The Increased Effectiveness of Platelet Concentrates Prepared in Acidified Plasma

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PLATELETS concentrated in small volumes of plasma are required for freezing and storage, and for transfusion therapy where fluid restriction and multiple units are necessary. When blood is collected in acid-citrate-dextrose anticoagulant (ACD Formula A*), platelet concentrates (PC) prepared by centrifugation of platelet rich plasma (PRP) at 1500–4500 g are only 35–40 per cent as effective per unit as PRP.1 Such concentrates resuspend poorly and contain numerous macroscopic clumps which are trapped in the plastic pack and filter of the administration set. This loss of platelets is a significant factor in the decreased effectiveness of PC. The use of ethylenediamine tetra-acetic acid anticoagulant (EDTA) eliminates the platelet clumping. Such EDTA platelets disappear rapidly from the circulation after transfusion, are sequestered in the liver, and later recirculate with a maximum recovery of less than 30 per cent.2 Therefore, the need for an improved method of production of platelet concentrates remains.

Both mechanical and chemical factors appear to be involved in platelet adhesion during manipulation. Repeated centrifugation of platelets releases increasing amounts of a clumping factor shown to be adenosine diphosphate (ADP), and the adhesiveness of these platelets is increased.3 However, platelet concentrates prepared utilizing ADP aggregation give posttransfusion increments comparable to those following transfusion of PRP.4 When ADP is added to PRP in a final concentration of 10 µg./ml, the macroscopic platelet aggregates formed are efficiently removed from the plasma by centrifugation at 50 g for 5–10 minutes. The reduced gravitational force may be important in the success of these concentrates, for the platelet aggregation is reversible in this situation. Although ADP concentrates give good platelet recovery, a method which does not require additives other than those for anticoagulation would be more acceptable.

Using a Cr51 label Aster and Jandi have reported that platelets handled in an acid medium resuspend without clumping and circulate well following transfusion.2 At a plasma pH of 6.5 irreversible clumping is prevented despite the use of high gravitational forces. We have, therefore, undertaken a study of
the effect of acid pH on platelet aggregation by ADP. A method of preparation of platelet concentrates from fresh whole blood which employs an acid medium to reduce adhesiveness is described. Finally, the results of a transfusion study are reported in which such concentrates are compared to PRP and PC.

MATERIALS AND METHODS

Platelet Rich Plasma (PRP)

The method of collection, preparation and transfusion of PRP from whole fresh blood collected in ACD-A anticoagulant has been previously described.

Platelet Concentrates

Acid concentrates (AC) were prepared by two methods. In method A, 0.25 molar citric acid was added to PRP in a ratio of 1.0 cc. to 100 cc. PRP. The individual units of PRP were then centrifuged (International PR-2 centrifuge) at 2500 rpm (1500 g) for 15 minutes at room temperature. The supernatant plasma was expressed, leaving approximately 25 cc. of native plasma for platelet resuspension. Five such concentrated units were pooled for transfusion. Platelet counts were done on the PRP, platelet poor plasma (PPP) and pooled concentrates. In method B, the same amount of citric acid was added and the PRP was centrifuged at 2500 rpm for 20 minutes. The PPP was expressed, leaving approximately 20 cc. for platelet resuspension in 4 units and 50 cc. in the fifth. During the pooling procedure the 50 cc. concentrate was used as a "wash" in combining the other 4 units in an attempt to reduce platelet loss in the bags and tubing. Platelet counts were performed on the PRP, PPP and on duplicate samples of the pooled concentrate. Standard concentrates (PC) were prepared by method A without the addition of citric acid.

Transfusion Study

To evaluate posttransfusion recovery and viability of AC, 10 patients with thrombocytopenia received 13 sets of transfusions, including PRP and PC as controls. The 3 different preparations were given in random order to each recipient; each type was given first, second, and third in the set an equal number of times. An additional group of 16 AC transfusions was given controlled only with PRP. Platelet counts were done on the recipients prior to transfusion, 1 hour posttransfusion and daily in the morning thereafter. The median pretransfusion count was 16,000/mm.3 (range 4000–28,000) for all transfusions with similar values for each type of preparation. Transfusions were repeated upon return to the original baseline level. The posttransfusion increment was calculated from the 1-hour count using the formulation: Increment per 10^11 platelets transfused multiplied by the square meters of body surface area (Inc./10^11/M2) as previously described. The calculated increments were based on the total number of platelets in the PRP prior to concentration in all instances and therefore include all platelet losses incurred during manipulation. A second calculation in the case of AC was made using the actual platelet count of the pooled concentrate (AC corrected). Platelet clumping precludes an accurate count in the case of PC. Body surface area (BSA) was computed from a nomogram to the nearest 0.1 M2. For the adult patients studied the median BSA was 1.7 M2 with a range 1.5–1.9 M2; for the children the median was 0.9 M2 and the range 0.7–1.0 M2. Blood volume was estimated to be 2500 ml./M2 BSA.

The survival of circulating platelets posttransfusion was evaluated by calculating the percent of maximum increment remaining on the first and second day posttransfusion. Only those transfusions were included which resulted in a posttransfusion platelet count at least double the pretransfusion count and greater than 20,000 platelets.

ADP Aggregation

The effect of acid medium on the aggregation of platelets in vitro was evaluated by a turbidimetric method using a photonephelometer. To adjust the pH, two-tenths ml. of the
EFFECTIVENESS OF PLATELET CONCENTRATES

appropriate concentrations of citric acid or lactic acid was added to 10 cc. aliquots of PRP, followed in 1 minute by ADP in a final concentration of 5 \( \mu \)g/ml. To increase the level of ionized calcium, calcium chloride dihydrate in the proper aqueous dilution was added with the excess acid. The photonephelometer measures light reflected by particles in suspension. As the particle size increases, less light is reflected. The degree of aggregation is reported in nephelometer units from 0–100, where 100 units represents the maximum dispersion of light by PRP and zero units represents the dispersion by PPP.

**pH Determination**

The pH changes resulting from the addition of citric acid to PRP for clinical use were done 4–6 hours after collection (Beckman Model 76 Expanded Scale pH Meter) at 4 C. Determinations were done on fresh whole blood, PPP following addition of citric acid and removal of platelets, and whole blood following reconstitution of the packed RBC with acidified plasma. pH determinations in the in vitro studies were performed at room temperature (Beckman Glass Electrode pH Meter Model G).

**RESULTS**

As PRP is rendered more acid by the addition of excess citric acid, the aggregating effect of ADP is progressively inhibited. A similar effect is obtained with lactic acid using sufficient amounts to produce a comparable change in pH (Fig. 1). The addition of these acids to plasma at the time of maximum ADP aggregation results in accelerated reversal of platelet clumps. Below pH 6.7–6.8 aggregation is markedly inhibited and further addition of acid produces little increase in inhibition.

The addition of subclotting amounts of calcium to citrated plasma enhances ADP clumping, the point of maximum aggregation, as recorded in the nephelometer, occurring in 2–6 minutes. The clotting time after recalcification of PRP is greater than 10 minutes and it is possible to evaluate platelet aggregation prior to clot formation. The degree of ADP aggregation is always reduced at acid pH even in the presence of sufficient calcium to result in clot formation (Table 1). The addition of calcium can only partially reverse the inhibition by acid.

At 24 C, the addition of 0.25 molar citric acid (pH 1.8) to PRP in the ratio of 1.0 cc./100 cc. plasma lowers the pH from 7.0 to 6.5. This proportion of acid was used in the production of acid concentrates where the centrifugation takes place at 24 C. Assuming a hematocrit of 50 per cent and a volume of PRP of 200 cc, the excess citric acid increases the final molarity of citrate by less than 6 per cent. The pH of whole blood stored at 4 C prior to the addition of excess citric acid is approximately 7.1. Following the return of acidified PPP (pH 6.5) to the packed RBC, the resulting drop in pH of the whole blood minus platelets is less than 0.1 pH unit. The effect of the excess acid has been diluted and buffered by the volume of RBC.

Figure 2 shows the platelet yield from individual units of acidified PRP after centrifugation for 15 minutes as in Method A. The median yield of platelets from the single units is 86 per cent, giving a predicted yield of 85 per cent for a 5-unit transfusion prior to pooling. Another 10 per cent of the platelets is lost in the pooling procedure (Observed Recovery Acid Concentrate). By increasing the duration of centrifugation to 20 minutes (Method B), 92 per cent of the platelets are recovered in the single units. In Method B the use of a plasma
"wash" during pooling did not result in improved conservation of platelets. The final yield of platelets from PRP in pooled concentrate form was 76 per cent (Method A) and 83 per cent (Method B).

The final volume of the platelet concentrate from 5 units varied from 90 to 130 cc. This amount of acid plasma could be administered in 15 minutes or less without untoward effect. Concentrates previously made at pH 6.1 had elicited local pain on injection, and one instance of possible phlebitis was recorded. At pH 6.5 these local symptoms were not encountered. No effort was made to match ABO types for concentrate administration, but pooled units were always of a single type.

Figure 3 shows the result of 13 controlled sets of transfusions in which each patient received PRP, PC and AC. The median increment for PRP was $12,500/10^{11}/M^2$ which agrees closely with past experience. For AC the median was $10,000/10^{11}/M^2$, compared to $2500/10^{11}/M^2$ for PC. At a 95 per cent confidence interval the difference between AC and PC was significant; the difference between PRP and AC was not. The calculated increments for AC using the actual concentrate platelet count (AC corrected) have a median value of $17,000/10^{11}/M^2$. Because of the superiority of AC, no further PC’s were given and 16 additional AC transfusions were controlled only with PRP. For the total 29 transfusions the AC’s ($11,000/10^{11}/M^2$) again compare favorably with PRP ($12,500/10^{11}/M^2$), as shown in Figure 4. The median increment of AC corrected ($15,500/10^{11}/M^2$) is higher than that of PRP but not significantly
Table 1.—Maximum Platelet Aggregation in Nephelometer Units

<table>
<thead>
<tr>
<th>Final Conc. of Excess Ca**</th>
<th>pH</th>
<th>6.5</th>
<th>6.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>53</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>.005 Molar</td>
<td>55</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td>.01 Molar</td>
<td>56 c</td>
<td>33 c</td>
<td>18 c</td>
</tr>
<tr>
<td>.02 Molar (ADP 5 µg./ml final conc.)</td>
<td>65 c</td>
<td>42 c</td>
<td>25 c</td>
</tr>
</tbody>
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Maximum ADP aggregation in nephelometer units as affected by changes in calcium concentration and pH. ADP added in 5 µg./ml final concentration. Plasma acidified with lactic acid.

different. There was no detectable difference related to the two methods of concentrate preparation. For each adult transfusion the mean platelet dose per M² of BSA was: PRP—2.3 × 10¹¹; AC—3.4 × 10¹¹; PC—3.7 × 10¹¹. Similarly, for the children the dose was: PRP—2.4 × 10¹¹; AC—7.0 × 10¹¹; PC—7.4 × 10¹¹.

Assuming a blood volume of 2500 cc. per square meter of BSA, the number of infused platelets recovered in the circulation was calculated for each transfusion. The median per cent recovery for PRP was 32 per cent; for AC, 29 per cent; for AC corrected, 39 per cent; and for PC, 5 per cent. The calculation of platelet survival on the first and second day posttransfusion as a per cent of the maximum increment is recorded in Figure 5. For PRP and AC, approximately one-third of the transfused platelets are lost in each 24-hour period and the half-life is 36 hours.

DISCUSSION

Platelet concentrates prepared in acid plasma are superior to concentrates prepared by standard methods and are 80–90 per cent as effective as PRP. This small difference can be accounted for by the platelets lost during the concentrating procedures. Calculated from the actual platelet count of the concentrate, the posttransfusion increment and circulating platelet recovery of acid concentrates appear equal to and possibly superior to those of PRP. The reasons for this possible superiority are not clear. Perhaps those platelets remaining in the PPP after spins of 1500 g are small, less viable cells which have poor recovery, giving reduced estimates of the effectiveness of PRP. The existence of microscopic clumping in either PRP or AC could result in underestimation of the number of platelets present. Successful infusion of these small clumps and their subsequent dispersion would result in overestimation of the platelet increment and recovery. Such minor clumping was seen occasionally in AC and may be of greater degree in this preparation than in PRP.

Aster and Jandl² have reported that platelets prepared in an acid medium for Cr⁵¹ tagging resuspend without clumping and have a median recovery of
Fig. 2.—Platelet yield from single units of platelet rich plasma (PRP) in Method A (1500 g for 15 minutes). Predicted yield is calculated for a 5-unit transfusion after centrifugation but prior to pooling and does not include the losses inherent in the pooling procedure. Observed yield is calculated from the actual platelet count of the final concentrate.

62 per cent. This recovery is superior to values reported in other methods. Their studies were done in normal subjects where platelet recovery might be expected to be higher than in thrombocytopenic subjects with and without obvious hemorrhage. A per cent recovery of 30-40 per cent as in the present study has been previously reported and cannot be directly compared to recoveries in normal volunteers. The posttransfusion survival noted here agrees with the previous finding that acidified platelets appear to be undamaged and circulate normally in vivo. There is no evidence of sequestration as with EDTA anticoagulant.

In the preparation of acid concentrates for radioactive labeling and for therapy in thrombocytopenia, Aster collected blood in an ACD solution containing citrate of 50 per cent higher molarity than ACD-A. The resulting drop in pH to 6.5 included the red blood cells. Any deleterious effect of excess acidity on the erythrocyte is avoided in the present method. The relatively small amount of excess citric acid added to PRP (less than 6 per cent increase in citrate molarity) is easily buffered by the red cell, giving a final whole blood pH that remains at pH 7.0. The addition of 7.5 cc. of ACD-A solution to 100
Fig. 3.—Platelet increment $10^{11}/m^2$ measured 1 hour after transfusion of acid concentrates (AC), platelet rich plasma (PRP) and standard concentrate (PC) controls. "AC corrected" represents the increment calculated from the actual platelet count of the acid concentrate.

cc. of plasma will also lower the plasma pH from 7.0 to 6.5. This represents an increase in citrate molarity of slightly less than 20 per cent in the final whole blood product when ACD-A is used in place of 0.25 molar citric acid. The same pH effect can be obtained with lactic acid which will not alter the level of ionized calcium in the whole blood. This may be an important consideration in the presence of citrate toxicity.

Three mechanisms have been suggested by which pH might affect platelet adhesiveness.2 At low pH platelets are less sensitive to thrombin-induced aggregation. Second, acid pH may reduce the metabolism of ATP, as in the erythrocyte resulting in less ADP release. Finally, change in pH may affect electrostatic forces at the platelet surface. As with thrombin, the aggregation of platelets by ADP is affected by change in pH. With an increase in acidity below pH 6.7–6.8, there is substantial but not complete inhibition of aggregation as recorded in the photonephelometer. The aggregating effect of ADP is calcium-dependent, and it is difficult to differentiate directly the pH effect from simultaneous changes in the level of ionized calcium. However, the dissociation of calcium proteinate in human serum is increased at acid pH.10 The dissociation of citric acid is such that the citrate ligand-metallic ion complex is reduced at acid pH.11 Both of these effects cause an increase rather
than a decrease in ionized calcium with increasing acidity. Two factors in this study would also support the conclusion that change in pH, although interrelated with change in ionized calcium, has another and different effect on ADP aggregation. First, equal inhibition at the same pH is obtained with both lactic and citric acids despite a difference in their binding of calcium. Second, the addition of excess calcium in the presence of lactic acid cannot overcome the pH effect through a complete range of calcium concentrations to the point of clotting. Therefore, inhibition of ADP aggregation is yet another factor in the decreased platelet adhesiveness in acid plasma.

In the preparation of standard concentrates, irreversible clumping is not found in all units. In fact, after vigorous rubbing during resuspension, only 40 per cent or less of the units remain clumped. Pooling of clumped and unclumped units tends to promote more generalized clumping. The variation in adhesiveness of platelets in different units of PRP is probably related to several factors, including (1) the final citrate concentration, (2) the plasma pH, and (3) differences in the release of ADP and thrombin. The addition of citric acid in the present method of handling platelets allows a margin of safety in regard to these factors and eliminates clumping in most units.

The preparation of platelet concentrates in acid plasma results in an excel-
Fig. 5.—Per cent of maximum platelet increment surviving on the first and second day posttransfusion comparing AC and PRP. Maximum increment ≥ 20,000 platelets and greater than pretransfusion count.

It remains to develop a practical method of preparation of such concentrates from fresh whole blood. Studies are underway in this respect in the use of increased ACD-A anticoagulant in blood collection. Perhaps an intermediate pH will result in satisfactory concentrates and yet remain acceptable for storage of whole blood. A modification of the method described may prove feasible. A selected amount of 0.25 molar citric acid (2.0 cc.) can be added to the second bag of a double pack and can be sterilized during manufacture. Directly after bleeding into the primary bag containing the standard amount of ACD-A anticoagulant, PRP is prepared by centrifugation at 1500 g for 3 minutes. Two-hundred cubic centimeters of this PRP is expressed into the second bag of the closed system containing the citric acid. An acid concentrate can now be made from the PRP, centrifuging at 1500 g for 20 minutes. The acid PPP, when expressed back into the red cells, will result in little change in pH, and a unit of blood will be acceptable for storage minus platelets. If ACD Formula A is used in place of the 0.25 molar citric acid, 15.0 cc./200 cc. of PRP is sufficient in the second pack to accomplish the same result.

SUMMARY

Platelet concentrates prepared in acidified plasma (pH 6.5-6.7) are superior to concentrates prepared by standard methods, and are 80-90 per cent as effective as platelet rich plasma (PRP). The use of excess citric acid to acidify
plasma promotes resuspension of the concentrate by eliminating clumping, which is a major factor in the decreased effectiveness of standard concentrates. Analysis of posttransfusion recovery and survival of platelets reveals no evidence of platelet injury in an acid medium.

Acidification of PRP inhibits the aggregation of platelets by adenosine diphosphate (ADP). The presence of endogenous ADP may be an important factor in clumping during standard concentrate preparation.

A method of acidification of PRP using citric acid is described which allows preparation of an effective concentrate from fresh whole blood without subjecting the red cells to acid pH. Reconstitution of the acidified platelet poor plasma and its native red cells increases the citrate molarity by less than 6 per cent and results in minimal decrease in pH of the whole blood.

SUMMARIO IN INTERLINGUA
Concentratos de plachettas preparate in plasma acidificate (a un pH de 6,5 a 6,7) es superior a concentratos preparate per methodos standard. Lor efficacia amonta a inter 80 e 90 pro cento de illo de plasma nc in plachettas. Le uso de un excesso de acido citric pro acidificar le plasma promove le re-suspension del concentrato per eliminar le formation de aggregatos lo que es un factor major in le reduce efficacia de concentratos standard. Le analyse del restablimento e del longevitate plachettal post le transfusion revela nulle evidentia de un lesion del plachettas in un medio acide.

Le acidification de plasma ric in plachettas inhibi le aggregation de plachettas per diphosphato de adenosina. Le presentia de endogene diphosphato de adenosina es possibilemente un factor importante in le formation de aggregationes durante le preparation standard del concentratos.

Es describite un methodo pro le acidification de plasma ric in plachettas, le qual utilisa acido citric e permite le preparation de un efficace concentrato ab sanguine total fresc sin que le erythrocytos debe esser subjicite a un pH acide. Le reconstitution del acidificate plasma a paucitate de plachettas e de su erythrocytos native augmenta le molaritate citratic per minus que 6 pro cento e resulta in un minime declino in le pH del sanguine total.

ACKNOWLEDGMENT
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