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Research Article

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Platelet Sequestration in Man. I. Methods *

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Although the average lifespan of human platelets has been reported to be 8 to 11 days (1–5), the mechanism by which these cells are normally destroyed is controversial. Most investigators, favoring the concept that platelets die chiefly by a process of senescence, find the elimination of transfused platelets to be a simple linear function of time (1–5). On the basis of animal experiments, it has been suggested that effete platelets are sequestered in the reticuloendothelial system (6–8). Other workers have found that the platelet disappearance pattern is nonlinear and have proposed that platelet destruction is random, possibly a result of the action of coagulation factors (9–10).

Studies of these aspects of platelet physiology have been limited by the fact that the current method of labeling these cells with the gamma-emitting radioisotope chromium^{51} causes nearly all the platelets to disappear from the circulation for several hours after transfusion and permits only about one-third of them to survive normally thereafter (2, 4). This phenomenon of transient sequestration and the low viability achieved have been assumed inevitable consequences of manipulating platelets *in vitro*.

This report describes the use of a method for labeling platelets with Cr^{51} that permits improved viability and eliminates the initial transient sequestration. In addition to studies in normal subjects of the disappearance of platelets from the circulation, the results of external scintillation scanning are presented, permitting observation of the sites of platelet sequestration.

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Methods

Preparation and labeling of platelet concentrates with Cr^{51} for transfusion. Platelet-rich plasma was prepared using plastic equipment throughout the procedure.[1]

Chromium^{51}-labeled "EDTA platelets" were prepared according to the method of Aas and Gardner (2) except that no surface-active agent was used. In some experiments platelets were labeled in isotonic NaCl rather than plasma, or the procedure was carried out at room temperature rather than at 4° C. These changes were found not to affect the platelet survival pattern.

Chromium^{51}-labeled "citrate platelets" were prepared as follows. A freshly made solution containing 0.085 M trisodium citrate, 0.065 M citric acid, and 2% dextrose was sterilized by Seitz filtration. After 90 to 95 ml was injected into an empty blood pack,[1] 500 ml of blood was collected from the donor. The first 10 ml of blood to enter the collection tubing was discarded before mixing blood with the anticoagulant.[2] The pH of the collected blood was about 6.5; the final whole blood concentration of citrate was 0.022 M, about 50% greater than with the usual acid-citrate-dextrose (ACD) anticoagulant. All procedures were carried out at room temperature. Blood was centrifuged at 275 *g* for 14 minutes.[3] The supernatant platelet-rich plasma (PRP) was removed to a 300-ml transfer pack and centrifuged at 400 *g* (1,500 rpm) for 5 minutes to remove residual erythrocytes and leukocytes. The PRP was separated and centrifuged for 15 minutes at 1,000 *g* (2,300 rpm). Most of the supernatant platelet-poor plasma so derived was removed to a sterile pack and the platelet button resuspended with 15 to 20 ml of the remaining plasma. Approximately 250

[1] Obtained from Fenwal Laboratories, Morton Grove, Ill.

[2] This was done by cutting the needle, along with several inches of tubing, from the blood pack and inserting it on the tubing of a transfer set (Fenwal) under sterile conditions. After the venipuncture, the first blood was discarded, and the transfer set was then inserted into the blood pack.

[3] At 1,200 rpm in an International Equipment Co. (Boston, Mass.) size UV centrifuge. This is a slower speed than is required when blood is anticoagulated with EDTA or when citrated blood is centrifuged at 5° C. Platelets sediment more readily under the conditions used in this procedure.

μc of Cr^{51} in the form of Na_2CrO_4 [4] was added, and the mixture was incubated for 15 minutes with occasional shaking.[5] Most of the previously separated plasma was then added along with 30 mg of ascorbic acid to reduce excess chromate. The platelets were next centrifuged at 1,000 *g* (2,300 rpm) for 15 minutes. The supernatant plasma was poured off as completely as possible and discarded. The platelet button was suspended in 30 to 50 ml of remaining plasma and transfused to the recipient, either directly from the transfer pack or with a 50-ml siliconized syringe, using a siliconized needle already in place. The method is similar to that for EDTA platelets (2) except for the anticoagulant, temperature, speed of centrifugation, and omission of a surface-active agent. The entire procedure requires about 2 hours.

Determination of platelet radioactivity in transfused platelets. The transfused platelets contained plasma Cr^{51}, platelet Cr^{51}, and a small amount of radioactivity (usually 2 to 10% of the platelet value) in the red cells present in the preparation. To determine platelet radioactivity, two 0.4-ml fractions were taken from the final preparation. One was suspended in 3 ml of isotonic NaCl and the other in 3 ml of 1% ammonium oxalate. The latter compound hemolyzed red cells and released their Cr^{51} into the supernatant fluid.[6] The two mixtures were then centrifuged for 30 minutes in siliconized tubes in an angle-head centrifuge [7] at 3,400 rpm. Buttons and supernatant fluids were counted separately. The platelet radioactivity was represented by the ammonium oxalate button, and erythrocyte radioactivity was the difference between the two buttons. The value for the ammonium oxalate button was used to calculate counts per minute of platelet Cr^{51} injected into the recipient.

Recovery of circulating platelet Cr^{51} activity in recipients. To determine circulating platelet radioactivity, 12 ml of blood was drawn from the recipient with a siliconized syringe and anticoagulated with 0.35 ml 5% EDTA. Two ml was placed directly in a counting tube. Five to 6 ml of isotonic NaCl was added to the remaining blood, mixed, and the tube centrifuged at 380 *g* (1,500 rpm) for 7 minutes. The supernatant PRP was harvested, and platelets remaining in the cell fraction were "extracted" once more with isotonic NaCl. In this way, 98% of the platelets could be recovered. The PRP-isotonic saline "extracts" were pooled in a siliconized tube and centrifuged at full speed in an angle-head clinical centrifuge [8] for 30 minutes. The radioactivity of the platelet button, representing the platelets from 10 ml of whole blood, was determined and divided by 10 to obtain circulating platelet radioactivity per milliliter. The harvest of platelets from blood was $95 \pm 1\%$ complete, with some platelets being lost from incomplete extraction, adherence to glass surfaces, etc. To correct for this, the observed radioactivity value was divided by 0.95.[9] Duplicate determinations done in the same subject did not vary by more than 5%. The 2-ml sample of whole blood provided an additional check on accuracy. All determinations of radioactivity were made in a well-type scintillation counter with a radiation spectrometer to reduce background. Sufficient counts were usually obtained to reduce error to 1%.

Percentage of recovery of transfused platelets in the recipient was determined by comparing total counts per minute of platelet Cr^{51} injected per milliliter to counts per minute recovered per milliliter (above). No obese subjects were studied, and none had diseases that would be expected to alter blood volume. Therefore, blood volume was assumed to be 7.0% of body weight (11). Some investigators have used larger values for blood volume; this would increase the calculated recovery of transfused platelets proportionately.

Surface scanning of body organs was done with a directional scintillation counter using a radiation spectrometer [10] to reduce background to less than 100 cpm. General techniques for surface scanning have been described previously (12). Preliminary studies showed significant radioactivity only over the liver, spleen, precordium, and lung after transfusion of Cr^{51}-labeled platelets, although on the first day a small amount of activity was also present over the bladder. Once a site was selected for scanning, it was marked on the skin surface, and subsequent measurements were made over the same area. Sufficient counts were recorded to reduce the counting error to less than 5%. All counts were corrected for physical decay of Cr^{51} from the time of transfusion.

Units used to define organ surface radioactivity. Usually, the platelet suspensions injected contained 10 to 20 μc of Cr^{51}. However, variation in platelet concentrations labeled led to variation in the amounts of platelet Cr^{51} injected in different experiments. To standardize the body surface radioactivity data, the surface counts per minute were expressed as a proportion of the microcuries

[4] Rachromate, Abbott Laboratories, North Chicago, Ill. Specific activity about 120 mc per mg of chromium.

[5] The percentage of Cr^{51} incorporated into platelets was directly proportional to the platelet concentration, as noted by Baldini, Costea, and Dameshek (4). With a concentrate containing 3×10^6 platelets per mm^3, about 8 to 10% of the Cr^{51} was utilized.

[6] In preliminary experiments, we had found that this procedure releases all the erythrocyte Cr^{51} without affecting platelet radioactivity.

[7] Serofuge, Clay Adams, Inc., New York, N. Y.

[8] International Equipment Co., Boston, Mass. The combination of a siliconized tube and an angle-head centrifuge produced the most complete centrifugation of platelets.

[9] No error was introduced by ignoring the small amount of erythrocytes in the platelet button, since red cell radioactivity never constituted more than 1% of the total.

[10] Nuclear-Chicago (Des Plaines, Ill.) Ds-301 scintillation detector with 3-inch diameter crystal and collimator 3 inches wide $\times$ 3 inches deep and 132 B spectrometer-scaler with window set at 100 kv. As long as the "window" was centered each day on the chromium^{51} gamma peak, counts could be reproduced with an error of less than 5% from day to day.

of platelet Cr^{51} transfused. Accordingly, the surface counts per minute were divided by microcuries of platelet Cr^{51} transfused × 10. These units denote organ surface radioactivities in Figures 1 to 4. We subsequently found that a value of 11 to 14 for the liver curve indicates that nearly 100% of the transfused Cr^{51} is localized in that organ. A range of 45 to 60 on the splenic curve indicates nearly 100% splenic localization (13).

Selection of normals. The "normal" subjects ranged in age from 30 to 72 and included patients hospitalized for minor illnesses or for sociologic reasons. None had diseases of the blood or blood-forming organs. Some were alcoholics undergoing rehabilitation, but they showed no palpable enlargement of the liver or spleen or abnormality in liver function tests.

Platelet counts were done by phase microscopy using the method of Brecher and Cronkite (14).

Results

EDTA platelets. When autologous EDTA platelets were transfused to normal persons, they disappeared rapidly from the blood stream, as also noted by other investigators (2, 4). Ninety to 95% of platelet Cr^{51} was removed from the circulation in less than 10 minutes. At the same time, there was a marked increase in surface radioactivity over the liver (Figure 1). Splenic activity increased only slightly during this period, and there was no localization of Cr^{51} in the lungs. During the subsequent 24 hours, there was a gradual decrease in hepatic radioactivity to about one-half the initial value and a reciprocal increase in splenic radioactivity usually continuing for 24 hours. The latter changes coincided with the reappearance of Cr^{51}-labeled platelets in the circulation to a maximum of about 30% of the total transfused. After 24 hours, blood platelet radioactivity declined in a rectilinear fashion over an 8-day period with no further significant changes in organ surface radioactivity. After 8 days, the slope of the survival curve decreased to form a tail to the curve, suggesting that a small percentage of platelets survived 10 to 14 days. This sequence of events is shown for a typical subject in Figures 1 and 2. Similar survival curves and changes in organ radioactivity were observed in six other normal persons. In several experiments, platelets returned to the circulation more rapidly or more slowly, but the changes in peripheral blood and

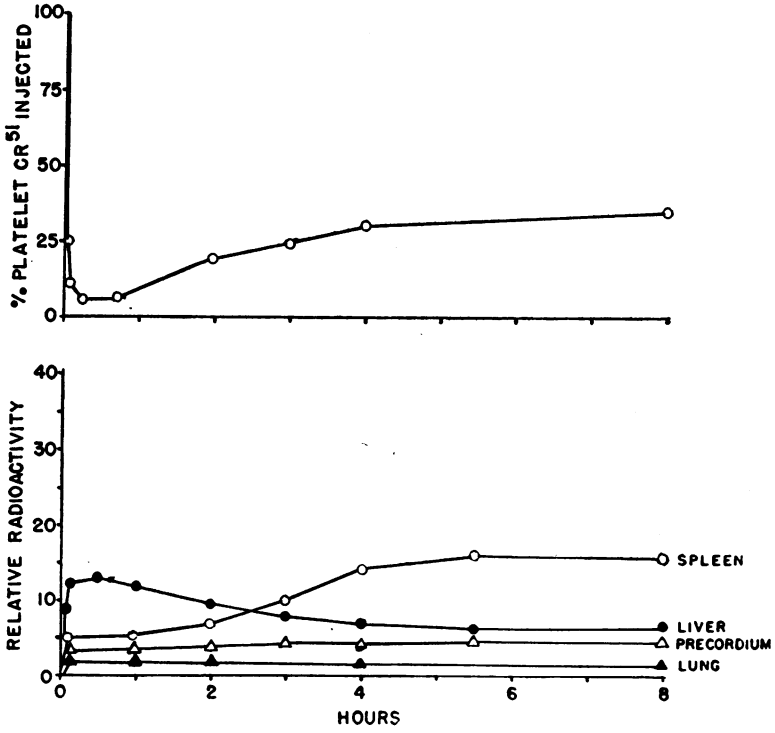

FIG. 1. THE SEQUESTRATION OF Cr^{51}-LABELED "EDTA PLATELETS" DURING THE FIRST 8 HOURS AFTER INJECTION INTO A NORMAL SUBJECT.

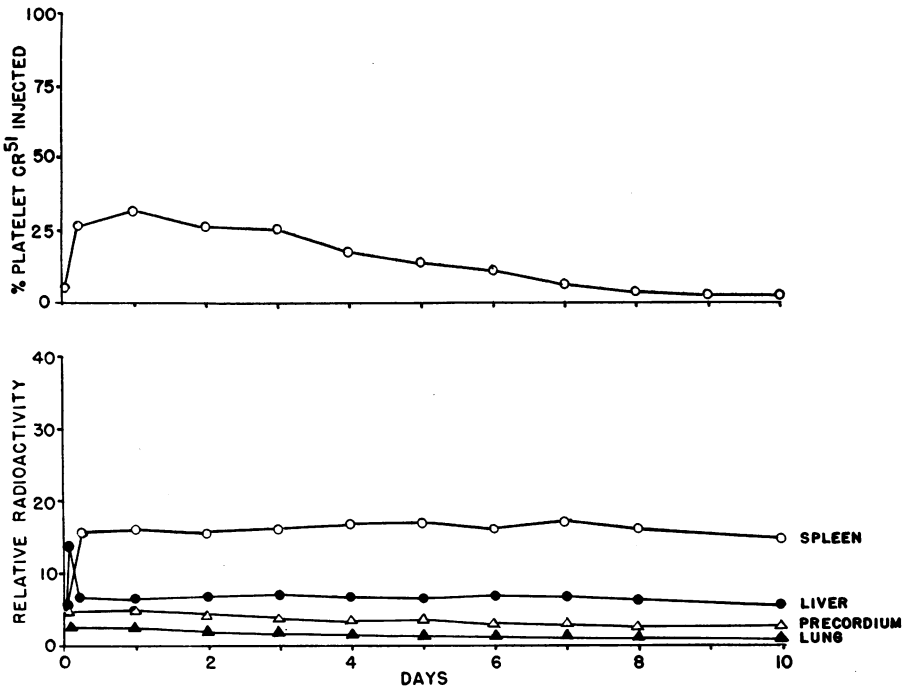

FIG. 2. THE SEQUESTRATION OF "EDTA PLATELETS" DURING THE 10-DAY PERIOD AFTER INJECTION INTO A NORMAL SUBJECT. The acute changes in radioactivity levels of the first hour, shown in Figure 1, are omitted.

organ Cr^{51} activity were qualitatively similar. In each of 10 experiments the maximal platelet Cr^{51} activity in the peripheral blood was reached at about 24 hours. Recovery values ranged from 15 to 35% of the total radioactivity transfused (average, 28%).

These findings suggest that the Cr^{51}-labeling procedure damaged platelets in some way so that they were sequestered in the liver immediately after transfusion. About one-half of these platelets returned to the circulation but were partially destroyed in the spleen so that less than one-third survived. This transient sequestration and poor recovery of transfused platelets caused by what might be termed the "lesion of collection" generally has been attributed to trauma received in the labeling procedure.

Effect of several variables on platelet viability. Initially, several other possibilities were considered. Since results using no surface-active agent were comparable to studies where such agents were used (2, 4), this factor did not seem important. It has been suggested that contamination of shed blood with tissue thromboplastin may damage platelets by provoking incipient coagulation (15), but there was no change in the platelet survival pattern when the first 10 to 15 ml of blood entering the collection tubing was discarded. No disadvantage was noted in preparing EDTA platelets at room temperature rather than at 4° C. The standard EDTA solution used is quite acidic (1.5% Na_2 EDTA, pH 3.85), although the final pH of the anticoagulated blood is about 7.40. It seemed possible that exposure of the first 200 to 300 ml of shed blood to an acidic, concentrated anticoagulant solution might affect platelet viability. When a neutralized EDTA solution was added gradually from a side-tube as blood was drawn from the donor, however, no improvement in platelet survival was observed. Thus, surface-active agents, tissue thromboplastin, temperature, and the effect of a high initial concentration of hydrogen ions or anticoagulant seem to be eliminated from consideration.

Development of a citrate-labeling technique. The possibility that the anticoagulant used, EDTA, might in itself be injurious to platelets was suggested by the *in vitro* observations of

Zucker and Borrelli (16). These workers showed that EDTA reduces the ability of platelets to retain their normal disc-like configuration and causes them to be converted to spheres. Other anticoagulants such as oxalate, citrate, and heparin do not readily cause "sphering" but have not been used for Cr^{51}-labeling studies because in the presence of these substances platelets adhere to one another when centrifuged. In our own attempts to use the usual ACD anticoagulant,[11] platelet clumping occurred as expected, causing much of the platelet radioactivity to be lost in the blood filter. Only 30% of the transfused platelets were viable. *In vitro* studies were then undertaken which led to the observation that a reduced pH results in a marked decrease in platelet adhesiveness. As long as the pH of platelet-rich plasma was less than 6.8, platelets could be resuspended easily after centrifugation. Between pH 6.8 and 7.2, some clumping occurred. At a pH greater than 7.2, platelets adhered to each other and even to the walls of the centrifuge tube. Platelet adhesiveness was not affected when the molarity of the citrate was varied between final concentrations of 14 and 60 mmoles per L, but rather seemed to depend on pH. Under phase microscopy, acid-citrate platelets were disc-shaped. After centrifugation and resuspension they transiently assumed a spherical form but reverted to discs in 1 to 2 minutes. After repeated centrifugations, aberrations in platelet shape occurred, and sickle-shaped and elongated forms developed. When the temperature of acid-citrate platelets was reduced to 5° C, they became spherical in 10 to 15 minutes, and slight clumping sometimes occurred that could be reversed by warming to 37° C (16). Aberrant forms appeared after one such treatment, however, and increased with repeated cooling and warming.

Survival of citrate platelets in normal subjects. When citrate platelets were transfused to normal persons, their survival pattern differed from that of EDTA platelets in two respects: platelet via-

[11] N.I.H. formula A, 75 ml + 500 ml whole blood. The pH of the separated platelet-rich plasma is approximately 7.1.

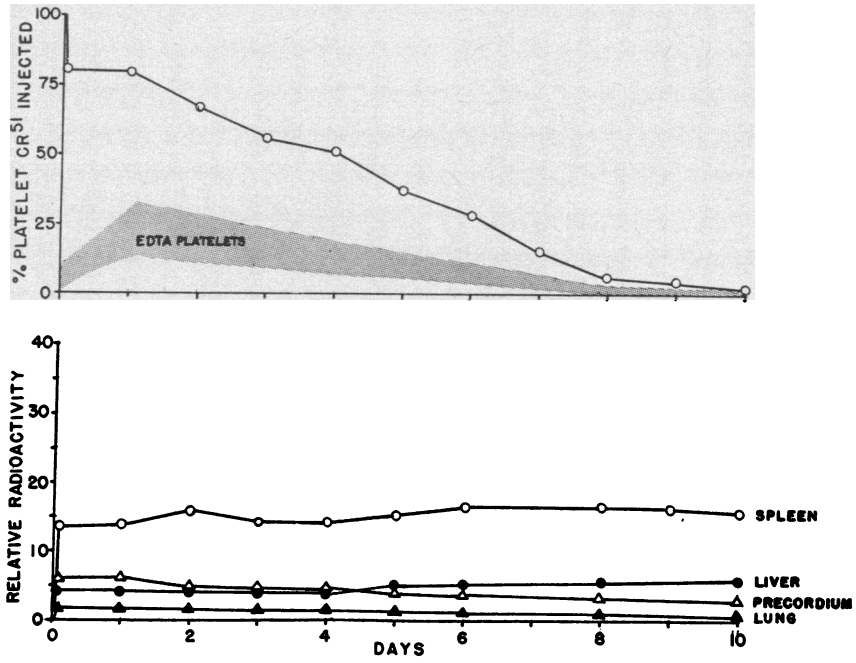

FIG. 3. THE SEQUESTRATION OF "CITRATE PLATELETS" DURING THE 10-DAY PERIOD AFTER INJECTION INTO A NORMAL SUBJECT. There was no acute rise in liver radioactivity immediately after injection. The shaded area of the upper portion denotes the range of blood platelet radioactivity after the injection of Cr^{51}-labeled "EDTA platelets" on 10 occasions in 7 normal subjects.

bility as measured by Cr^{51} recovery was much greater (Figure 3), and temporary sequestration of platelets was either absent or minimal, involving less than 10% of the total platelets infused and lasting only 30 to 60 minutes. Destruction of the nonviable fraction of transfused platelets was complete in less than 30 minutes. After 90 minutes, circulating platelet Cr^{51} activity was stable. It then declined steadily for 8 days except for a plateau sometimes observed during the first 24 hours. On each of 17 occasions, the disappearance curve of Cr^{51}-labeled platelets was a straight line, as was the case with EDTA platelets. Again, a small fraction of transfused platelets survived 10 to 14 days. In normal persons studied, platelet radioactivity was usually at maximal levels 2 hours after transfusion.

In 23 autologous platelet transfusions given to eleven normal subjects using citrate platelets labeled by the above technique and variations thereof, recoveries ranged from 36 to 85% of the transfused platelets. The average recovery in all studies with citrate was 62%. More recent studies (five patients) using the technique described in the Methods gave recoveries from 62 to 85% with an average of 72%. In general, donors with high platelet counts (greater than 400,000 per mm^3) gave the highest recoveries. Various alterations of the Cr^{51}-labeling technique that were used in early studies appeared to affect platelet viability. Reduction of pH to 6.0, decrease in the molarity of citrate used in the anticoagulant to 14.0 mmoles per L, labeling of platelets in isotonic NaCl, or failure to adequately separate erythrocytes and leukocytes before preparing platelet concentrates resulted in lower recoveries of transfused platelets. Variation of pH between 6.45 and 6.7, increase in molarity of citrate to 30 mmoles per L, or omission of glucose or ascorbic acid did not appear to affect viability. These findings should be interpreted with caution because the number of observations is not great enough for statistical analysis.

The effect of temperature on platelet viability was studied more systematically. In each of four subjects, autologous platelets were transfused on several occasions by the same technique except that wherever possible platelets were kept at 4° C in one experiment and at room temperature in the other. This eliminated factors peculiar to the platelet donor or recipient. Table I shows that a lesser recovery was obtained on 4 of 5 occasions when platelets were handled at 4° C. In one experiment where platelets were kept at 37° C wherever possible, recovery was reduced 15% below that achieved at room temperature.

TABLE I

*Viability of "citrate platelets" related to temperature maintained during the period of labeling in normal subjects**

Subject	Temperature	
	4° C	Room temperature
1	60	85
2	57	75
3	57, 55	62, 60
4	69	65

* Values are the percentage of transfused platelets circulating 2 hours after injection.

Organ radioactivity after transfusion of citrate platelets to normal subjects. In normal subjects after transfusion of citrate platelets, changes observed in organ radioactivity were different than with EDTA platelets. Corresponding to the removal of nonviable platelets from the circulation there was a rise in splenic radioactivity but very little increase over the liver or lungs. This suggested that damage to platelets caused by the labeling procedure was perceived chiefly by the spleen (Figure 3). Furthermore, in a splenectomized patient a recovery of transfused platelets of greater than 90% was achieved, the highest value of any experimental study (Figure 4). The survival curve was normal in this patient. In normal subjects over the subsequent 8 days, splenic radioactivity changed very little, whereas hepatic activity usually increased about 50% above the initial value. Precordial and pulmonary activity always declined. A pattern typical of 10 normal subjects is given in Figure 3.

To determine more quantitatively the extent to which normal platelets underwent sequestration in either the liver or the spleen, the rate at which platelet Cr^{51}, once deposited, was eliminated from these organs was determined in five subjects over a period of 20 to 30 days after circulating Cr^{51}-labeled platelets had disappeared from the blood stream (Figure 5). The decrease in Cr^{51} activity from each organ was approximately first order with time. The "biological half-life" of Cr^{51} in the spleen ranged from 20 to 30 days (average,

26 days), giving an average decay constant of 0.027 days^{-1} (2.7% per day). Removal of Cr^{51} from the liver was slower and more variable, with a half-disappearance time ranging from 25 days to 60 days. The biological half-life observed for platelet Cr^{51} is consistent with that reported for red cell Cr^{51} deposited in the liver or the spleen [biological decay constant (k) = 2 to 4% per day] (12, 17, 18).

The effect of EDTA on platelet viability. That platelets prepared in citrate at pH 6.5 were not appreciably sequestered after transfusion and that their viability was increased two- to threefold over that of EDTA platelets suggested that acid-citrate more effectively opposed the lesion of collection, or that EDTA per se was harmful, or both. Comparisons of the two anticoagulants were made at various pH levels. Citrate at pH 7.4 prevented the sphering effect of EDTA for 1 to 2 hours, whereas, at pH 6.5 in citrate, EDTA was almost totally ineffective in producing sphering. Platelet viability under these conditions was then studied in normal subjects. In the first experiment, sodium citrate was added to the usual EDTA anticoagulant to give a 0.022 M citrate concentration when 500 ml of whole blood was added. The final pH was 7.3. The platelets were labeled with Cr^{51} in the usual way, and at the time of transfusion, about 30% were in the disc form. A typical EDTA survival pattern was observed with 90% of platelets being temporarily sequestered and only 34% remaining viable. In two other experiments EDTA in the usual anticoagulant concentration was added to the ACD mixture. The plasma pH was 6.45. These platelets were all disc-shaped at the time of transfusion. After transfusion, transient sequestration occurred but was less marked than with EDTA alone, since 25% of the transfused platelets were circulating 30 minutes later. The final platelet recoveries, albeit higher than in other studies where EDTA was used, were still low: 43% and 53%. Thus, EDTA appeared to impair platelet viability even at a low pH. A third study was done to determine whether the EDTA lesion could be reversed *in vitro* before transfusion. Platelets prepared in the usual way with EDTA were incubated in autologous acid-citrate plasma

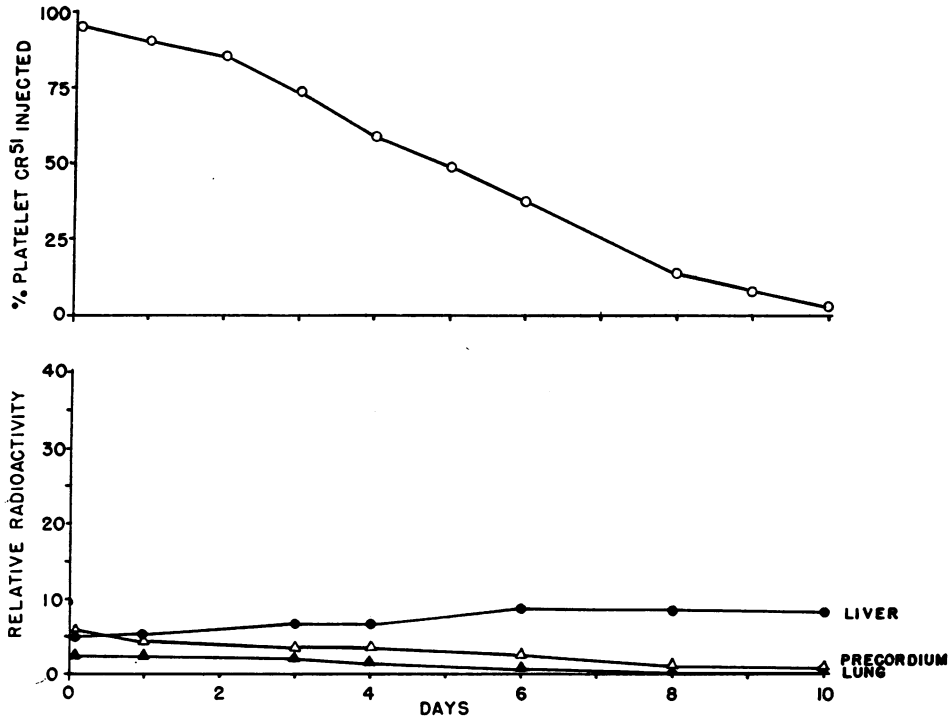

FIG. 4. THE SEQUESTRATION OF "CITRATE PLATELETS" DURING THE 10-DAY PERIOD AFTER INJECTION INTO A SPLENECTOMIZED SUBJECT.

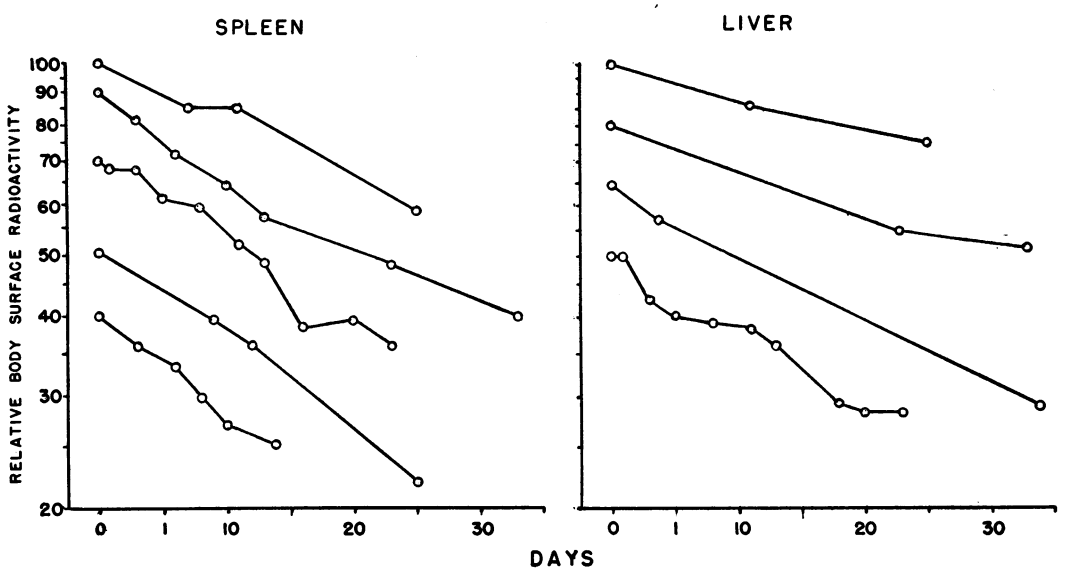

FIG. 5. ELIMINATION OF Cr^{51} FROM THE SPLEEN (left) AND LIVER (right). Sequestration of Cr^{51}-labeled platelets as determined by surface scanning in normal subjects. Initial values were assigned to prevent overlapping of the curves.

at pH 6.5 for 15 minutes before transfusion. During this time about 30% reverted from the spherical to the disc shape. After transfusion, however, 95% of these platelets were temporarily sequestered, and only 32% recovery resulted.

Validity of Cr^{51} as a platelet label. The following studies were carried out to determine whether the Cr^{51} activity recovered from the peripheral blood accurately measured the survival of transfused platelets.

Elution of Cr^{51} from platelets labeled with acid-citrate plasma did not occur *in vitro*. Platelets could be kept at 37° C for 4 hours or at 4° C overnight with no detectable loss of Cr^{51} activity. That the survival curves are straight lines strongly suggests that elution does not occur *in vivo*.

Transfer of label from transfused platelets to the recipient's own platelets also did not occur, since when all donor platelets were removed from the circulation after reacting with transfused isoantibody, no circulating platelet Cr^{51} activity remained (13).

Error could also result if the platelet Cr^{51} label were not homogenously distributed between viable and nonviable platelets. On 4 occasions when Cr^{51}-labeled platelets were transfused to thrombocytopenic patients it was possible to compare the actual increase in platelet levels determined by direct counting of platelets with the expected increase determined from Cr^{51} activity. Table II shows that the two values were in good agreement for six different determinations. The Cr^{51} technique apparently does measure the true survival of transfused platelets.

TABLE II

Increase in platelet levels after transfusion of Cr^{51}-labeled "citrate platelets" compared with the increase expected from circulating Cr^{51} activity

Patient	Base-line counts*	Increase in platelets by direct count	Increase expected from circulating Cr^{51} activity
1	46,000	24,000	20,000
2	3,000	35,000	30,000
3	15,800	7,400	6,500
4	10,000	68,000	70,000
		67,000	70,000
		62,000	66,000

* Based on a 3-day observation period for Patients 1, 3, and 4 during which platelet levels did not vary more than 10%.

Discussion

Effects of EDTA and citrate on platelet shape and viability. The EDTA lesion. Our results suggest that the sequestration and low recovery

of Cr^{51}-labeled platelets usually observed (2, 4, 19) are due to the use of EDTA as anticoagulant. The body surface scanning data show that this sequestration of platelets prepared in EDTA takes place in the liver. That EDTA might reduce platelet viability was suggested earlier by Kissmeyer-Nielsen, who observed that three transfusions given with EDTA blood produced a lower recovery of platelets than when blood was anticoagulated with ACD (20). These findings suggest that EDTA should not be used as an anticoagulant for the transfusion or preservation of human platelets.[12]

The effect of EDTA on platelets may be related to its affinity for magnesium [13] so that this ion is unable to act as a co-factor for enzymes required to catalyze the transfer of cations across the cell membrane. The sphering effect of EDTA may be due to a reduction in active membrane transport. The superiority of the citrate anticoagulant could, on the other hand, be due to citrate's lower affinity for magnesium, which is of the same order of magnitude as that of adenosine triphosphate.[14] In the presence of citrate, then, high energy phosphate at the cell membrane could retain magnesium required for it to function effectively in cation transport. These assumptions are supported by platelets retaining their disc-like configuration when exposed to the magnesium salt of EDTA (25). That the situation may not be this simple is suggested by the observation that, *in vitro,* citrate protects platelets from the sphering effect of EDTA. This "protection" is partial at pH 7.4 and nearly complete at pH 6.5. It seems paradoxical that citrate, which can only reduce the concentration of magnesium ion still further, should assist platelets in retaining their shape in the presence of EDTA. The three *in vivo* studies presented indicate, however, that preservation of platelet shape in EDTA-citrate does not prevent EDTA from reducing platelet viability except for some improvement with platelets prepared at pH 6.5. This could relate to the fact that EDTA binds magnesium much less readily at a lower pH (22). The EDTA lesion could not be reversed by suspending platelets for 15 minutes in acid-citrate plasma, although many platelets changed their shapes to discs. On the other hand, acid-citrate platelets prepared at 4° C contained many spherical forms at the time of transfusion, yet little or no transient sequestration was observed, and recoveries were only slightly reduced. Morphologic changes alone apparently do not permit a prediction of the *in vivo* behavior of transfused platelets.

The effect of reduced pH on platelet adhesiveness and viability. The mechanism by which a lowered pH reduces platelet adhesiveness, permitting platelets to be centrifuged and resuspended, is unclear. At a low pH platelets do not readily undergo "viscous metamorphosis" (26) and are less sensitive to thrombin-induced aggregation (27). The latter effect may in part account for the decreased adhesiveness of platelets in citrate plasma at pH 6.5. A second possibility is that a reduced pH affects platelet metabolism as with erythrocytes, where it prolongs the retention of high energy phosphates (28). That breakdown of ATP may cause platelets to become adhesive is suggested by the fact that low concentrations of ADP cause them to aggregate (29, 30). Platelet adhesiveness is reduced very quickly, however, when the pH of citrate platelet-rich plasma is lowered to 6.5. Therefore, electrostatic changes at the platelet surface may also be involved.

Survival of citrate platelets in normal subjects. The survival curves of platelets transfused to normal persons have been shown by various techniques to be straight lines for the first 6 to 8 days (1–5). Cohen, Gardner, and Barnett pointed out, however, that with Cr^{51} the observed curve could also reflect the release of platelets from some site of sequestration (31). The present method of labeling eliminates transient sequestration and reduces the fraction of nonviable platelets, but the survival curves were still straight lines for the first 8 days in normal subjects over a wide range of platelet recovery values. These data support the concept that platelets are removed from the circulation chiefly by a process of senescence. There has been argument over the significance of the "break" in the curve that appears at about 8 days, allowing a few platelets to survive 10 to 14 days. This can be accounted for reasonably by

[12] With animal platelets, the choice of anticoagulant may be less important (21).

[13] Stability constant of magnesium EDTA $\cong 10^8$ at pH 7.4 (22).

[14] Stability constant of magnesium citrate $= 10^{3.2}$ (23) and of magnesium ATP, 10^4 (22, 24).

assuming a certain variability in the predestined lifespan of individual platelets as predicted by Dornhorst for red cells (32). Platelet survival curves that are approximately first order (random destruction) have been obtained by some investigators using P^{23} orthophosphate (9) or C^{14} serotonin (33). Recent reports have emphasized the hazard of labels that are subject to metabolic turnover and loss by the cell (34, 35).

Sites of platelet sequestration [15] *in normal subjects.* In animals there is suggestive evidence that platelets die in the reticuloendothelial system (6–8), but the site at which platelets are destroyed in man has not previously been determined. Najean and colleagues (19) attempted to find this site by using EDTA platelets and comparing radioactivity levels over the spleen and liver at 8 days with those observed at 24 hours. They felt that the slight changes observed do not warrant conclusions as to the site at which surviving platelets are destroyed in normal subjects.

With citrate platelets, less activity appeared initially in the liver and spleen, and a relatively greater amount of radioactivity remained in circulating platelets. Therefore, any gradual accumulation of circulating platelets in an organ was more readily apparent. As in Figure 3 there was always a decrease over the lungs paralleling that over the precordium in normal subjects. Since studies in patients with pulmonary hemosiderosis have shown that pulmonary sequestration of labeled cells can be demonstrated with Cr^{51} (36, 37), it seems unlikely that the lungs are an important site of platelet sequestration. The changes in the spleen were variable, but there was never more than a 30% increase or a 10% decrease in radioactivity. This suggested that a small percentage of platelets might be sequestered in the spleen. That the amount is not large was indicated by later studies where a 200 to 300% increase in splenic radioactivity was observed after circulating platelets were destroyed there upon reacting with a specific isoantibody (13). The liver presented a more difficult problem because only a small part of its volume could be collimated with an external scanner, and hepatic blood flow represented a significant fraction of the surface radioactivity. In all patients, liver radioactivity increased to a value 10 to 70% above the initial level. This occurred despite a decrease of whole blood radioactivity and biological removal of any Cr^{51} already stored in the organ. Subsequent experiments in which known amounts of Cr^{51}-labeled platelets were selectively deposited in the liver and spleen (13) made possible the correlation of the surface activity as determined by a standard scanning technique with the number of microcuries of Cr^{51} actually present in these organs. In the liver, 1 μc of Cr^{51} produced about 134 cpm over the surface, whereas 1 μc in the spleen produced about 512 cpm. This indicates that splenic radioactivity was detected about 4 times as efficiently as liver radioactivity with the collimation involved. Similar observations have been made with Cr^{51}-labeled red cells (17, 18, 38). Differences observed in the ratio of spleen : liver counts per minute probably result from variability in the equipment used, particularly the collimation angle of the scanning device. Factors used for converting body surface activity to quantitative organ uptake are crude and do not apply in patients with abnormal organ sizes or body builds. With these reservations it was possible to estimate from external scanning the radioactivity present in the liver and spleen of normal subjects 8 days after the transfusion of citrate platelets. When appropriate corrections were made,[16] we found that the Cr^{51} present in these

[15] In the following discussion, "sequestration" refers to removal of platelets from the circulation subsequent to the 2-hour period after transfusion.

[16] Studies with Cr^{51}-labeled red cells showed that radioactivity over the spleen caused by the blood flow through the organ itself was very small compared to that caused by deposition of nonviable Cr^{51}-labeled platelets. Therefore, no correction was introduced for this factor.

Surface scanning done 2 hours after transfusion of citrate platelets showed that 80 to 100% of the nonviable platelets could be accounted for in the spleen. If the biological half-life of this radioactivity is 26 days, giving a decrease of 2.7% per day (Figure 5), 20% would be removed in 8 days. Therefore, 80% of the 2-hour splenic activity was subtracted from the 8-day radioactivity to determine the net splenic radioactivity caused by platelet sequestration. As platelet Cr^{51} is deposited in the spleen throughout the 8-day period, it is probably also removed with the same biological half-life. The correction factor for this removal was derived as follows.

If an amount of platelet Cr^{51}, Po, is deposited in the spleen over time T and the deposition is linear with time (i.e., proportional to the over-all removal of platelet Cr^{51}

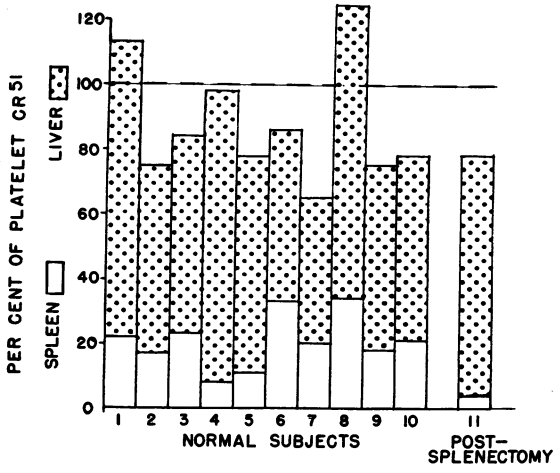

FIG. 6. THE RELATIVE SEQUESTRATION IN THE LIVER AND SPLEEN OF PLATELETS DURING THEIR PHYSIOLOGIC DESTRUCTION IN NORMAL SUBJECTS. These rough estimates are based upon experimentally derived factors for expressing body surface Cr^{51} activity quantitatively (see text).

two organs accounted for between 65 and 125% of the Cr^{51} known to have disappeared from the blood stream (Figure 6). The danger of calculating absolute radioactivity from external scanning in organs other than the thyroid is recognized. Nevertheless, the correlation between organ radioactivity and surface radioactivity holds fairly well in different normal subjects over a wide range (13), and the values in Figure 6 probably represent a good approximation to the true ones. Although areas such as the marrow and lymph nodes may play a role in the removal of effete platelets from the circulation, in man the majority of platelets seems to normally die in the reticuloendothelial cells of the liver.

from the blood), then the rate of deposition is $dp/dt = -Po/T$. At the same time, this radioactivity is being removed at the rate of the biological decay constant k. The net increment of radioactivity deposited in time interval dt that will still be present at time T is then $-Po/T\ e^{-k(T-t)}dt$, and the net amount of Cr^{51} in the spleen at time T is $\int_0^T -\frac{Po}{T} e^{-k(T-t)}\,dt = 0.92\ Po$, when $T = 8$ days and $k = 0.027$ days^{-1}.

This means the net increase in splenic radioactivity caused by platelet sequestration should be increased by 8% to obtain the true Cr^{51} deposition.

For the liver, the biological half-life was less well defined (Figure 5). The surface counts present at 8 days were converted directly to microcuries and entered in Figure 6.

Use of citrate at low pH in the preparation of platelet concentrates for transfusion therapy. In the past, platelet concentrates used for therapeutic purposes in thrombocytopenia have been prepared using either EDTA (39, 40) or the usual ACD anticoagulant (41–43). Although often therapeutically effective, they have given relatively poor recoveries in the recipients. In several large studies using ACD, less than 50% and often less than 25% of transfused platelets were circulating 2 hours after transfusion (42, 43). The low recoveries with ACD concentrates are very likely related to platelet clumping which, in our experience and in that of others (4, 21), almost invariably occurs, either microscopically or macroscopically, when the recommended ratio of anticoagulant : whole blood is used. Reduced recoveries of platelets in EDTA, on the other hand, probably are related to the direct deleterious effect of this compound on platelet viability. The present data indicate that when used at a pH of about 6.5, citrate abolishes platelet clumping *in vitro,* preserves platelet morphology, and permits most platelets to survive thereafter *in vivo.* Preliminary experience in the treatment of thrombocytopenic subjects has shown that such platelets are highly effective in producing hemostasis (44). Much more experience will be required, but it seems possible that this medium, or some modification of it, may find application in platelet transfusion therapy by improving either the efficiency or economy of such treatment.

Summary

Current methods of labeling platelets with chromium^{51} using ethylenediamine tetraacetate (EDTA) as an anticoagulant were found to cause rapid hepatic sequestration of nearly all the transfused platelets. About 50% returned to the circulation but then were partially destroyed in the spleen. Less than one-third survived. Evidence is presented which indicates that EDTA is injurious to platelets.

When an acid-citrate medium that buffered plasma at pH 6.5 was used as an anticoagulant, 36 to 85% of the labeled platelets survived (average, 62%), and marked temporary sequestration did not occur. Studies in thrombocytopenic persons showed that circulating platelet Cr^{51} activity

measures the true survival of such transfused platelets.

The survival and sequestration of platelets were studied in normal subjects. The survival data support the concept that platelets die chiefly by a process of senescence. External scintillation scanning of the various organs suggests that normally the majority of platelets is destroyed in the liver.

Addendum

Since this manuscript was prepared, Davey and Lander (Brit. J. Haemat. 1964, **10**, 94) also concluded that the initial sequestration of platelets prepared in EDTA occurs chiefly in the liver. They too observed a decline in hepatic radioactivity and an increase in splenic radioactivity during the first few hours after transfusion.

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