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PLATELET PRESERVATION. I. PRESERVATION OF CANINE PLATELETS AT 4° C*

By PHIN COHEN and FRANK H. GARDNER

From the Richard C. Curtis Hematology Laboratory, Peter Bent Brigham Hospital; and the Department of Medicine, Harvard Medical School, Boston, Mass.)

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Transfusions of fresh whole blood, platelet-rich plasma, or platelet concentrates have been used to control hemorrhagic manifestations of thrombocytopenia (1, 2). As yet, lyophilized platelets, phospholipids, or gelatin-preserved platelets have not been found to be adequate substitutes for fresh, viable platelets in arresting thrombocytopenic hemorrhage (3, 4). Viable platelets can participate in viscous metamorphosis (5, 6) which is essential for "hemostatic plugging" (7).

The replacement of viable platelets by fresh, whole blood administration is wasteful of blood donors and frequently is dangerous because of overloading the circulation. When the platelet level is depressed disproportionate to the loss of, or need for, red blood cell or plasma replacement, whole blood administration is limited by the amount of red cells or plasma given along with the platelets (8). This laboratory, therefore, has placed primary emphasis on deriving information about the viability of platelet concentrates prepared from platelet-rich plasma, immediately, or after storage of the latter for various time intervals. A platelet transfusion derived from one 500 ml blood donation can be administered in a 30 ml volume (8). Platelet concentrates prepared from 15 U of blood can be administered in a 450 ml volume, which is less than the volume of a standard phlebotomy.

In addition, separation and storage of viable red blood cells and viable platelets from fresh, platelet-poor plasma will achieve the goal of blood-component therapy. Blood banks will derive more effective use from blood donations, as well as obtaining a larger therapeutic arsenal of fresh plasma and viable platelets for use in coagulation-protein deficiencies and thrombocytopenia, respectively. Only when platelets have been made available on demand will their general therapeutic usefulness be assessable (9). The paucity of adequate data relating to the variability of platelet isoimmunization (10, 11) likewise does not permit the generalization that this phenomenon will severely limit the usefulness of platelet transfusion therapy (9).

Platelet viability is evaluated best by life-span measurement, since clot-retracting and thromboplastic properties of platelets are retained even when the platelets are not viable *in vivo* (9, 12–15). The present study was initiated to determine more precisely the duration of time that whole blood, platelet-rich plasma and platelet concentrates could be stored at 4° C and still contain viable platelets. The basis for assessing *in vivo* viability of platelets has been the life-span measurement using Cr^{51} labeling of the platelet concentrates. An attempt has been made to correlate the preservation of clot retraction with the life span of platelets in platelet-rich plasma stored at 4° C for varying time intervals.

Multiple experiments utilizing radioactivity are difficult to justify in healthy human subjects. This investigation, therefore, was concerned with canine platelets with the hopes that the information derived would have application in man.

MATERIALS AND METHODS

Life-span measurements. Mongrel dogs were used after a proper period of observation in the animal farm. Canine platelets can be labeled with radioactive sodium chromate by a modification of the technique described for human platelets by Aas and Gardner (16). In the studies reported here, a colony of 11 dogs was used, each dog serving as its own control in each variation of the storage studies. The animals received autologous platelets in all life-span measurements. A minimum of 6 experiments (in 6 separate animals) was performed to evaluate each time interval of storage with whole blood, platelet-rich plasma and platelet concentrates.

Preparation of platelets. Three hundred to 350 ml of whole blood was collected from the femoral artery through

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a hemo-repellent-coated no. 17 gage needle. The blood flowed into a polyvinyl plastic bag [1] containing 45 ml acid citrate dextrose (ACD-NIH formula). Disodium ethylenediamine tetraacetate (EDTA; 1.5 per cent) in 0.7 per cent saline (30 ml) also was used as an anticoagulant. Two of 11 animals exhibited consistent clumping of platelet concentrates prepared in ACD but not in EDTA. Two other animals occasionally showed clumping of ACD concentrates, such as is seen in man. The whole blood was centrifuged at 2° C for 15 minutes at 200 G. After centrifugation the platelet-rich plasma was transferred to another plastic bag (Fenwal, TA-2) containing 3 ml of 2 per cent Triton [2] in 0.85 per cent saline. A pneumatic press was used in the separation of plasma from the red cells and leukocytes (buffy coat). The plastic bag containing platelet-rich plasma was distended with air to prevent platelet trapping in creases of the bag and was centrifuged at 2° C for 30 minutes at 1,000 G. The platelets collected as a creamy button at the bottom of the bag. The supernatant platelet-poor plasma was withdrawn under sterile conditions and saved, leaving approximately 3 ml of residual plasma with the platelet button.

Labeling of platelets with Cr^{51}. Through the plastic inlet tubing attached to the bag, 3 ml of 0.2 per cent Triton in physiologic saline was injected by syringe. The bag was kneaded between the fingers to resuspend the platelet button until no macroscopic clumps were visible. One hundred fifty μc of sterile radioactive sodium chromate ($Na_2Cr^{51}O_4$) with a specific activity of 25 to 35 mc per mg of chromium was injected through the plastic tubing into the platelet suspension. After mixing, the platelet suspension was incubated for 15 minutes at room temperature (20 to 22° C). Ten per cent of the Cr^{51} was firmly bound to the platelets, 90 per cent to the surrounding plasma proteins. Thereafter, the suspension was centrifuged again at 2° C for 30 minutes at 1,000 G. The radioactive plasma-Triton solution was drained from the platelet button by gravity and saved to determine residual Cr^{51} activity. Seven ml of nonradioactive autologous plasma saved earlier in the experiment and 7 ml of 0.2 per cent Triton in saline were added to the platelet button and the platelets were resuspended. One hundred mg of 5 per cent ascorbic acid solution was added to the suspension to prevent red cell binding in the recipient by excess $Na_2Cr^{51}O_4$ and about 100 cc of air was added to the bag to facilitate the complete infusion of the labeled platelet suspension. The infusion was made through a short piece of plastic tubing attached to the outlet of the bag. The final volume averaged 25 ml with a platelet count of 2×10^6 per mm^3. The labeled platelet concentrate was injected into the jugular vein of the dog (autologous recipient) after a careful venipuncture with syringe and disposable no. 18 gage needle [3] had been made.

To estimate the life span of the labeled platelets, 12-ml blood samples were obtained from the jugular vein at 0.5 and 2 hours and daily thereafter until no further radioactivity was detected in the recipient's platelets. The blood was processed by differential sedimentation with 6 per cent dextran, as outlined in a recent report from this laboratory (10).

Life-span measurements in the recipient were plotted in two ways: *1)* counts per minute per platelet button; *2)* highest platelet button radioactivity count as the 100 per cent value and all other values related to it as a percentage. The latter method permitted easier standardization of graphing, and more effective comparison of multiple studies in the same animal. Figure 5 was plotted as counts per minute in order to illustrate the application of this method. The highest platelet button radioactivity in normal canine Cr^{51} studies averaged 3,000 to 5,000 counts per minute. *The same labeling dose of Cr^{51} was used for all platelet concentrates, permitting direct comparison between studies.* All of the data in this report, whether listed as per cent activity (Figures 1–4) or counts per minute (Figure 5) were plotted from studies in which the counts per minute yield was in the normal range (3,000 to 5,000 counts per minute in the platelet button derived from the 2-hour sample).

The platelet viability index (17) was not used to plot platelet life span. The advantages and disadvantages of this procedure in routine Cr^{51} life-span studies have been discussed in a recent report from this laboratory (10).

The platelet viability index (17) represents an attempt to quantitate the percentage of platelet-bound radioactivity in the labeled concentrate (transfusion) that can be harvested from the peripheral blood. The range of normal values for the platelet viability index has been 25 to 50 per cent in our hands. This wide range of normal has applied even when identical experiments in the same animal have been compared. Reliance on a true quantitative expression of platelet viability by this particular method, therefore, may be misleading. A 25 per cent viability index may represent a 100 or a 50 per cent yield in a storage experiment, depending on what the value for the index would have been with a control experiment.

Variations in platelet preparation for storage studies. *1)* Whole blood collected in plastic bags with ACD anticoagulant was stored at 4° C. At varying time intervals the platelets were harvested by differential centrifugation and labeled with $Na_2Cr^{51}O_4$. Much difficulty was experienced in separating platelets after hours of chilling. Only by warming the blood for an hour before centrifugation could adequate platelets be obtained for labeling with Cr^{51}. The time required to warm blood markedly impaired the survival of whole blood platelets.

2) Platelet-rich plasma was prepared by differential centrifugation from blood collected in ACD anticoagulant. The plastic bag containing the platelet-rich plasma was

[1] TA-5, Fenwal Lab., Somerville, N. J.

[2] Triton is a trademark of Rohm and Haas Co., Philadelphia, for oxyethylated tertiary octyl phenol formaldehyde polymer and is supplied as Entsufon (WR-1339) by Winthrop Laboratories, New York, N. Y. It is used to effect more rapid resuspension of platelet concentrates after centrifugation.

[3] Supplied as Monoject needles by Roehr Products Inc., Deland, Fla.

stored at 4° C for varying intervals. At the end of the designated storage period, the platelet concentrates were prepared and labeled with Cr^{51}, and the platelet transfusions were given to the autologous recipient.

3) Platelet-rich plasma was stored as in *1.* Instead of further centrifugation to prepare a platelet concentrate, the platelet-rich plasma was labeled directly with a large dose of Cr^{51}. A massive labeling dose of Cr^{51} was necessary because of the large ratio of plasma to platelets in platelet-rich plasma as compared with plasma concentrates. The plasma proteins ordinarily accept 90 per cent of the label even with platelet concentrates. An effective platelet label was achieved by a large labeling dose in the range of 600 to 800 mc. These latter studies allowed evaluation of unmanipulated (uncentrifuged) platelet-rich plasma.

4) Platelet concentrates were labeled with Cr^{51} and stored at 4° C for varying time intervals.

Clot retraction observations. A modification of several available techniques for measuring clot retraction has been developed in an effort to semiquantitate the procedure (12, 18–20).

1) Technique. Acid-cleaned round-bottom test tubes (100 × 15 mm) were fitted with hand made stainless steel wire coils. The bottom inch of the coil was wound tightly in a spiral of 4 mm diameter while the remaining upper portion of the coil had the same internal diameter as that of the tube (Figure 1). The bottom part of the coil was 4 mm from the bottom of the tube and was supported at the center by the tension of the upper coil against the wall of the tube. This arrangement permitted suspension of the clot at the geometric center of the tube, thus lessening its chance of sticking to the sides of the tube.

Various concentrations of canine platelets from 150,000 to 5,000 mm^3 were prepared by mixing platelet-rich plasma and platelet-poor plasma in varying proportions in a total volume of 2.9 ml. Seven dilutions were made for each study and platelet counts were made of each dilution. The counts were done by phase microscopy by the method of Brecher and Cronkite (21). One-tenth ml of $CaCl_2$ (0.02 M) solution was added to each plasma dilution and the steel coil was inserted.

The tubes were placed in a 37° C water bath for 2 hours to allow clot retraction. At this time the retraction was estimated qualitatively as 1 to 4 + for each tube in the series (Figure 1). These observations were expressed in a semiquantitative manner by measurement of the residual serum in each tube (22). The wire coils with the attached clots were removed gently from the tubes and the remaining serum measured after pouring into a graduated centrifuge tube. The clot retraction was expressed as the ratio of residual serum to the total plasma volume (3 ml). The results were graphed with platelet count on the ordinate and the percentage of clot retraction on the abscissa (Figure 4).

2) Storage studies. Platelet-rich plasma was prepared and divided into two aliquots. One portion was stored at 4° C for varying intervals. The other aliquot was prepared immediately in dilutions of varying platelet concentrations, as described above, and the clot retrac-

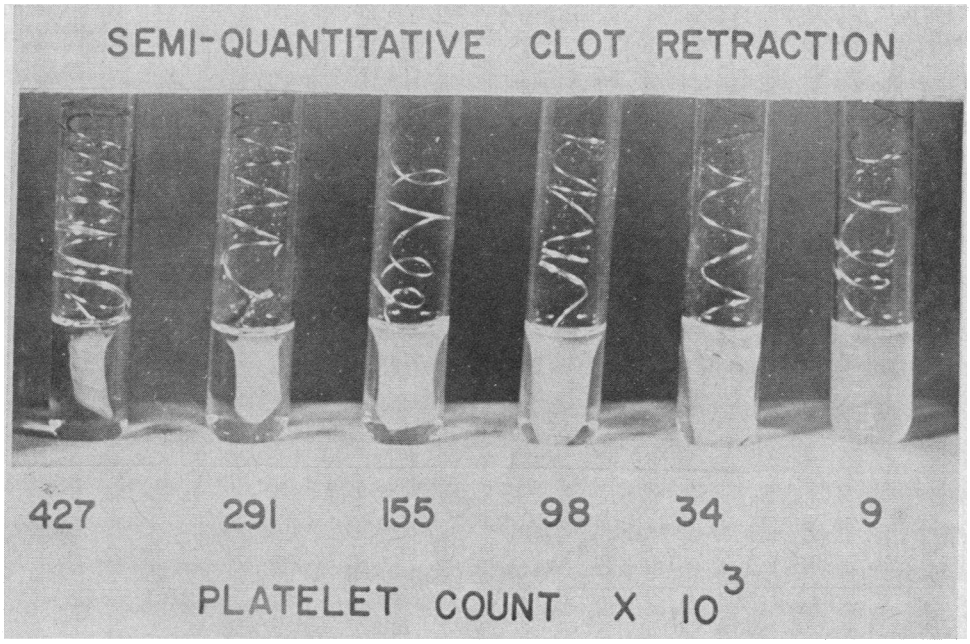

FIG. 1. THE METHOD FOR SEMIQUANTITATIVE CLOT RETRACTION. Clot retraction is estimated qualitatively by inspection and semiquantitatively by withdrawal of the wire coil and adherent clot and measurement of residual serum. The serum-plasma ratio may be expressed as a per cent value for clot retraction at any level of platelet count.

tion estimated. After storage, the first aliquot was similarly prepared to determine whether storage influenced the clot-retraction function of the platelet. No centrifugation or manipulation of the platelet-rich plasma was done other than that described above (see *Clot retraction observations*). In some instances the platelet-rich plasma at 4° C was labeled directly with large labeling doses of Cr^{51} and the lifespan determined for comparison with clot retraction.

Platelet yield in recipients. To determine the recovery of the labeled platelets, anesthetized dogs were depleted of platelets by multiple phlebotomies and plasmapheresis (23). The blood was collected in EDTA, centrifuged, and the platelet-rich plasma removed. The red cells suspended in platelet-poor plasma (autologous) were returned to the animal quickly. After 11 to 13 350-ml bleedings over a 3- to 4-hour period, the animal became thrombocytopenic. During the early part of the thrombocytopheresis, two bags of platelet-rich plasma were withdrawn from the exchange and stored at 4° C for 5 hours. When the induced thrombocytopenia appeared to have stabilized, Cr^{51}-labeled platelet transfusions were prepared from the stored platelet-rich plasmas and given to the animal.

The main purpose of this procedure was to determine the quantitative yield of platelets in the thrombocytopenic recipient. Labeling the platelets with Cr^{51} permitted comparison between the radioactivity disappearance curve and the platelet counts (Figure 5). A 7 per cent body weight-blood volume ratio was used in deriving the yield of circulating platelets from the labeled transfusions (1).

RESULTS

Measurements of canine platelet life span. In 33 studies of normal autologous platelet transfusions, a Cr^{51} platelet life span of 7 to 8 days was observed (Figure 2). When platelet concentrates were resuspended readily in ACD there was no apparent difference in the life-span pattern of Cr^{51}-labeled platelets derived from ACD and EDTA blood. In almost all instances of fresh autologous platelet transfusions, the half-hour platelet button radioactivity was lower than the 2-hour radioactivity. This was similar to the sequestering that has been noted with labeled human platelets (10, 16, 24) except that the latter have shown the highest radioactivity after 24 hours.

Inspection of the plotted curves revealed a curvilinear downslope of platelet-bound radioactivity during the platelet life span. Similar curves have been noted after transfusions of canine platelets

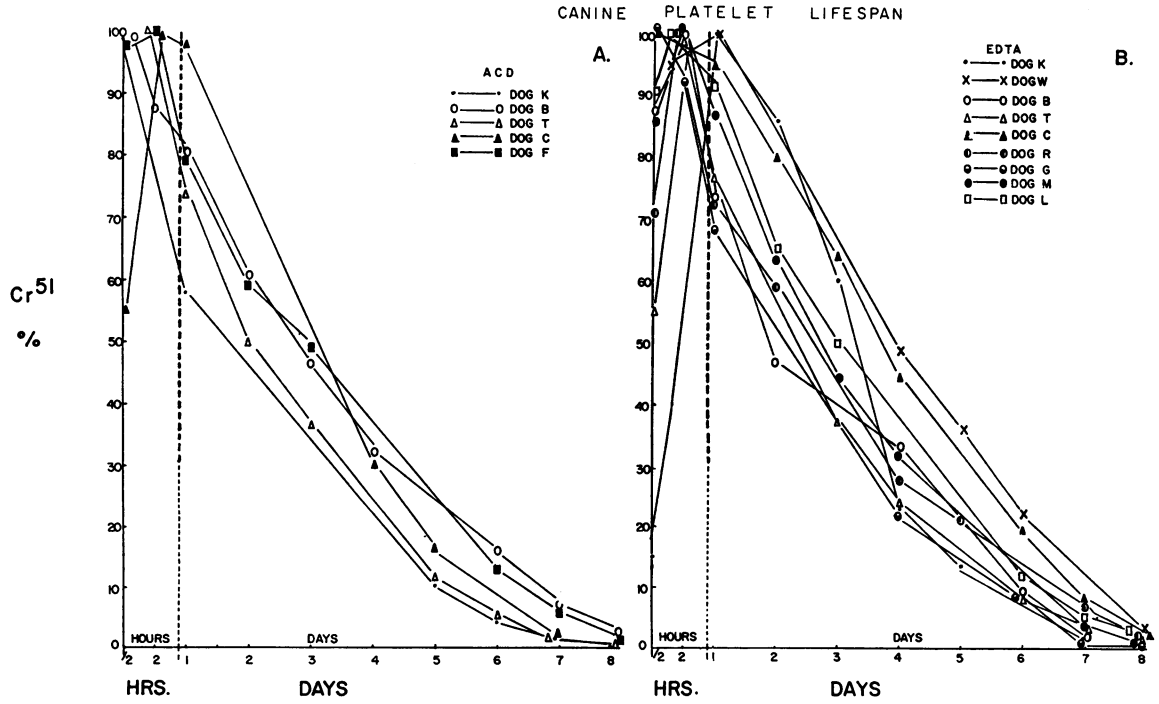

FIG. 2. THERE WAS NO APPRECIABLE DIFFERENCE BETWEEN THE LIFE SPAN OF CANINE PLATELETS DERIVED FROM ACD AND EDTA BLOOD. Canine platelets have a 7- to 8-day life span. Platelet sequestration was observed in that the platelet button radioactivity was lower on the half-hour than on the 2-hour sample. The downslope of platelet-bound radioactivity was curvilinear and was reproducible through many studies in the same animal.

labeled with $Na_2P^{32}O_4$ (24). The platelet life span was remarkably reproducible, since the results were repeated six times in each animal.

Storage observations. *1*) Storage of platelet-rich plasma in ACD anticoagulant for 4 hours did not alter markedly the life span of labeled plate-

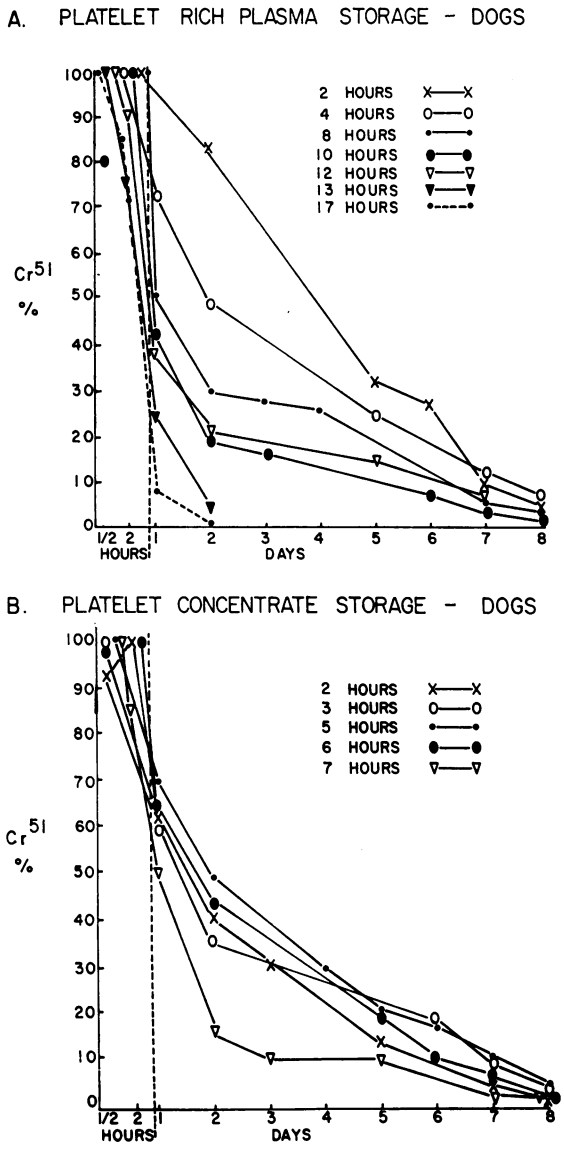

FIG. 3. **A.** Cr^{51} LIFE SPAN OF PLATELET CONCENTRATES DERIVED FROM PLATELET-RICH PLASMA STORED AT 4° C FOR VARYING INTERVALS. A progressive decline in recoverable platelet-button radioactivity was seen with increasing periods of storage from 2 to 17 hours. **B.** Cr^{51} LIFE SPAN OF PLATELET CONCENTRATES STORED AS SUCH FOR VARYING INTERVALS AT 4° C. A progressive decline in recoverable platelet button radioactivity was seen with increasing periods of storage from 2 to 7 hours.

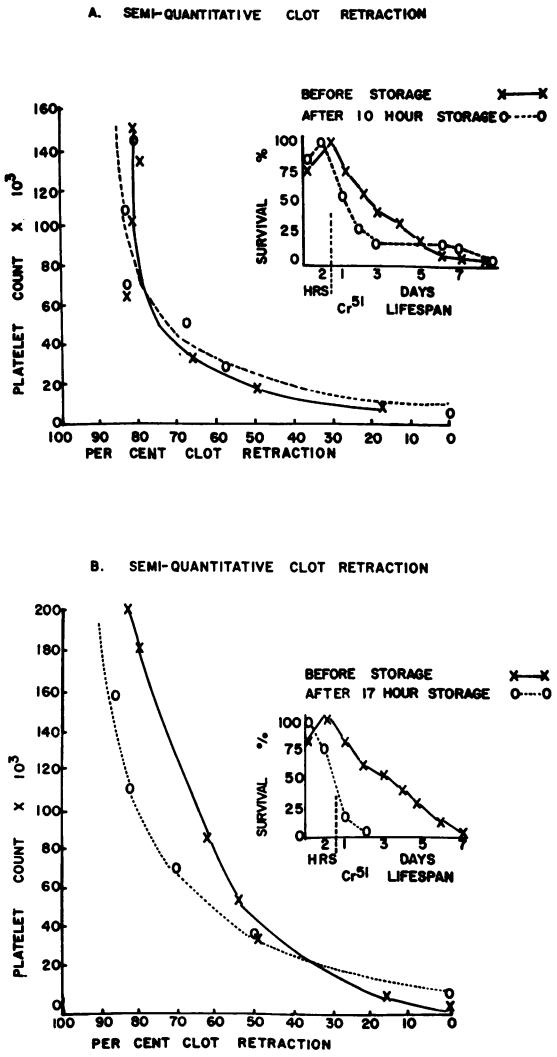

FIG. 4. **A.** A COMPARISON BETWEEN CLOT RETRACTION AND Cr^{51}-PLATELET LIFE SPAN OF PLATELET-RICH PLASMA STORED FOR 10 HOURS. The large portion of the graph shows curves of semiquantitative clot retraction before and after 10 hours of storage. The insert shows a graph of the Cr^{51}-platelet life spans of the same platelet-rich plasmas before and after 10 hours of storage. Although there was no difference in clot retraction, the Cr^{51} life-span curve after 10 hours was diphasic. This is illustrated both by gross inspection of the curve and by comparison of the per cent recovery of radioactivity in the platelet buttons at 24 hours. The 10-hour storage experiment showed 50 per cent activity at 24 hours compared with the baseline study which showed 75 per cent activity at 24 hours. **B.** A COMPARISON BETWEEN CLOT RETRACTION AND Cr^{51}-PLATELET LIFE SPAN OF PLATELET-RICH PLASMA STORED FOR 17 HOURS. Note the markedly shortened Cr^{51} life span after 17 hours of storage despite the maintenance of normal clot retraction function *in vitro*.

lets in the recipient. Longer storage, up to 12 hours, progressively impaired the survival of labeled platelets. The platelets that remained, however, had a 7-day life span (Figure 3). Beyond 12 hours of storage virtually no viable platelets remained to circulate in the recipient.

2) The storage of platelet concentrates in small volumes of plasma had a deleterious effect on viability even after storage intervals of only 2 to 3 hours (Figure 3). After 7 hours' storage a sharp decrement in recovery of labeled platelets was noted, the first day's platelet button showing only 15 per cent of the peak radioactivity.

3) The prolonged chilling of whole blood at 4° C altered the viscosity so that a poor plasma harvest was obtained after centrifugation at 200 G, compared with the yield following immediate centrifugation of freshly shed blood. Only by warming the blood at room temperature (22° C) for 1 hour was a representative yield of platelet-rich plasma obtained. The life-span curves of platelets harvested from chilled, stored whole blood, even after storage intervals of 1 to 2 hours, were very poor compared with the results on platelets derived from platelet-rich plasma stored for the same intervals (Figure 3) and were not graphed.

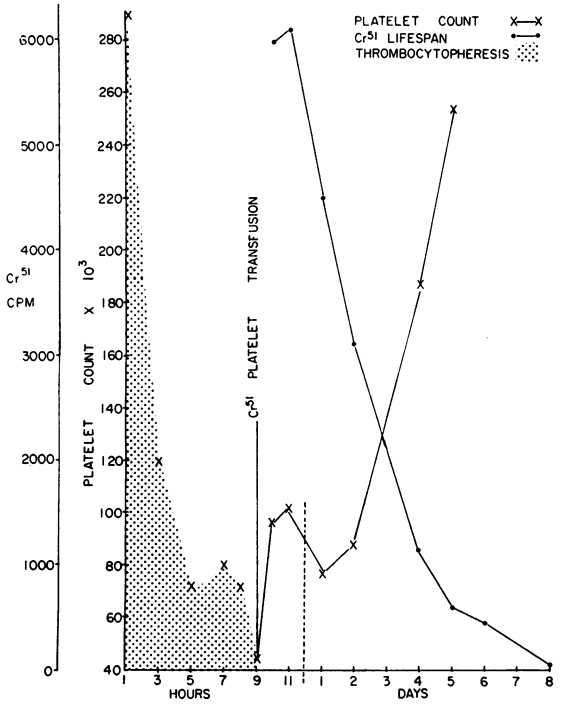

FIG. 5. THE SHADED AREA INDICATES THE DURATION OF THE THROMBOCYTOPHERESIS AND THE DEVELOPMENT OF THE INDUCED THROMBOCYTOPENIA. At the trough of the platelet depression, a Cr^{51}-labeled platelet concentrate derived from 2 U of platelet-rich plasma stored for 5 hours at 4° C was given to the animal. The platelet counts as well as the Cr^{51}-platelet life span then were followed. Note the correlation between the sharp rise in platelet counts and the normal Cr^{51}-platelet life span. The second sharp rise in platelet count which began on Day 2 of the experiment was a reflection of bone marrow recovery from the insult of thrombocytopheresis.

Clot retraction. Some platelets have clot-retracting properties even after 54 hours' storage. These results contrasted sharply with the *in vivo* viability of platelets stored for the same time intervals. After 10 hours of storing platelet-rich plasma, a biphasic Cr^{51} life-span curve was obtained while clot retraction was perfectly normal (Figure 4). After 17 hours' storage, when virtually no platelets were viable by the Cr^{51} life-span measurement, clot retraction function was still retained.

Platelet yields. After thrombocytopheresis, a pronounced thrombocytopenia was produced. When this thrombocytopenia had stabilized over a 1-hour observation period with three platelet counts at the same level, an animal was given Cr^{51}-labeled platelet concentrates which had been derived from 2 U of platelet-rich plasma stored for 5 hours (Figure 5). A 100 per cent recovery of these platelets was demonstrated by platelet counts. The viability of platelets after 5 hours' storage also was demonstrated by the high level of circulating Cr^{51} platelet activity noted on the first day (75 per cent of the peak radioactivity level after transfusion). A companion thrombocytopheresis study of platelet-rich plasma stored for 3 hours was done, which showed an 84 per cent yield of circulating platelets by direct enumeration. In this instance, however, the level of Cr^{51} after 24 hours was only 53 per cent of the peak sample, suggesting some decreased platelet viability. One platelet concentrate stored 8 hours had only a 26 per cent yield of platelets by direct enumeration.

DISCUSSION

A canine model has been developed for the study of platelet life span by the Cr^{51}-labeling method. The characteristics of the Cr^{51}-platelet life-span curve in the dog are similar to those in

man (10, 16). The phenomena of sequestration of platelets and the curvilinear downslope of the Cr^{51} life-span curve have been discussed in a recent report from this laboratory (10). The human and canine Cr^{51}-platelet life-span curves have differed mainly in the rapidity of release from sequestration, the canine showing the highest platelet-bound radioactivity at 2 hours, compared with 24 hours in man (10, 16).

Canine platelet concentrates prepared from ACD blood were not clumped in the majority of animals (9 out of 11) and almost uniformly had Cr^{51} life-span curves which were similar to those seen with concentrates prepared from EDTA blood. This contrasts with the experience of this laboratory in preparing human platelet concentrates which are almost always clumped when derived from ACD blood.

Our lack of success in resuspending human ACD platelet concentrates is at variance with the work of Minor and Burnett (25) and with the views expressed in a more recent summary report on the transfusion of whole blood and its components (26). Our work, indeed, emphasizes a fundamental difference between the canine and human platelet in their capacity to be resuspended in these two anticoagulants. An explanation of the mechanism for this difference may help to explain certain phenomena of platelet membrane physiology and viscous metamorphosis (5, 6).

Furthermore, the discovery of this difference between canine and human platelets has been exploited here in order to provide the first extensive experience with 4° C storage of platelet concentrates in an anticoagulant-nutrient mixture. Certainly, red blood cell storage experiments with anticoagulation alone indicate a markedly reduced shelf-life at 4° C (26–29). The failure to employ an anticoagulant-nutrient mixture in control studies vitiates the results of recently reported platelet storage experiments in rabbits and man (17). Perhaps an EDTA-glucose, anticoagulant-nutrient mixture (30) would have been a better choice for 4° C storage experiments with human platelets.

The studies reported here indicate that canine platelets have very limited viability at 4° C. Storage of platelet concentrates for 2 hours at 4° C resulted in a biphasic Cr^{51}-platelet life-span curve, indicating an immediate loss of a portion of the transfused platelets. As the storage time was increased from 2 to 7 hours by 1-hour increments, loss of platelet viability was progressive. Platelets stored at 4° C as platelet-rich plasma and then processed to derive platelet concentrates showed a longer period of viability by the Cr^{51} life-span measurement and by direct enumeration in the animal rendered thrombocytopenic by thrombocytopheresis. The better results with storage of platelet-rich plasma when compared with storage of platelet concentrates may be related to the greater availability of nutrient (glucose) (31, 32) or plasma-protein coating (plasmatic membrane) (33, 34) in the former. Both the nutrient and the plasmatic coating may be vital to the glycolytic (31) and enzymatic functions of the platelet (32, 35). The use of adenosine and inosine may support adenosine triphosphate to extend platelet viability beyond the time observed in these experiments (36). If the experience with red blood cells proves to be analogous, however, enzymatic and nutrient additives are not likely to add a sizable increment to 4° C storage time of platelets (26).

Jackson and Krevans have demonstrated normal viability of canine platelets after 24 hours of storage at 4° C (37). This is not in basic conflict with our findings. These authors used direct enumeration of circulating platelets after transfusion of whole blood into thrombocytopenic recipients. The Cr^{51} life-span method used in these studies involves three centrifugations for preparation of the labeled platelet transfusion. This extensive *in vitro* manipulation decreases platelet viability somewhat. This should encourage comparison between storage times using the same technique of platelet manipulation and life-span measurement rather than accentuate the differences in results.

It also has been reaffirmed here that the *in vitro* phenomenon of clot retraction is related only negatively to platelet viability (12). If platelets cannot retract a clot they are not viable. If platelets can retract a clot they still may not be viable as determined by the Cr^{51} life-span measurement. In a similar manner other clotting functions of platelets dependent on their thromboplastic activity are of no value in predicting platelet viability or hemostatic capacity *in vivo* (3, 4, 14).

SUMMARY

The experience gained with 4° C storage of canine platelet-rich plasma and platelet concentrates has emphasized the following points:

1. Simple refrigerator (4° C) storage of platelets in any concentrated form for use on demand is not feasible. If experience with the red blood cell is any guide, nucleosides or other nutrient additives are not likely to extend the 4° C shelf-life of platelets to the point of logistical usefulness.

2. The preservation of platelets by freezing methods is likely to provide the only possible means for their banking. Because of the volume relationships in platelet-replacement therapy the freezing of platelet concentrates seems to be the most fruitful avenue of approach to this problem.

3. The use of any criteria other than life-span measurement in platelet preservation procedures is to be discouraged, since clot retraction and thromboplastic functions may be retained by platelets which are incapable of survival in the circulation.

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CORRECTION

On pages 2077 and 2078 of the article entitled "A Quantitative Study of Recycling of Isotope from Glycine-1-C^{14}, α-N^{15} into Various Subunits of the Uric Acid Molecule in a Normal Subject" by R. Rodney Howell, Melinda Speas and James B. Wyngaarden (J. clin. Invest. 1961, **40**, 2077), two numbers are incorrect because of typographical errors in the manuscript. On page 2077, right-hand column, line 27 should read: "The total N^{15} administered was 923.0 mg," instead of 293.0 mg. On page 2078, left-hand column, the figure given four lines from the bottom of the page should be "0.360 per cent," not 0.260 per cent.