 125,000 platelets per cu. mm. gave similarly low field errors of 0.90 $\sqrt{m}$ and 0.94 $\sqrt{m}$. Berkson excluded visual errors in his own data by photographing the entire field. This we found impracticable because the small size of the platelet would have required separate photographs for each block of small squares. It may be noted, however, that the visual errors that could reduce the field error are due either to selection of fields or due to unconscious equalization of counts. Selection of fields cannot be incriminated since identical blocks of squares were counted in all chambers throughout this study. Unconscious equalization of counts is unlikely with low cell densities as Berkson’s data on WBC counts show. The question whether factors other than crowding or visual errors may reduce the Poisson error slightly must remain open. Fortunately the deviation from Poisson here reported is small enough not to alter the total error seriously.

Our chamber and pipette errors, though slightly lower than Berkson’s, did not differ significantly from his values of 4.7 and 4.6 per cent respectively. Berkson’s figures are based on a larger series, and are therefore more firmly established. They are used in the subsequent computations.

The total error of a single count using one pipette and one chamber, $V_t$, is 11.4 per cent, if the count is 250,000 and 50 cells are counted in each half of the chamber. For an estimate of the error of the mean individual counts presented in table 1, the 5 separate days may be pooled since we have shown
that the observed day-to-day variation in an individual is probably due to pipette errors. The average values of cells counted was 1954, the number of chambers 4, the average number of different pipettes per individual 4.1\(^e\), and \(V_t = 3.9\) per cent. The observed value of 2.9 per cent is in excellent agreement with the expectation.

The data of table 1 thus show that the total error of measurement of the platelet level can be accounted for by the field, chamber and pipette errors which are inherent in any method using hemocytometers. This makes it doubtful that fragility or adhesiveness of platelets are a major source of error. This conclusion is substantiated by the result of our experiments in which a 1:100 dilution of blood with one per cent ammonium oxalate gave identical platelet counts for three consecutive days. In that case, the diluted blood was kept in the cold room. However, in the experiments in which siliconed and non-siliconed pipettes were compared, no loss occurred for at least 8 hours at room temperature, once the blood had been suitably diluted. The time during which any platelets might be lost would thus be during venepuncture and dilution. If such a loss of platelets occurs it could hardly be expected to be of the same order at each sampling, and should manifest itself in an otherwise unexplained error of measurement. Since this was not the case, the possibility of a significant loss of platelets during venepuncture is remote. There can be little doubt, however, that platelet clumping will occur with incipient coagulation and the dilution of the sample should therefore be accomplished with speed. Our data indicate that the siliconing of pipettes might be dispensed with. Siliconing of the syringe appears a worth-while precaution, since retardation of clotting until the dilution is made is desirable. However, chilling of the syringe and rapid work may be expected to achieve the same results. The use of oxalated blood is not advisable, since the customarily used amounts of oxalate are insufficient to abolish platelet adhesiveness (13). This factor is presumably responsible for the lower platelet counts from oxalated blood reported above.

Comparison of the foregoing figures with errors of measurements of RBC and WBC counts show that the average single platelet count \((V_t = 11.4\%)\) is measured with the same accuracy as the single leukocyte count \((V_t = 11.6\%)\). If 2 chambers, each filled from a different pipette, are counted \(V_t\) becomes 8.1 per cent which is comparable to the error of a single erythrocyte count \((V_t = 7.8\%)\). The increase in the number of pipettes and chambers used will increase the accuracy further, as can be seen from the \(V_t\) of about 3 per cent for 4 chambers filled from each of 5 pipettes. Increasing the number of cells counted in a single chamber is less efficient in increasing accuracy than the use of multiple pipettes and chambers (17).

\(^e\) When 10 pipettes are used randomly on 5 different days, the most probable number of different pipettes assigned to an individual is 4, and the average number 4.1.
The counts presented in table 1 indicate, as already noted, that there are marked differences in platelet levels from person to person, and that individual levels stay within narrow limits. The period of observation was too short to determine whether seasonal variations of any magnitude exist (20).

**SUMMARY AND CONCLUSIONS**

Platelet morphology is a function of contacting surface, the properties of the anticoagulant, its concentration, thickness of the preparation, and probably of other factors. The customary anticoagulants change platelet morphology rapidly and markedly. Morphologic changes reported in the past as characteristic of platelet behavior during the clotting process must be interpreted with caution because the techniques of examination in themselves influence platelet morphology and behavior. The changes in platelet morphology observed after addition of anticoagulants do not necessarily interfere with the ready identification of platelets as separate blood elements. Data are presented which show that the direct platelet count performed on fresh venous blood with a suitable diluent, e.g. 1% ammonium oxalate, is subject only to those errors inherent in any hemocytometer count. Platelet counts performed on oxalated blood within an hour of bleeding are significantly lower than on fresh blood. The platelet count is thus reproducible with an accuracy comparable to that of the RBC or WBC count. Platelet counts vary substantially between individuals. Platelet counts of any given individual remained within narrow limits from day to day during the period of observation.

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