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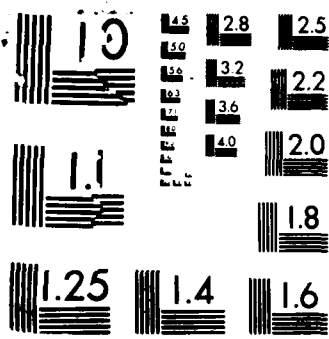
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Feasibility Study for Long-Term Storage and Hemostatic Effectiveness of Platelet Membrane Fractions Prepared From Human Platelet Concentrates

Final Report

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products and fibrinogen in animals transfused with membrane were unchanged over controls, suggesting a direct effect of platelet membrane on bleeding time sites. Greater correction of bleeding times in animals with moderate rather than severe levels of thrombocytopenia also suggested potentiation of intact platelet function by transfused membrane. Administration of platelet membrane also stimulated platelet production in thrombocytopenic animals who otherwise would not have resumed platelet production. The data show that platelet membrane and lysed platelets in transfusion products may contribute to a hemostatic response and that corrections in bleeding times are not always associated with an increase in circulating platelet count. Production of platelet membrane concentrates by freezing and thawing is simple and allows storage of a product capable of short-term hemostasis for months. In massive transfusion situations associated with dilutional thrombocytopenia, platelet membrane concentrates could provide an important role as an adjunct to fresh platelet transfusions.

FORWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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ABSTRACT

This project studied the possible role of platelet membrane on hemostatic function in vivo. Platelet membrane concentrates were prepared from fresh rabbit platelets, stored for up to 6 months at -60C, thawed and transfused into thrombocytopenic rabbits. Significant reductions in microvascular bleeding times were observed up to 24 hours posttransfusion, with the greatest corrections at 4 hours. Platelet counts, Factor V, Factor VIII, fibrin degradation products and fibrinogen in animals transfused with membrane were unchanged over controls, suggesting a direct effect of platelet membrane on bleeding time sites. Greater correction of bleeding times in animals with moderate rather than severe levels of thrombocytopenia also suggested potentiation of intact platelet function by transfused membrane. Administration of platelet membrane also stimulated platelet production in thrombocytopenic animals who otherwise would not have resumed platelet production. The data show that platelet membrane and lysed platelets in transfusion products may contribute to a hemostatic response and that corrections in bleeding times are not always associated with an increase in circulating platelet count. Production of platelet membrane concentrates by freezing and thawing is simple and allows storage of a product capable of short-term hemostasis for months. In massive transfusion situations associated with dilutional thrombocytopenia, platelet membrane concentrates could provide an important role as an adjunct to fresh platelet transfusions.

INTRODUCTION

Some of the earliest clinical studies of platelet transfusions noted cessation of bleeding and decreases in bleeding times following transfusion of platelets stored for up to twelve months in gelatin (Tullis, 1953). Similar results were obtained with lyophilized platelets (Klein, et al., 1956) or frozen platelets (Djerassi, 1966). These studies noted that the morphological integrity of platelets was not preserved and that they did not produce increases in counts. It was noted, however, that they decreased the tendency for bleeding. In contrast, other studies indicated that blood bank platelets must be intact and circulate for a hemostatic response to be achieved (for reviews see Baldini, et al., 1960 and Kaplan, 1980). These studies concluded that the hallmark of a successful platelet transfusion was an increase in circulating platelet count.

Techniques for storage of intact platelets in the liquid state or cryopreservation yields many lysed platelets and membrane fragments (Spector, et al., 1979; Ward et al., 1982). In order to investigate the possible clinical effect of damaged platelets and determine the potential of lysed platelets and platelet membranes for hemostasis, thrombocytopenic rabbits were transfused with platelet membrane concentrates prepared from rabbit platelets. Effects of platelet membrane concentrate

transfusions on rabbits at various levels of thrombocytopenia are reported.

MATERIALS AND METHODS

Platelet Membrane Concentrate Production

New Zealand white rabbits were used as platelet donors and for the thrombocytopenic animal model. For all blood collections and transfusion procedures animals were anesthetized with ketamine hydrochloride (35mg/kg) and Xylazine (5mg/kg). 30 - 40 mL of whole blood was collected into sodium citrate (0.35% final concentration) at ten day intervals by cardiac puncture. Platelet rich plasma prepared from 50 mL pools of whole blood was centrifuged to pellet fresh platelets and pellets were frozen without cryoprotectant at -65°C , within four hours of collection. Pellets were stored frozen for up to six months. Immediately prior to transfusion pellets were thawed, refrozen and thawed twice, rinsed in platelet free plasma (stored at -65°C) and resuspended in 20 mL of plasma by vigorous syringing through a 23 gauge needle. Examination by light and electron microscopy indicated that all platelets were lysed. White blood cell counts in platelet rich plasma used for platelet membrane concentrate production was less than $0.5 \times 10^3/\mu\text{L}$. The number of platelets used for production of one membrane concentrates averaged 5.23×10^{10} or the number of platelets equivalent to that contained in one-half of the total blood volume of an adult rabbit.

The Thrombocytopenic Rabbit Model

Our thrombocytopenic animal model was taken largely from two previous models: those described by Blajchman, et al. (1979) and Kitchens (1977). Baseline values for microvascular bleeding time (MBT = time required for a 6mm, full-thickness incision of the ear to stop bleeding while submerged in saline at 37°C), platelet count and hematocrit were determined in normal, untreated rabbits (Table 1). Thrombocytopenia was induced with busulphan (Burroughs Wellcome Co., New Jersey) suspended in polyethylene glycol 400 at a final concentration of 10 mg/mL. Doses of 30-70 mg/kg were administered by subcutaneous injection. Doses for each experiment are shown in results. Tetracycline (final concentration = 800 mg/L) was given continuously in drinking water and gentamycine (4.4 mg/kg) was administered intramuscularly during test and transfusion procedures in all busulphan treated rabbits. Platelet concentrates were administered through ear veins as a single bolus using 21-gauge needles. Microvascular bleeding times, manual platelet counts and hematocrits were determined for up to 72 hours posttransfusion. Other thrombocytopenic rabbits were transfused with platelet poor plasma alone, as a control. Plasma volume transfused was 15 mL. Plasma for transfusion was frozen at -65°C within four hours of collection.

Coagulation Studies

Tests for fibrin degradation products (FDP), Factor V, Factor VIII and fibrinogen in control and treated rabbits were performed to evaluate possible effects of platelet membrane concentrates on consumption of coagulation proteins. FDP was tested with the Thrombo-Wellcotest (Burroughs Wellcome Co., N.C.) utilizing latex particles coated with antibody to fragments D and E of human fibrinogen. The antibody was crossreactive with rabbit fibrinogen (Burroughs Wellcome Co., personal communication). Factor V and Factor VIII activities were measured using activated partial thromboplastin time tests (Dade Diagnostics, Inc., Puerto Rico) and deficient plasma. Fibrinogen was tested with the thrombin clotting time test (Dade Diagnostics, Inc., Puerto Rico). Pretransfusion and 4 hour posttransfusion blood samples for coagulation studies were collected from ear veins. The 24 hour samples were collected by cardiac puncture.

Transmission Electron Microscopy of Microvascular Bleeding Time Lesions

Full thickness, circular plugs containing MBT lesions were fixed in glutaraldehyde and osmium tetroxide, dehydrated in alcohol and flat embedded in modified Luft embedding materials 812. Tissue blocks were trimmed to allow cross sectioning of cut portions of MBT lesions. Normal, prolonged and corrected MBT were fixed immediately following cessation of bleeding.

Statistical Analysis

MBT test results in animals transfused with platelet membrane concentrates were compared to pretransfusion values by the rank sum test. Possible differences in MBT between animals transfused with platelet membrane concentrates and platelet free plasma were evaluated by repeated measures analysis of variance and life table analysis of the two groups over time (Winer, 1982; Hollander and Wolfe, 1973; Kalbfleisch and Prentice, 1980).

RESULTS

In Vitro Test Values in Rabbits Pretransfusion

Mean (\pm SD) hematocrit and platelet count values in control animals were 39.1 ± 3.0 and $489 \pm 122 \times 10^3/\mu\text{L}$ ($n = 21$), respectively (Table 1). The mean (\pm SD) MBT was 94 ± 23 seconds. Twelve days after injection of busulphan (range = 11 - 15 days) platelet counts reached their nadirs and MBT increased to 551 ± 100 seconds (after 30 mg/kg busulphan) and 586 ± 27 seconds (after 70mg/kg busulphan). Seven of nine busulphan treated rabbits had MBT of greater than 600 seconds pretransfusion. In general, MBT were inversely proportional to platelet counts and reached greater than 600 seconds at counts of less than 100,000 per μL . Two animals with pretransfusion MBT of less than 600 seconds (350 and 539 seconds) had platelet counts of 91,000 and

93,000, respectively. Two animals with MBT greater than 600 seconds had platelet counts of greater than 100,000 per uL (102,000 and 112,000). The other five (with MBT > 600 seconds) had platelet counts ranging from 12,000 to 91,000/uL.

MBT in Thrombocytopenic Rabbits Transfused with Platelet Membrane Concentrates

Platelet membrane concentrates were transfused into each of nine rabbits with varying degrees of thrombocytopenia. The effects of these transfusions on MBT and platelet count are summarized in Tables 2 and 3. The data in Table 2 show that marked reductions in MBT occurred in all but one animal at 15 and/or 60 minutes posttransfusion. In four animals (1,3,5,9) MBT showed maximum decreases in responses to transfusions at 60 minutes while four others (2,4,6,8) showed lowest MBT at four hours. At 24 hours, MBT were lower than pretransfusion values in seven of nine animals. With few exceptions platelet counts remained unchanged up to 24 hours posttransfusion (Table 3). At 48 hours, however, membrane transfusions affected platelet counts in an unexpected way (Table 3). Platelet counts in five animals increased. This increase resulted in a decrease in MBT over 24 hour values in 4 of 5 animals. In animals unable to make platelets posttransfusion MBT returned to pretransfusion levels at 48 hours. No significant changes in hematocrits were detected in transfused rabbits (data not shown).

Transfusion of 15 mL of platelet free plasma had no obvious effects on pretransfusion test values. MBT did not decrease or increase up to 24 hours posttransfusion in any of five animals (Table 4). For this reason MBT tests were not performed at 48 hours. Repeated measures analysis of variants was chosen for comparison of platelet membrane transfusions with plasma transfusions, despite the fact that MBT were censored data in that 600 seconds was accepted as the maximum value. Results indicated an overall difference between the groups ($p < 0.05$). The two groups also varied to different degrees over time ($p < 0.05$). Life table analysis was performed at each time interval. At pretransfusion no differences existed in MBT. At 15 minutes the platelet membrane transfused group stopped bleeding earlier than those transfused with plasma ($p = 0.07$). At 60 minutes, differences were similar ($p = 0.07$). Significant reductions over plasma controls occurred at four hours ($p = 0.02$) and at 24 hours ($p = 0.04$).

Coagulation Studies

Four other thrombocytopenic rabbits with pretransfusion platelet counts of $7,700 \pm 4,500$ /uL and MBT of 601 ± 0.0 seconds were transfused with standard membrane concentrates to evaluate effects of transfusions on FDP, Factor V, Factor VIII and fibrinogen (Table 5). No changes in FDP values were detected. Factor VIII levels were decreased at four hours but not at 24 hours posttransfusion. Factor V showed a small decrease at four

hours and some increase at 24 hours posttransfusion while fibrinogen remained unchanged at four hours and increased slightly at 24 hours posttransfusion.

Ultrastructural Morphology of MBT Lesions

Full thickness, circular plugs of ear tissue containing MBT lesions were taken from normal rabbits and thrombocytopenic animals showing significant corrections in MBT after transfusion with platelet membrane concentrates. All plugs were removed immediately following cessation of bleeding. Tissue was sectioned to reveal the ultrastructural morphology of the microvasculature and its contents at areas immediately adjacent cut surfaces. Figures 1 and 2 are micrographs from a normal rabbit with a MBT of 90 seconds. A venule or arteriole at the cut surface (Figure 1) reveals sections of intact platelets near an endothelial cell and a damaged area of the vessel. Figure 2 shows a mass of intact, aggregated platelets typical of those in normal thrombi. Sections from transfused rabbit 937 (Table 2 and 3) showed vessels containing platelet membrane (Figure 3) and other vessels containing both membrane and intact platelets (Figure 4).

DISCUSSION AND CONCLUSION

The objective of this project was to assess the hemostatic potential of platelet membrane and its effect on platelet function, by determining its effect on small vessel hemostasis. New Zealand white rabbits were chosen as the transfusion model (Blajchman, et al., 1979). Thrombocytopenia was induced with the cytotoxic drug busulphan (Kitchens, 1977; den Ottolander, et al., 1982). Normal values for hematocrit and platelet counts were consistent with that reported for New Zealand white rabbits. Our mean MBT of 94 ± 23 seconds in untreated controls was comparable to MBT reported by Blajchman et al., (1979) of 83 ± 13 seconds, despite the fact that his model induced thrombocytopenia by an immune mechanism. Our model developed a pancytopenia resembling aplastic anemia in humans (den Ottolander, 1982).

All animals transfused with platelet membrane showed significant reductions in MBT up to 24 hours posttransfusion. The greatest corrections were observed after four hours. Some animals began production of platelets 24 - 48 hours posttransfusion, which initiated a second decrease in MBT. Thus, platelet membrane concentrates may stimulate production of platelets in some animals, by mechanisms which are unclear. Taken together these data also show that, in the same animal, both intact autologous platelets and donor platelet membrane can initiate a hemostatic response. Transfusion of platelet free plasma had no effect on MBT and studies of coagulation proteins seemed to rule out the possibility that intravascular coagulation was initiated by membrane transfusions.

Transmission electron microscopic data suggested that platelet membrane interacted directly with damaged vessel walls and with intact platelets forming thrombi at MBT sites. This is consistent with earlier observations in our laboratory (unpublished data) which showed that lysed platelets and membrane from cryopreserved platelet concentrates reacted directly with vessel wall subendothelium under blood flow conditions. Larger corrections in MBT occurred in animals with lesser degrees of thrombocytopenia, indicating that corrections in MBT may be achieved by platelet membrane mainly by potentiating the hemostatic effect of intact platelets. Platelet membrane concentrate doses were determined arbitrarily and probably were too low to achieve significant hemostatic effects by direct involvement of membrane with MBT lesions alone.

TABLE 1

Mean (\pm SD) Pretransfusion Test Values
In New Zealand White Rabbits

Rabbits	n	Hematocrit %	Platelet Count ($\times 10^3/\mu\text{l}$)	MBT (sec)
Normal	21	39.1 \pm 3.0	489 \pm 122	94 \pm 23
Busulphan Treated				
30 mg/kg	5	35.4 \pm 3.0	89.4 \pm 19.7	551 \pm 100*
70 mg/kg	4	32.3 \pm 1.3	56.5 \pm 34.1	586 \pm 27

*Four of 5 animals had MBT > 600 seconds. Calculations of mean MBT used 601 seconds for MBT > 600. Subjects with MBT > 600 routinely showed MBT > 900 seconds.

TABLE 2

Effect of Platelet Membrane Concentrate Transfusions On
Microvascular Bleeding Times In Thrombocytopenic Rabbits

Animal	MBT (sec)					
	Pretransfusion	Posttransfusion				
		15 min	60 min	4 hrs	24 hrs	48 hrs
No.						
1	>600	256	256	235	363	>600
2	>600	260	321	206	234	221
3	350	204	140	225	275	>600
4	>600	495	414	139	475	166
5	>600	470	375	535	513	200
6	>600	>600	440	213	420	204
7	>600	>600	>600	>600	>600	>600
8	539	580	245	145	224	425
9	>600	300	120	415	>600	>600
Group Mean	600	470	313	302	412	402
	±84	±59	±161	±171	±147	±202

TABLE 3

Effect of Platelet Membrane Concentrate Transfusions On
Platelet Count ($\times 10^3 \mu\text{l}$) In Thrombocytopenic Rabbits

Animal	Posttransfusion						
	No.	Pretransfusion	15 min	60 min	4 hrs	24 hrs	48 hrs
1		60	n.d.	n.d.	n.d.	69	50
2		117	n.d.	121	133	131	161
3		91	n.d.	97	120	236	117
4		77	n.d.	52	48	69	135
5		102	n.d.	143	108	141	216
6		86	n.d.	74	68	60	152
7		12	n.d.	13	9	23	11
8		93	n.d.	90	110	91	95
9		35	n.d.	39	41	32	17
Group Mean		75	n.d.	79	80	95	106
		± 34		± 43	± 44	± 66	± 69

TABLE 4

Effect of Platelet Membrane Concentrate Transfusions* On
Microvascular Bleeding Times In Thrombocytopenic Rabbits

Animal	MBT (sec)					
	Pretransfusion	Posttransfusion				
No.		15 min	60 min	4 hrs	24 hrs	48 hrs
1	>600	>600	>600	>600	480	n.d.
2	>600	>600	>600	>600	576	n.d.
3	398	589	317	600	600	n.d.
4	320	395	403	411	515	n.d.
5	489	360	530	496	400	n.d.
Group Mean	482	509	490	542	514	
(± SD)	±124	±121	±126	±86	±80	

*Volume of plasma infused was 15 mL

TABLE 5

Effects of Platelet Membrane Concentrate Transfusions* On
Coagulation Protein Activity In Thrombocytopenic Rabbits**

Coagulation Tests	Pretransfusion	Posttransfusion	
		4 hr	24 hr
FDP	>2	>2	>2
VIII	224±17	111±50	285±64
V	2557±483	2077±413	3626±398
Fibrinogen	139±22	101±27	272±27

*Platelet membrane concentrates were prepared from an average of 4.2×10^{10} platelets

**Mean (±SD) pretransfusion platelet count and MBT values were $7,700 \pm 4,500/\mu\text{L}$ and 601 ± 0.0 seconds, respectively.



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