Preservation of hemostatic and structural properties of rehydrated lyophilized platelets: Potential for long-term storage of dried platelets for transfusion

(platelet adhesion/platelet agglutination/thrombocytopenia/thrombosis)

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Contributed by Kenneth M. Brinkhous, September 22, 1994

ABSTRACT Currently, therapeutic platelet concentrates can be stored for only 5 days. We have developed a procedure that permits long-term storage of fixed and lyophilized platelets that retain hemostatic properties after rehydration. These rehydrated lyophilized platelets (RL platelets) restore hemostasis in thrombocytopenic rats and become incorporated in the hemostatic plug of bleeding time wounds of normal dogs as well as von Willebrand disease dogs with partially replenished plasma von Willebrand factor. Ultrastructurally, these platelets are well preserved and are comparable to control normal washed platelets. Flow cytometry analysis shows that RL platelets react with antibodies to the major surface receptors, glycoprotein (GP)Ib and GPIIb/IIIa. These receptors are involved in platelet agglutination, aggregation, and adhesion. *In vitro* functional tests document the ability of RL platelets to adhere to denuded subendothelium and to spread on a foreign surface. Circulating RL platelets participated in carotid arterial thrombus formation induced in normal canine subjects. The participation of RL platelets in these vital hemostatic properties suggests that with further development they could become a stable platelet product for transfusion.

To promote effective hemostasis, platelets must respond quickly to changes in normal blood flow or vessel injury (1, 2). After vascular injury, platelets adhere to exposed subendothelium, aggregate, and form a primary platelet plug. Platelet activation and initiation of coagulation follow with stabilization of the platelet plug by the formation of fibrin. The initiation of a thrombus at a site of vascular injury is mediated through platelet membrane glycoprotein (GP) receptors (3, 4). Platelet adhesion to a damaged vessel wall and its extracellular matrix at high shear is primarily mediated through the specific interaction of the platelet membrane GPIb-IX complex and bound von Willebrand factor (vWF) (5–7), which is synthesized and released into plasma and the vessel wall by endothelial cells (1). Platelet adhesion at low shear rates is mediated by several interactions, including collagen with the $\alpha_2\beta_1$ integrin (7). Platelet adhesion stimulates a spreading of the platelet (8). Although the mechanism of platelet spreading has not been completely characterized, recent *in vitro* studies have shown that platelets will spread on surfaces coated with fibrinogen (9) or polymerized fibrin (10). The activation of the GPIIb/IIIa receptor by agents such as ADP results in a conformational change in the receptor (11–13). The activated receptor binds fibrinogen, which forms a “bridge” between the platelets, and causes aggregation (1, 14, 15). Activated platelets provide the phospholipid surface for the assembly of blood clotting en-

zyme complexes, and the concentration and localization of activated coagulant proteins at sites of vessel wall injury may be facilitated by adherent platelets (16). Internal storage granules in platelets release clot-promoting contents in response to activation of biochemical systems triggered by platelet-platelet or other interactions. Interactions of adherent platelets with neutrophils, mediated through platelet integrins, specifically P-selectin receptor (17), may contribute to hemostatic and other cell functions (13).

The control of hemorrhage due to thrombocytopenia often requires transfusion of multiple units of fresh platelets. In transfusion medicine, platelets cannot be replaced by other blood products or artificial media. Maintenance of critical membrane GPs during storage is crucial to platelet function *in vivo*. With the storage life of fresh platelets limited to 5 days, there has been considerable study to lengthen platelet shelf life and enhance stored platelet response (18–24). Investigators have addressed storage conditions that preserve platelet integrity and responsiveness (25, 26). The effects of preservatives on platelet activation and expression of membrane GPs have also been investigated (27). In a recent review, the use of inhibitors of platelet activation to extend the shelf life and enhance the quality of liquid stored platelets is discussed (28). Cryopreservation of platelets extends the shelf life to 1 year but requires extensive washing and processing to remove cryoprotectant agents (29). In other blood cell studies, red blood cells washed with saline followed by lyophilization retain metabolic activities similar to red blood cells stored under blood bank conditions (30). Lyophilization of platelets or platelet-rich plasma as previously attempted (31–33) neither preserved the structural integrity of the platelets nor provided adequate hemostasis when infused into thrombocytopenic pediatric patients or hemorrhagic animal models.

We have successfully prepared a paraformaldehyde-treated, lyophilized and rehydrated platelet product (RL platelets) with intact morphology and agglutinating properties (34–36). Efforts to refine this process have produced platelet preparations that are structurally stable and capable of undergoing activation. We report here on the hemostatic properties displayed by our RL platelets as tested *in vitro* and in canine and rat animal models. In this study, we have administered RL platelets to normal and von Willebrand disease (vWD) dogs and to thrombocytopenic rats. Our results indicate that RL platelets retain many essential biologic properties and promote hemostasis. The successful preparation of a dried transfusion platelet product without loss of hemostatic capabilities

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Abbreviations: RL platelet, rehydrated lyophilized platelet; BT, bleeding time; vWD, von Willebrand disease; vWF, von Willebrand factor; GP, glycoprotein.

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suggests potential for the development of this product as a blood banking resource.

MATERIALS AND METHODS

Preparation of RL Platelets. Lyophilized human and canine platelets were prepared as described (34). Human platelets were obtained from the American Red Cross 3–6 days after collection. Canine platelets were obtained from normal dogs ($n = 5$) from the Francis Owen Blood Research Laboratory (University of North Carolina). Washed platelets were incubated for 1 hr with paraformaldehyde at concentrations of 1.8% for human platelets and 0.68% for canine platelets. Washed paraformaldehyde-free platelets in citrated saline (0.006 M trisodium citrate/0.154 M NaCl, pH 6.8) with 5% bovine serum albumin were frozen in 1-ml aliquots containing 8×10^8 platelets per ml and lyophilized at -20°C to -40°C for 20–24 hr. Dried platelets were stored at -80°C until used. Dried platelets were rehydrated in 1.0 ml of imidazole buffer (IB; 0.084 M imidazole, pH 7.35) and centrifuged at $1000 \times g$ for 8 min to pellet the platelets. The rehydrated platelets were freed of albumin and imidazole by three washes in citrated saline. For use, the platelet pellets were resuspended in platelet-poor plasma or in a modified Hanks' buffered salt solution (mHBSS; 0.17 M NaCl/6.7 mM KCl/1.0 mM MgSO_4 /0.5 mM K_2HPO_4 /2.8 mM Na_2HPO_4 /13.8 mM dextrose, pH to 7.2 with 1.4% NaHCO_3) for *in vitro* studies and in normal saline for *in vivo* studies. Gas chromatography was used to document the absence of formaldehyde in washed canine and human RL platelet solutions. A detection limit of 0.002% was used (National Medical Laboratories, Willow Grove, PA).

RL platelets were labeled with the fluorescent dye Zynaxis PKH 26 as a marker for platelets in infusion studies. The pelleted rehydrated platelets were washed once by resuspension in 1.0 ml of acid citrate dextrose, centrifuged at $600 \times g$ for 8 min, and resuspended in 0.1 ml of mHBSS. The mixture was incubated for 20 min in the dark and centrifuged ($600 \times g$; 8 min). The labeled platelets were washed once by resuspending in 1.0 ml of mHBSS containing either 0.1% canine serum albumin or 0.1% bovine serum albumin. No label was transferred or lost from fluorescent platelets incubated at 25°C – 37°C for several hours in whole blood.

Hemostatic and Other Methods. The saline bleeding time (BT) in canines was performed as described (37). The BT wound sites were excised and prepared for fluorescence and light microscopy (38). For the rat toenail BT, rats were anesthetized with ketamine hydrochloride (Ketaset)/ProMACE, and a foot was antiseptically cleansed and warmed in a 37°C bath. A sterile scalpel blade was used to excise the distal 1.0 mm of the vascular nail bed from one nail. Blood was blotted onto filter paper for BT measurements. Platelet adhesion of fresh and RL human platelets was compared in an annular perfusion chamber (39). Adhesion studies were carried out at high shear (flow rate, 125 ml/min; 37°C) using porcine arterial subendothelium that had been denuded by exposure to air. After platelet solutions containing fresh or RL platelets were exposed to the subendothelium, segments were removed and processed for scanning electron microscopy to visualize platelet adhesion. Citrated blood, platelet-rich plasma, platelet-free plasma (PFP), and red blood cell fractions were isolated as described (34). Adhesion of fresh platelets was determined after passing whole citrated blood over the subendothelium. Adhesion of RL platelets was determined after PFP enriched with RL platelets and the red blood cell fraction was passed over the subendothelium. To confirm the absence of platelets in PFP prior to the addition of RL platelets, phase-contrast microscopy was used. To examine platelet spread, RL platelets were reconstituted in IB, washed once in HBSS (40) to remove albumin, and spread on Formvar-coated grids (41). Spread platelets were examined

with a Cambridge autoscan scanning electron microscope at 20 kV.

For morphological studies, fresh and RL platelet pellets were processed for transmission electron microscopy as described (38). Platelets were examined with a Zeiss 10A microscope. Rehydrated platelet surface antigen distribution and overall light scatter properties were analyzed on a Becton Dickinson FACS 440 flow cytometer. Monoclonal antibody binding to rehydrated platelets or to fresh platelets resuspended in citrated plasma was evaluated by indirect immunofluorescence 488-nm excitation as described (42). Control antibody was used to identify nonspecific IgG binding to fresh and rehydrated platelets. In each run, 10,000 events were measured and analyzed.

Infusions of RL Platelets. The animals used were normal dogs ($n = 3$), a vWD dog ($n = 1$), and Sprague–Dawley rats ($n = 3$). The vWD dog was from the closed colony at the Francis Owen Blood Research Laboratory. Normal Sprague–Dawley rats were obtained from the Division of Laboratory Animal Medicine (University of North Carolina, Chapel Hill). All animals were treated according to published standards (43). Thrombocytopenic rats were given 4.5×10^9 and 3.4×10^{10} human RL platelets, respectively, through the tail vein. Toenail BTs were performed immediately after infusion of RL platelets. Normalization of BT was taken as an indicator of RL platelet hemostatic function.

Fluorescence-labeled canine RL platelets were infused into three normal dogs. After infusion of these platelets, plasma levels of vWF (44), coagulation factor IX (45), coagulation factor VIII (46), platelet counts (Unopette; Becton Dickinson), and serum fibrin degradation products (Thrombo-Wellcotest; Wellcome) were determined prior to and at the

FIG. 1. Transmission electron microscopy of fresh (A) and RL (B) platelets. Both fresh and RL platelets have intact and randomly distributed organelles and some pseudopod formation. ($\times 7760$.)

Table 1. GPIb, GPIIb/IIIa, and GPIb/IX on the surface of fresh and RL platelets

Antigen	Antibody	% labeled platelets	
		Fresh	RL
GPIb	AN-51	98	92
GPIb	SZ-2	92	85
GPIb/IX	SZ-1	98	92
GPIIb/IIIa	10ES	98	98

Monoclonal antibodies to GPIb (clones AN-51 and SZ-2) were obtained from Dakopatts (Glostrup, Denmark) and AMAC (Westbrook, ME), respectively. The anti-GPIb/IX complex antibody (SZ-1) and the anti-GPIIb/IIIa complex antibody (clone 10E5) were obtained from AMAC and Barry Collier (State University of New York, Stony Brook), respectively. There were <5% labeled platelets in the positive gates using a control nonimmune mouse IgG-2a antibody (Coulter Immunology). Fresh platelets were washed with citrated saline.

following intervals postinfusion: 1, 5, 15, 30, and 60 min during the first hour, and 2, 4, 6, 8, and 24 hr thereafter. BTs were performed postinfusion of labeled RL platelets and wounds were excised after cessation of bleeding for examination by fluorescence and light microscopy (38). A similar infusion was

performed with a vWD dog after partial replacement of vWF by treatment with cryoprecipitate. RL platelets represented 51.4% of the vWD dog's normal platelet count based on the number of RL platelets infused. Cryoprecipitate was prepared as described (47) and assayed for vWF content (44). A modified Folts procedure (48) was used to produce carotid arterial thrombosis in dogs ($n = 3$).

RESULTS AND DISCUSSION

Structural Features of RL Platelets. Transmission electron microscopy showed that RL platelets are morphologically similar to fresh washed platelets (Fig. 1). RL platelets are partially activated, similar to fresh washed platelets. Flow cytometry using anti-GPIb and anti-GPIIb/IIIa monoclonal antibodies indicated that both GPs were present on the surface of RL platelets (Table 1). The number of RL platelets with antibody recognition of receptors is expressed as a percentage of the platelets with specific immunofluorescence.

Functional Characteristics of RL Platelets *in Vitro*. Platelet adhesion and platelet spreading are shown in Fig. 2. A comparison of platelet adhesion with fresh and RL platelets shows that RL platelets adhere in numbers similar to fresh platelets, with irregular shapes and with multiple pseudopodia,

FIG. 2. Scanning electron microscopy (SEM) of vessel segments from an annular perfusion chamber with RL platelets (A) and fresh platelets (B). Vessel subendothelium exposed to platelet-free blood was free of platelets (data not shown), while segments exposed to platelet-containing blood was carpeted with platelets. SEM of spread RL platelets shows that paraformaldehyde-stabilized platelets adherent to Formvar-coated grids formed dendritic patterns with multiple pseudopodia and are fully spread (C), similar to fresh platelets spread on the same substrate (D). (A, $\times 960$; B, $\times 2320$; C, $\times 2080$; D, $\times 1600$.)

Table 2. Infusions of human RL platelets shorten the prolonged BTs in thrombocytopenic rats

Animal	BT, min	Normal	BT, min	Thrombocytopenic	BT, min	Thrombocytopenic with RL platelets
		Platelet count per μl , $\times 10^{-3}$		Platelet count per μl , $\times 10^{-3}$		Platelet count per μl , $\times 10^{-3}$
1	0.5	685	>15	25	0.5	220
2	2.0	580	>15	32.5	1.5	237

Thrombocytopenia was induced in two Sprague–Dawley rats by treatment with 1 ml of a 1:10 dilution of anti-rat thrombocyte polyclonal antibody (Accurate Chemicals). Platelet counts and toenail BT measurements were used to monitor the level of circulating rat platelets 10 min after treatment with the antibody. RL platelets were infused immediately after a BT of >15 min was established. In a control rat without treatment with RL platelets, bleeding times were >15 min, and platelet counts were <50,000 platelets per μl for >12 hr.

although pseudopodia are present to a lesser extent in RL platelets (Fig. 2 *A* and *B*). Neither RL platelets nor fresh platelets were present in areas where the endothelium remained intact (data not shown). A comparison of platelet spreading of fresh and RL platelets showed both having a similar flattened or “pancake” morphology (Fig. 2 *C* and *D*). Multiple pseudopodia were found associated with both fresh and RL platelets, which were not completely spread. Few

discoid forms were present, suggesting that paraformaldehyde-stabilized platelets retained sufficient metabolic activity for platelet spreading to occur. Earlier studies have demonstrated that functional GPIb is preserved in lyophilized platelets (49). While GPIIb/IIIa epitopes are identified in RL platelets, minimal platelet aggregation was observed in preliminary studies with ADP (A.P.B., unpublished data).

Functional Characteristics of RL Platelets *in Vivo*. RL platelets were labeled with a fluorescent dye to distinguish rehydrated platelets from circulating native platelets in infusion experiments. BT studies in rats with human RL platelets and in normal and vWD dogs with canine RL platelets were conducted. The results of two separate RL platelet infusion experiments using thrombocytopenic rats are shown in Table 2. After administration of human RL platelets, toenail BTs in two rats treated with an anti-rat thrombocyte antibody decreased from >15 min to normal. One rat was tested at 30 min and had a toenail BT of 3.5 min, which remained corrected for 1 hr, at which time the rat was sacrificed. Treatment of normal rats with diluted anti-rat thrombocyte antibody depleted circulating rat platelets to <33,000 platelets per μl and lengthened the rat toenail BT to >15 min. Normal rats treated with experimental levels of the anti-rat thrombocyte antibody without additional RL platelets had elongated BTs (>15 min) and low autologous platelet counts (<50,000 platelets per μl) for 12 hr. The toenail BT is a simple and reproducible method of measuring BT in the rat and requires only that the rat be anesthetized before testing. In 10–12 normal rats, the BTs ranged from 30 sec to 3 min.

FIG. 3. Fluorescence microscopy (*A*) and a schematic representation (*B*) of an ear BT wound of a normal dog after infusion of labeled rehydrated platelets. Clumps of labeled platelets adhere to the cut surfaces of the ear wound. (*C*) Fluorescence microscopy of a carotid artery with deep vessel wall injury in a normal dog after administration of fluorescent RL canine platelets. Internal elastic lamina (*I*) is disrupted and deep medial injury is present. Platelet thrombi (*T*) in the lumen (*L*) and in the superficial and deep media (*M*) contain labeled rehydrated platelets. In these models, with a mixture of native and RL circulating platelets, RL platelets represented between 18.4% and 43.8% of the total platelet count based on the number of RL platelets infused. ($\times 280$.)

RL platelets were infused into three normal dogs and one vWD dog in order to determine whether the RL platelets were incorporated into the hemostatic thrombi of BT wounds. The vWD dog was infused with cryoprecipitate, which raised the vWF level to 38% of normal; the BT of the vWD dog was reduced from >15 min to 8 min. RL platelets were then administered to the vWD dog, and there was no significant change in the BT. The BT remained in the normal range (6 min) for the other three dogs. RL platelets circulated for the duration of the experiments (up to 4 hr). They were found to be part of the hemostatic plug in normal dogs (Fig. 3A and B). In the vWD dog, RL platelets were observed at the same sites as in the normal dogs but in far fewer numbers (data not shown). Samples of liver, lung, and kidney were examined after sacrifice of these animals. No gross or microscopic changes were observed.

Carotid arterial thrombosis was induced by using a canine model of stenosis and injury in three normal dogs that had been infused with RL platelets (48, 50). All animals experienced occlusive thrombosis as indicated by the cessation of blood flow. When thrombosis occurred, the vessels containing the thrombi were harvested after >30 min of observation and examined by fluorescence microscopy. Fluorescent RL platelets were present in the induced thrombi and were also adherent to the exposed subendothelium. Single and aggregated platelets were present in areas of hemorrhage (Fig. 3C). In vessels where damage was minimal, fluorescent RL platelets were seen in the lumen and adhering to the luminal surface where the internal elastic lamina was disrupted. There was no evidence of RL platelets adhering to intact endothelium. The absence of disseminated intravascular coagulation was indicated by no change in fibrinogen level, no loss of coagulation factors VIII and IX, and no appearance of fibrin degradation products.

Platelet preparations can be stored for several days without the use of refrigeration. After this period, platelets lose many of their functions (51, 52). Lyophilization has the potential to extend blood cell (erythrocytes and platelets) shelf life from days to years. The fixation of platelets in paraformaldehyde followed by lyophilization has proven effective in maintaining some of the normal functions of the human platelet (34). We have shown that RL platelets retain many properties necessary for normal hemostasis. These observations suggest that this method of stabilization may offer a method for long-term storage of platelets.

We thank Stephen Pemberton for help in preparing the manuscript and the animal support staff at the Francis Owen Blood Research Laboratory. The research was supported in part by Navy Medical Research and Development Command Grants N00014-89-J-1712, N00014-92-J-1244, and N00014931034 and National Institutes of Health Grant HL 01648-47.

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