Effect of Anticoagulant and ABO Incompatibility on Recovery of Transfused Human Platelets

By Richard H. Aster

PLATELET-RICH PLASMA and platelet concentrates are frequently effective in the treatment of hemorrhage secondary to thrombocytopenia. The recovery of transfused platelets in the recipients is often low, however, the average ranging from 20–33 per cent in several series.

Previous work in this laboratory has shown that a citrate anticoagulant which buffers platelet-rich plasma (PRP) at pH 6.5 improves the viability of concentrated platelets by facilitating their resuspension without clumping. The same study suggested that EDTA, a substance commonly used for platelet transfusions, was toxic for these cells. This work implied that acid citrate was a superior anticoagulant for platelet transfusion therapy, but special manipulations were required to label the platelets with Cr51. In the present study, the effects of different anticoagulant solutions on platelet viability were investigated, utilizing technics comparable to those employed under clinical conditions.

Although A and B blood group antigens have been shown to exist on the platelet membrane, their importance for platelet transfusion therapy has not been clarified. It has been suggested that the recovery of group A or B platelets transfused to group O recipients is equal to that of group O donors. Other workers found a “slight, but consistently lower” recovery of “incompatible” platelets. In part, difficulty in assessing the role of ABO antigens in platelet transfusions may have arisen from the relatively low maximum platelet recovery achieved in most studies. The availability of a Cr51-labeling technic that gives a high recovery of platelets permits differences in the recovery of incompatible platelets to be more readily apparent. The use of normal subjects as platelet recipients eliminated variables such as hemorrhage and isoimmunization which might obscure the effect of ABO incompatibility in ill, thrombocytopenic subjects.

MATERIALS AND METHODS

Platelets were labeled with Cr51 and their recovery in recipients was calculated as described previously, with the following exceptions:

1. In most studies the anticoagulant mixture was prepared by adding 0.15 molar citric acid. From the Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

This work was supported by research grants HE-07652-01 and T1-AM-5391-01 from the National Institutes of Health, Bethesda, Md.

First submitted January 5, 1965; accepted for publication March 13, 1965.

Richard H. Aster, M.D.: Research Associate, Thorndike Memorial Laboratory, Boston City Hospital; Associate in Medicine and Tutor in Medical Sciences, Harvard Medical School, Boston, Mass.
acid directly to the collecting bag containing ACD formula A, rather than preparing fresh anticoagulant on each occasion. The amount of citric acid which produced the required pH of 6.5 when 500 ml. of whole blood was added to the anticoagulant mixture varied with the hematocrit of the donor due to the high buffering effect of red blood cells (RBC). The volume of citric acid required is given as a function of the donor hematocrit in figure 1.* Citric acid was autoclaved and stored at 5 C. for up to 6 months. The average recovery and range of recoveries of autologous platelets in vivo was the same as when freshly prepared anticoagulant was used.

2. During the labeling procedure, platelets were centrifuged at 14 C. rather than at room temperature.

3. After incubation of platelets with Cr51, centrifugation and removal of the supernatant plasma containing excess Cr51, the platelet button was gently covered with 5-10 ml. of platelet-poor plasma (PPP). This plasma in turn was poured off in order to remove remaining Cr51 not attached to cells. Platelets were then resuspended in PPP and injected. On the average, 95 per cent of injected Cr51 was attached to platelets.

4. High specific activity of platelets used for serial-transfusion experiments (see "Results") was achieved by incubating the concentrated platelets from 500 ml. of blood in a small (10-15 ml.) volume of plasma and adding 1 millicurie of Cr51 rather than the usual 300 microcuries. The Cr51, which is suspended in distilled water in the commercial preparation, was made isotonic by the addition of sterile 3 per cent sodium chloride before adding to platelets. In this way 50-100 microcuries of Cr51 could be incorporated into transfused platelets.

Recoveries of transfused platelets were based on the average of at least 2 determinations done 1-4 hours after transfusion except with "EDTA platelets," where the maximum value was used. This maximum sometimes occurred 24 hours after injection.

In some studies of the effect of ABO-incompatibility on platelet recovery, labeled platelets were divided into 4-6 aliquots which were given to an equal number of recipients. In this way the recovery of the same labeled platelet preparation could be compared in both ABO-compatible and ABO-incompatible recipients. When aliquots of the same platelet preparation were given to different ABO-compatible recipients, the range of recoveries was the same as for autologous platelets.

Body surface scanning was performed as previously described.7'12 Units of surface radioactivity (fig. 4) were standardized by dividing the number of counts per minute observed at the surface by 10 times the number of microcuries of Cr51 injected.

Isoagglutinin titers were determined by incubating 1 drop of 2 per cent washed RBC's with 3 drops of serially diluted serum for 30 minutes at room temperature. Agglutination was read after centrifugation for 20 seconds in a serologic centrifuge.

Subjects studied were normal volunteers or volunteer convalescent patients on the medical wards who did not have hematologic disease or diseases of the liver or spleen. Twenty-four of the 41 platelet recipients had not been transfused previously. The remainder had not received more than two transfusions each, and none had received blood during the previous two months. There was no difference in the transfusion history of subjects receiving ABO-incompatible platelets in comparison with those receiving compatible platelets.

RESULTS

Effect of Anticoagulant on Recovery of Transfused Platelets

Autologous platelets labeled with 50-100 μC. of Cr51 in the acid citrate anticoagulant were reinfused into the same normal donors. One day later, when 50

*Concentration of citrate or dextrose does not appear to be crucial for satisfactory results, but a pH of 6.5 is necessary before centrifugation of platelet-rich plasma. This can be achieved in a number of ways, including addition of acid to PRP after separation of red blood cells.
Fig. 1.—Relationship between donor hematocrit and ml. of 0.15 M citric acid added to 75 ml. NIH formula A ACD solution which produced pH about 6.5 in platelet-rich plasma. Values are for 500 ml. of donor blood.

to 63 per cent of the transfused platelets were still circulating (initial recoveries ranged from 55 to 70 per cent), 500 ml. of whole blood was obtained using the following anticoagulants:

a. 50 ml., 1.5 per cent EDTA in 2 per cent dextrose, final pH of platelet-rich plasma (PRP) 7.3 (3 subjects).

b. 95 ml., 0.15 M trisodium citrate in 2 per cent dextrose, final pH of PRP 7.4 (1 subject).

c. 75 ml., NIH formula A ACD plus 22 ml. 0.15 M citric acid, final pH of PRP 6.5 (1 subject).

Using procedures normally employed in platelet transfusion therapy, the whole blood was centrifuged at 1300 r.p.m. for 15 minutes and the PRP was transferred to a separate pack. An aliquot was removed for determination of Cr\(^{51}\) activity and the remaining PRP, containing 2–5 \(\mu\)c of Cr\(^{51}\), was transfused to a normal ABO-compatible recipient. Total time between phlebotomy and infusion of PRP was one hour. Platelet recoveries and survival curves are shown in figure 2. With EDTA, most platelets were transiently sequestered after transfusion, and maximum recovery was 34 per cent. With either acid or neutral citrate there was no temporary sequestration, and 70 and 75 per cent of transfused platelets remained in the general circulation in the respective studies. There was no significant difference in platelet recoveries at 1 hour as opposed to 4 hours. These recoveries calculated from the platelet Cr\(^{51}\) activity in venous blood probably represent true recoveries of 90–100 per cent.
Fig. 2.—Effect of anticoagulant on recovery and survival of Cr\(^{51}\)-labeled platelets transfused as platelet-rich plasma without concentration. The pH is that of the platelet-rich plasma after separation from RBC.

of the transfused cells, for 20–30 per cent of platelets appear normally to enter a splenic platelet pool.\(^{13}\) In all subjects platelet disappearance was approximately rectilinear with time over the next 7 to 8 days.

**Effect of ABO Incompatibility on Platelet Recovery**

Recoveries of platelets given to ABO-compatible versus incompatible recipients are given in figure 3. In compatible recipients recoveries were about the same as those observed with autologous platelets (average 63 per cent). When platelets from group A\(_1\) donors were given to group O recipients, the average recovery was much lower (19 per cent). When group B platelets were given to incompatible recipients, the average recovery was 57 per cent. When group A\(_1\)B platelets were given to two group O recipients, the average recovery was only 8 per cent. In two subjects, a second transfusion of group A\(_1\) incompatible cells given one week and four weeks later, respectively, resulted in the same low recovery as did the first transfusions. Thus, there was no evidence that "tolerance" following the first antigenic exposure improved the recovery of subsequently injected incompatible platelets.

The removal of a large fraction of ABO-incompatible platelets from the circulation occurred within the first 10 minutes after transfusion, too rapidly
to be accounted for by purely splenic sequestration.\textsuperscript{14} There was no corresponding increase in plasma radioactivity to suggest that elution of the Cr\textsuperscript{51} label might have occurred rather than actual destruction of the platelets themselves. Body surface scanning in three patients showed an increase in hepatic surface radioactivity after the infusion of A\textsubscript{1} incompatible platelets which was greater than that observed in any patient after the infusion of ABO-compatible cells (fig. 4). No significant increase was observed over the lungs. With the scanning device used in these studies, a given increase in surface counts per minute (cpm) over the liver represents 3–4 times as much Cr\textsuperscript{51} deposited in the organ as does the same increase in surface cpm over the normal-sized spleen.\textsuperscript{7} The increases in hepatic radioactivity after transfusion of ABO-incompatible platelets suggest that most of the platelets were removed from the circulation in the liver. The reduced surface radioactivity over the spleens of these subjects presumably was due to the smaller amount of platelet Cr\textsuperscript{51} remaining in the general circulation and contributing to the splenic platelet reservoir.\textsuperscript{13}

After the initial rapid destruction of a fraction of the ABO-incompatible cells, the remaining platelets usually disappeared from the circulation normal-
Fig. 4.—Organ surface radioactivity after transfusion of ABO-compatible and incompatible platelets. Units on the ordinate are adjusted so that values are independent of the dose of radioactivity administered.

ly over the next 8 days (fig. 5). In two instances, slightly shortened survival times were presumed due to previous isoimmunization.

The higher recoveries observed after transfusion of group B incompatible platelets suggested that the level of isoagglutinins might be a factor determining platelet recovery, since anti-B isoagglutinins are usually of lower titer than anti-A isoagglutinins. Figure 6 shows that a rough correlation of this sort did, in fact, exist. In general, recoveries were low in recipients with high titers of anti-A or anti-B. Conversely, relatively high recoveries were found in recipients with low isoagglutinin titers. When group A,B platelets were transfused to group O recipients, the anti-A and anti-B isoagglutinins appeared to act additively in destroying transfused platelets. No relationship could be established between isohemolysin activity and recovery of incompatible platelets.

The normal survival manifested by platelets which survived the period immediately after transfusion suggested that some transient reaction between the infused cells and the recipient’s plasma at or near the site of injection might be responsible for the destruction of a portion of the transfused cells. For example, the small number of RBC present in the platelet concentrate would be expected
Fig. 5.—Survival of group A₁ platelets in group O recipients. (Dashed lines)
Initial recoveries are low, but platelets remaining in the circulation disappeared at
the normal rate in most subjects.

to react immediately with isoantibody after entering the recipient’s circulation,
and might cause platelets to become involved in mixed cell aggregates or might
activate plasma factors deleterious to platelets. Several experiments were per-
formed to study these possibilities. In one group O normal subject, autologous
platelets were labeled with Cr₅¹. 0.25 ml. of washed red cells from a group A₁
donor was then added to the suspension of labeled platelets before they were
reinfused into the original donor. Platelet recovery (58 per cent) and survival
was identical to that seen in the same subject without added RBC. In another
group O subject, the same experiment was performed except that purified A
substance,* derived from hog stomach and equivalent to approximately 4 ml.
of packed group A, RBC in antigen content (as determined by in vitro neu-
tralization of anti-A), was substituted for the group A₁ red cells. Again, no de-
crease in recovery was observed. On two other occasions, A substance equiva-
ient to 10 ml. of packed RBC was infused to group O recipients whose platelets
had been labeled one day previously with Cr₅¹, and reinfused. In these sub-

*Obtained from Michael Reese Research Foundation. Chicago, Illinois.
Fig. 6.—Relationship between recovery of ABO-incompatible platelets and homologous isoagglutinin titers.

jects, both circulating Cr\textsuperscript{51} activity and total circulating platelets were reduced by about 25 per cent of the preinjection levels. Simultaneously there was a small but significant rise in hepatic surface radioactivity, but no change over the spleen. The survival time of the remaining labeled platelets was not shortened.

**Discussion**

Since the studies of Dillard and coworkers in dogs and guinea pigs,\textsuperscript{1} EDTA has been used frequently as an anticoagulant for platelet transfusions. It is clear that this agent is satisfactory for the preparation of platelets from most animal species. Early workers suggested that EDTA might be the anticoagulant of choice for platelet transfusions in man, reporting platelet recoveries of 65 per cent\textsuperscript{2} and 75 per cent\textsuperscript{3} with transfused PRP, and 25 per cent\textsuperscript{3} and 52 per cent\textsuperscript{2} with concentrates.

The present data indicating that EDTA reduces platelet recovery in man were obtained by using a Cr\textsuperscript{51} label. It has been shown, however, that the Cr\textsuperscript{51} technic does measure the true recovery of transfused human platelets,\textsuperscript{7} and the results obtained are in very close agreement with those of Kissmeyer-Nielsen and Madsen,\textsuperscript{15} who used an in vivo phosphate label. The latter workers
found that platelet recovery after 3 transfusions of EDTA whole blood was 30 per cent, about one-half the recovery obtained when blood was anticoagulated with ACD. Recently, Freireich and coworkers have confirmed by direct platelet counts after transfusion of concentrates, that EDTA reduces the viability of transfused platelets to about one-half that achieved with citrate at pH 6.5.16

Figure 2 shows clearly that EDTA does reduce the viable fraction of transfused platelets to about half that obtained with citrate anticoagulants. Both neutral citrate and acid citrate gave comparable recoveries. Thus, the reduced recoveries with EDTA result from a direct toxic effect of this substance, rather than from the effect of handling platelets at a neutral rather than an acid pH. The number of observations are few, but the differences observed are so striking and so clearly consistent with previous work7,15 that it was felt that further experiments were not justified in view of the risks inherent in cross-transfusion studies. It is also apparent that EDTA is chiefly responsible for the temporary sequestration of platelets during the first few hours after transfusion, a phenomenon that has been assumed to be an inevitable consequence of handling platelets in vitro, and which is due to trapping of these cells in the liver.7,17 Thus, during the first few hours after transfusion to a thrombocytopenic recipient, circulating levels of platelets prepared in citrate may be expected to be 5-10 times greater than when platelets are prepared in EDTA. Figure 2 shows that if platelets are not concentrated, the pH of the citrate anticoagulant is of little consequence. If concentration is required, however, it is impossible to resuspend platelets in citrate at pH 7.4 because of adhesion between cells. With the usual ACD solution, which buffers platelet-rich plasma at about pH 7.1, suspension is easier, but aggregation, at least on a microscopic scale, remains a problem and reduces recoveries. At pH 6.5, concentrated platelets can be resuspended readily without detectable clumping.7 Recoveries of such platelets, even after two centrifugations and resuspensions, ranged from 50-80 per cent (fig. 3).

Preliminary studies strongly suggest that the ease with which concentrated platelets can be resuspended at pH 6.5 is due to interference with the action of adenosine-diphosphate (ADP), which is known to increase platelet adhesiveness at very low concentrations,18 and which is probably present in shed blood as a result of cell injury. At pH 6.5, the amount of ADP required to cause platelet aggregation in platelet-rich plasma is about 100 times that required at pH 7.4.19

The mechanism by which EDTA reduces viability of human platelets remains unclear. It is known that platelets rapidly assume a spherical configuration in EDTA,20 suggesting that this substance may interfere with ion and water transport across the cell membrane. Ironically, this very effect which smooths the platelet's shape may have stimulated the use of EDTA as an anticoagulant for transfusion purposes.

The Effect of ABO Incompatibility on Platelet Recovery

It is known that ABO incompatible platelets which remain in the circulation after transfusion survive normally.11,21 The effect of incompatibility on platelet
recovery has not been extensively studied, although Baldini, Costea, and Ebbe noted “slight, but consistently lower recoveries” of ABO-incompatible platelets in an undisclosed number of subjects. Freireich et al. found no effect of ABO groups on platelet recoveries in one patient under treatment for leukemia. Figure 3 indicates that incompatibility with respect to the ABO antigen system is capable of reducing the recovery of transfused platelets. This is particularly true when platelets from group A₁ or group A₁B donors are given to incompatible recipients. When platelet preparations were subdivided and given to several recipients, the dose of platelets was less than that which would be administered in the treatment of thrombocytopenia. However, on six occasions when an entire unit of platelets \((5 \times 10^{10} - 8 \times 10^{10})\) platelets) was transfused to a single recipient, the same low recoveries of incompatible cells were observed as when smaller numbers of platelets were used. Thus, the recoveries in figure 3 do not appear related to platelet dose. Figure 6 indicates that the recovery of incompatible platelets is inversely related to the corresponding isoagglutinin titer. It is apparent that this relationship is not a linear one.

The mechanism by which a fraction of ABO-incompatible platelets are destroyed is not yet clear. The normal survival of cells that escape destruction during the first minutes after transfusion suggests two possible explanations: (1) “A” substance may elute from a portion of the cells after transfusion or may be distributed nonhomogeneously on the platelets themselves. (2) Platelets may be secondarily injured following the reaction between red blood cells or “A substance” present in the transfusion mixture and isoantibody in the recipients’ plasma. The first alternative seems unlikely since “A substance” is very stable on platelets in vitro, and, if the label were unevenly distributed among cells, it would be surprising to find a relationship between antibody titer and platelet recovery. The second alternative gains support from the fact that platelets may be injured by the presence of unrelated antigen-antibody complexes in their vicinity. The present studies do not reveal the mechanism by which the recovery of ABO-incompatible platelets is curtailed. It is of interest, however, that infusion of “A” substance to group O recipients does produce hepatic sequestration of platelets and that a fraction of ABO-incompatible platelets also were destroyed in the liver after infusion (fig. 4). It may be that failure of “A” substance or group A₁ RBC added to group O platelets to affect recovery of such platelets relates to the fact that a limited range of antigen-antibody ratios produces a secondary type of platelet damage.

**Implications for Platelet Transfusion Therapy**

The present study indicates that for maximum effectiveness, ABO-compatible platelets prepared in a citrate anticoagulant should be used in the treatment of thrombocytopenia. If platelet-rich plasma is transfused, the composition of the citrate medium appears unimportant. If platelets are to be concentrated, the addition of sufficient citric acid to reduce the pH of platelet-rich plasma to about 6.5 will facilitate platelet resuspension and improve platelet recoveries.

It must be kept in mind that in thrombocytopenic patients, recoveries of transfused platelets may be reduced by other factors such as isoimmunization, hemorrhage, or the patient’s disease process itself.
SUMMARY

The effects of anticoagulant solutions on the recovery of transfused platelets were studied. Citrate anticoagulants at pH 7.4 or pH 6.5 were found to be equally effective in preserving the viability of platelets when centrifugation of the cells was not required. When centrifugation is required, as in most platelet survival studies, citrate at pH 6.5 gives maximum recoveries. Ethylene diamine tetraacetate (EDTA) caused temporary sequestration of nearly all transfused platelets and reduced maximum recoveries by about 50 per cent.

Platelet recovery was lowered by ABO-incompatibility between donor platelets and recipient serum, but survival time of remaining platelets was not altered. Lowest platelet recoveries resulted when group A_1 or A_1B platelets were given to group O recipients with high isoagglutinin titers.

It is suggested that ABO-compatible platelets prepared in citrate should be used wherever possible in the treatment of thrombocytopenic disorders.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the technical assistance of Miss Nancy Mann, Mrs. Una Tuck and Miss Hollister Nash.

REFERENCES

5. Djerassi, I., Farber, S., and Evans, A. E.: Transfusions of fresh platelet


Effect of Anticoagulant and ABO Incompatibility on Recovery of Transfused Human Platelets

RICHARD H. ASTER