Recommended Methods for Radioisotope Platelet Survival Studies

By The Panel on Diagnostic Application of Radioisotopes in Hematology, International Committee for Standardization in Hematology

THE EXPERT PANEL on diagnostic applications for radioisotopes in hematology at its recent meetings in Ulm (1972), Leiden (1973), and Annapolis (1975) discussed recommendations for the standardization of the measurement of platelet survival. The following document was prepared at these meetings and provides an outline of the technical and analytical aspects of platelet survival determination.

1. GENERAL

The study of platelet survival has proved of value in the investigation of: (a) the mechanism of thrombocytopenia and the quantitative evaluation of the factors contributing to it; (b) the effects of various diseases, environmental factors, and therapies on platelet survival; (c) the effect of collection and storage on platelet survival; and (d) the effect of extracorpuscular factors on the survival of allogeneic platelets.

Autologous platelets should be used in (a) and (b) above except when the yield is inadequate. On rare occasions a normal allogeneic survival study may help identify an intrinsic platelet defect rather than an extrinsic immunologic or consumptive mechanism as a cause of thrombocytopenia. Allogeneic platelets should be derived from HBs antigen-negative and ABO- and Rh-compatible donors. If the recipient has had previous transfusions or pregnancy, the use of allogeneic platelets may compromise the interpretations (due to rapid immune destruction of the allogeneic platelets on the basis of prior sensitization). Allogeneic platelets may themselves induce sensitivity. When platelet survival is determined to evaluate the effect of collection and storage, autologous studies in volunteers are most applicable since the difficulties in interpreting allogeneic studies are eliminated.

1.1 Labeling. Two methods have been employed to label platelets for survival determinations. In one, a cohort of platelets is labeled in vivo; in the other, the label is attached randomly to circulating platelets in vitro or in vivo ("random" or "population" labeling). Cohort labeling yields survival data
more readily and accurately analyzed. However, no satisfactory cohort label for platelets has yet been found.

1.2 Cohort labeling. Attempts have been made to use $^{35}$S- or $^{75}$Se-methionine for this purpose but they have not met with success largely because the period during which these labeled compounds remain available for labeling megakaryocytes or platelets is long compared with the mean platelet survival.

1.3 Random labeling. $^{14}$C-serotonin and $^{32}$P-orthophosphate have been used as random labels but have proved unsatisfactory owing to their rapid exchange or elution. Diisopropyl phosphofluoridate (DFP) labeled with $^{32}$P, $^3$H or $^{14}$C has been widely used; it has provided valuable information about platelet survival and is a useful aid in clinical investigations. It has the advantage of being an in vivo label, which obviates any possible damage during the procedures associated with in vitro labeling. However, the DFP survival curve does not reach zero, but shows a plateau or even a secondary peak of radioactivity amounting to 10%-15% of the initial value and persisting for 2 or more weeks. It is not clear whether the secondary peak is due to labeling of megakaryocytes, to reutilization of the label, or to both. Furthermore, when DFP is injected, red and white cells take up the label to a greater extent than do platelets. Since $^{51}$Cr was first used for this purpose as an in vitro label, $^{51}$Cr-chromate has been widely used and a number of technical improvements relating to its use have been recently introduced. These reduce damage to the cells during labeling, increase the efficiency of labeling, and make it possible to apply the method to studying the fate of autologous platelets even when the platelet count is $50 \times 10^9$/liter or less. In view of the foregoing, these recommendations have been restricted to methods involving the labeling of platelets in vitro with $^{51}$Cr.

2. MATERIALS

All solutions must be sterile and pyrogen free.

2.1. The blood is drawn into a sterile plastic bag (with two satellite bags) (containing ACD* or CPD†) as used in blood transfusion practice.

2.2. A centrifuge large enough to allow bags containing 500 ml of blood to be centrifuged at 1500 g and with facilities for ensuring that the temperature does not rise above 25°C should be used. A smooth braking action is essential.

2.3. A “plasma extractor” (a device for squeezing a plastic bag) is required.

2.4. Sterile ACD solution or 0.15 M citric acid may be used for acidification of the platelet-rich plasma (PRP). It may be made up in batches of 10-20 ml volumes. The ACD may be put in the first satellite bag before sterilization.

2.5. Preservative-free $^{51}$Cr-sodium chromate solution, prepared in sterile saline (pH 6.5) and with a specific activity of more than 20 μCi/μg Cr is used for labeling.

*ACD, NIH solution A: 2.2 g trisodium citrate (dihydrate); 0.8 g citric acid (monohydrate); 2.5 g dextrose; water to 100 ml. Ratio of anticoagulant to blood, 15:100.

†CPD: 0.327 g citric acid (monohydrate); 2.63 g trisodium citrate (dihydrate); 0.222 g sodium dihydrogen phosphate (monohydrate); 2.56 g dextrose; water to 100 ml. Ratio of anticoagulant to blood, 14:100.
3. TECHNIQUES

3.1 Platelet isolation and labeling. All operations should be carried out with sterile techniques. All centrifugation should be carried out at 20°–25°C.

3.1.1. The patient is weighed, and the platelet count performed. After considering the patient’s age, weight, clinical condition, and platelet count an appropriate volume of blood is decided upon (200–500 ml). For every 100 volumes of blood to be taken, 15 volumes of ACD are measured into the sterile transfusion bag (with two dry satellite bags attached). The blood is then taken into the bag. Donor blood collected not more than 2 hr previously and maintained at a temperature between 20° and 25°C may be used.

3.1.2. The entire bag is then centrifuged at 300 g (as calculated at the bottom of the container) for 15 min, including acceleration but not deceleration.

3.1.3. The supernatant PRP is transferred with the help of the “plasma extractor” into the first satellite bag.

3.1.4. Approximately 5 ml of ACD or 1 ml of 0.15 M citric acid per 100 ml of PRP is added, bringing the pH of the PRP to 6.5 ± 0.2. As mentioned in section 2.4, the ACD may be put in the first satellite bag before sterilization.

3.1.5. The platelets are sedimented into a pellet by centrifugation at 1500 g for 15 min.

3.1.6. All but 5 ml of the supernatant platelet-poor plasma (PPP) is transferred into the second satellite bag without disturbing the pellet. The platelets in the pellet are resuspended by repeated gentle inversion of the bag.

3.1.7. One to two μCi of 51Cr/kg body weight as 51Cr-sodium chromate is added to the platelet suspension. The upper limit of activity should be used when autologous platelets are used in thrombocytopenic patients and the lower limit in studies on children.

3.1.8. The mixture is incubated without shaking for 30 min at a temperature between 20° and 25°C.

3.1.9. All but 40 ml of the PPP in the second satellite bag is returned to the incubated mixture. Two hundred ml of filtered air is introduced into the bag before centrifugation to produce a good air-plasma interface; this facilitates the complete removal of the supernatant.

3.1.10. The bag is centrifuged at 1500 g for 15 min to form a second platelet pellet. The radioactive PPP is then carefully removed, without disturbing the platelet pellet.

3.1.11. Approximately 20 ml of nonradioactive PPP is gently layered over the platelet pellet in such a way as not to disturb the pellet; the PPP is then carefully decanted and discarded. (The object of steps 3.1.10 and 3.1.11 is to remove as much unbound 51Cr from the bag as possible.)

3.1.12. The labeled platelets in the pellet are gently resuspended in 10–20 ml of nonradioactive PPP. It is desirable, especially if a whole-blood radioactivity measurement is to be used (3.3), to remove the contaminating 51Cr-labeled red cells before injecting the labeled platelet suspension. The red cells are sedimented by centrifuging the suspension in tubes at 200 g for 5 min. The labeled supernatant platelet suspension is collected by aspiration. While the
adequacy of red cell removal can frequently be judged visually, more objective assessment should be carried out by preparing standards in both saline and ammonium oxalate.

3.1.13. Standards are prepared in duplicate by adding 0.1 ml of the labeled platelet suspension to each of four counting tubes, two containing 2 ml of ammonium oxalate solution 10 g/liter and two containing saline. The four tubes are centrifuged at 200 g for 30 min. The supernatants are removed without disturbing the platelet pellet and discarded. The four tubes are then capped and saved for counting.

3.1.14. A known amount of labeled platelet suspension, usually 10–20 ml containing 5–15 μCi,* is injected intravenously and the time noted.

3.2 Sampling procedure and counting. The number and timing of the samples will be decided by the purposes of the study and the anticipated mean survival. Samples should be taken at 30-min and 2 hr after injection, and thereafter daily for 10 days, unless no significant counting rate above background remains in a sample measured before day 10. Where survival is expected to be short, additional samples should be taken on the first day. In all studies, daily samples should, whenever possible, be taken at the same time of day.

3.2.1. For each sampling 5–20 ml of blood are taken into a syringe containing 0.2 ml of 100 g/liter EDTA (K salt) per 10 ml of blood. The platelet count is determined in the aliquot of the sample. The sample is then diluted with an equal volume of saline and mixed.

3.2.2. The mixture is centrifuged at 300 g for 10 min and the platelet-rich supernatant (PRP) is transferred to a counting tube.

3.2.3. After the addition of another volume of saline to the packed cells the latter procedure is repeated and the two harvests of PRP are pooled, the platelet count is determined in an aliquot and the remaining volume is precisely measured.

3.2.4. The PRP is centrifuged at 200 g for 30 min and the supernatant is removed without disturbing the pellet and discarded.

3.2.5. The counting rates of the standards and samples are measured in a well-type scintillation counter, or other suitable gamma-ray measurement system, to a standard deviation of 2% or less.

3.3. Measurement of radioactivity in whole blood may be used as an alternative procedure if adequate platelet radioactivity is present in 5 ml of whole blood and if contaminating 51Cr-labeled red cells are removed by slow centrifugation before injecting the labeled platelet suspension (3.1.12). Labeled red cell contamination may be disregarded if the counting rates of the standards prepared with saline do not differ by more than 10% from those of the standards prepared with ammonium oxalate (3.1.13). In the whole blood procedure, 5-ml samples of EDTA-treated blood are placed in counting tubes and the blood cells are lysed by saponin or sodium dodecyl sulfate to ensure constant counting geometry. Standards are similarly prepared in 5 ml of water to which a lytic agent is added.

4. EXPRESSION OF RESULTS

4.1. The platelet-bound radioactivity, $N_i$ (per second per milliliter) of whole blood sampled at time $t_i$ is

$$N_i = \frac{C_i}{V_i}$$

where $V$ is the volume of the blood samples (ml) obtained and counted at time $t_i$ and $C$ is the counting rate of the total whole-blood sample in counts per second. If PRP is to be prepared and a platelet pellet from each whole blood sample is to be counted, extraction of platelets from the whole blood may be incomplete. In this case it may be desirable to measure the efficiency of collection of platelets ($E_i$):

$$E_i = \frac{\text{number of platelets per ml of PRP}_i \times \text{volume PRP}_i (\text{ml})}{\text{number of platelets per ml of whole blood collected at time } t_i \times \text{volume of that blood sample}}$$

Then $N_i$, corrected for any loss of labeled platelets during the preparation of the platelet pellet from the blood sample taken at $t_i$ is expressed as

$$N_i = \frac{C_i 1}{V_i E_i}$$

If it is demonstrated that $E_i$ remains constant throughout serial samplings, this constant value may be used in the above formula. If one wishes to express the results only relative to the initial sample and $E$ is constant, it may be omitted all together.

4.2. To express circulating platelet radioactivity at time $t_i$ as a fraction of the total platelet-bound radioactivity injected into the subject ($N_0$) the following calculations may be used: where $R$ is the total platelet-bound radioactivity injected into the subject (cps/ml standard $\times$ dilution factor of standard $\times$ volume in ml injected) and $B$ is the total blood volume of the subject (ml)*, then $N_0 = \frac{R}{B}$ and

$$N_i = \frac{C_i}{V_i} \frac{1}{E_i}$$

or

$$N_i = \frac{C_i}{V_i} \frac{B}{R}$$

5. ANALYSIS OF DATA

The validity of these methods of analysis is based on the assumption that the blood volume of the recipient is constant and the pattern of disappearance of each successive cohort of platelets from the circulation remains constant during the course of the study.

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*Estimates of blood volume based on height and weight have confidence limits of $\pm 15\%$ and therefore are less satisfactory than direct measurement. (See ICSH Report on Standard Techniques for the Measurement of Red Cell and Plasma Volume, Br J Haematol 25:801, 1973.)
5.1. If the physician is interested in estimating mean platelet survival in order to assess the diagnosis and prognosis in an individual patient, the disturbance of platelet survival is usually expected to be marked. In this event, the disappearance curves can be approximated by visual fitting, although this procedure is imprecise and subject to observer bias. The tangent to the curve at the point where it intersects the vertical axis will then intersect the horizontal axis at a distance from the origin equal to the mean platelet life span. Where survival time is exponential (as is easily verified by obtaining a straight line when the logarithm of the relative radioactivity is plotted against time), the mean may also be found by finding the length of time required for radioactivity to be reduced by 50% ($\tau/2$) and using the formula

$$\text{Mean survival} = 1.443 \times \tau^{1/2}.$$ 

Often more precise assessment may be necessary, for example, when studying effects of therapies, smoking, or dietary manipulations. In order to make the best use of the data, more complex procedures must then be carried out.

5.2. Where the main concern is only to obtain an estimate of the mean survival, the data may be analyzed by a simple method (method I, 5.3), calling only for a desk calculator and systematic computation. Where there is also interest in the shape of the survival curve, methods are available that require sophisticated computation facilities. One such procedure (method II, 5.4) is based on a model developed for red cell survival studies.* The other procedure (method III, 5.5) is based on a multiple hit model. Method I has been shown to give results with an acceptable correlation with those of method III. Method III depends on the use of iterative procedures for nonlinear estimation. It is essential that least-squares fitting procedures be used and the error in the estimated mean survival time be calculated from the variance of the data. Method III has the advantage of greater coherence and flexibility and offers the greater prospect of extracting information where data are scanty. It also yields confidence limits in the individual case. The properties of method II as applied to platelet survival have not been extensively explored.

5.3. Method I.

5.3.1. To the values of radioactivity at times $t_i$, $N_i$, is fitted the linear decay function

$$N_i = c - kt_i,$$

and the value $A$ of $t_i$ when $N_i = 0$ is estimated. This value is the "linear estimate" of the mean platelet survival $A$.

5.3.2. To the values is also fitted the function

$$\ln(N_i) = c^* - k^*t_i,$$

and the value $B$ of $1/k^*$ is estimated; this is the logarithmic estimate of the mean platelet survival $B$.

5.3.3. A weighted mean of $A$ and $B$ is now obtained and this is taken to be the mean survival time: Let $S_A$ be the sum of the squares of the deviations of

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the data points from the fitted straight line (see Appendix), and let \( S_B \) be the sum of the squares of the deviations of the data points from the fitted exponential curve (the deviations are the discrepancies between the fitted line and the data and not of the logarithms of the data values) (see Appendix); then the weighted estimate of the mean, \( M_w \), is

\[
M_w = \frac{A S_B + B S_A}{S_A + S_B}.
\]

In particular, for a strictly exponential decay, \( S_B = 0 \) and \( M_w = B \). For a strictly linear decay, \( S_A = 0 \) and \( M_w = A \).

5.4. Method II. As is well known, the mean survival may be determined even if the form of the probability distribution of survival is unknown. Thus if a decay curve fits the data well, the mean may be estimated even if neither the form of the curve nor the parameters it contains have any literal biologic interpretation. The equation originally described by Dornhorst in 1951 provides an appropriate function that is based on the assumption of a deterministic life span \( (T) \) that may be cut short by an exponential process. The corresponding population curve has the form

\[
Y_i = N_0 \left( \frac{e^{-ki} - e^{-kT}}{1 - e^{-kT}} \right)
\]

where \( k \) is the constant instantaneous rate of random destruction. An appropriate method of fitting such a curve to the data would be that of least squares, i.e., to find those values of \( N_0 \) (the \( y \) intercept), \( k \), and \( T \), that minimize \( S \), the residual sum of squares:

\[
S = \sum_{i=1}^{m} (N_i - y_i)^2,
\]

where \( N_i \) is the observed radioactivity and \( y_i \) the fitted activity at time \( t_i \). In general, this procedure will call for iterative methods and for practical purposes a computer will be necessary.

5.5. Method III. Those values of \( c, a \), and \( n \) that minimize the residual sum of squares of the \( m \) data points from the appropriate gamma function are found by iteration on a computer. The quantity minimized is

\[
\sum_{i=1}^{m} (N_i - H_i)^2,
\]

where

\[
H_i = c \sum_{i=0}^{n-1} \left( \frac{(n - i)}{i!} e^{-a} (at)^i \right)
\]

and \( N_i \) is the observed radioactivity at time \( t_i \). The constants have the following significance: \( a \) is the reciprocal of the mean waiting time between hits; \( n \) is the number of hits before the platelet is destroyed; and \( c \) is the \( y \)-intercept (the initial intensity of labeling). The mean platelet survival is \( n/a \) and the variance is...
The computer program for obtaining the estimates is available from the ICSH office.

APPENDIX I

METHOD OF CALCULATING WEIGHTED MEAN SURVIVAL

Let the times at which samples are taken be denoted by \( t_i \), where \( i = 1, 2, 3, \ldots, m \), and the relative radioactivities by \( N_i \), where \( y_i = \ln N \).

Compute the following quantities:

\[
\begin{align*}
\bar{F}_i &= (t_1 - t) \quad \bar{G} = (N_1 - N)^2 \\
\bar{H} &= \left( (N_1 - N)(t_1 - t) \right) \\
\bar{M} &= (y_1 - y) \quad (y_1 - y)(t_1 - t) = M
\end{align*}
\]

Then

\[
A = \frac{\bar{F} \cdot \bar{G}}{\bar{H}} \quad B = \frac{\bar{F}}{\bar{M}}
\]

The residual sum of squares, \( S_i \), associated with estimate \( A \) is given by

\[
S_A = \bar{G} - \frac{\bar{H}^2}{\bar{F}}
\]

and that \( (S_B) \) associated with estimate \( B \) is obtained by first computing

\[
C = \sum_i \frac{\bar{G}}{B}
\]

and then

\[
8 = \left( \sum_i \frac{1}{B} \right)^2
\]

The weighted average estimate of mean survival, \( \bar{M} \), is given by

\[
\bar{M} = A \cdot (S_B) + B \cdot (S_A)
\]
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