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AN INVESTIGATION OF THE CAUSES OF THE BLOOD PLATELET "STORAGE LESION"

ANNUAL SUMMARY REPORT

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Arthur P. Bode

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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

We have found evidence of the generation of thrombin in the preparation and storage of standard, citrated platelet concentrates. We have also identified a support mechanism in platelet concentrates for thrombin generation; namely, platelet activation and expression of procoagulant activity by stored platelets. These results were obtained through an examination of the levels of fibrinopeptide-A and platelet factor 3 in platelet concentrates prepared by standard techniques. We have shown in preliminary experiments that addition of agents that inhibit thrombin activity and inhibit platelet activation results in markedly improved platelet storage at least by in vitro markers. Follow-up studies are in progress.

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SUMMARY

This report is structured so as to inform the reader of the progress achieved by the authors on each specific objective as well as progress towards the overall goal of developing an understanding of the cause(s) of the blood platelet storage lesion. For the first phase of our work, the purpose has been to investigate evidence of thrombin generation and platelet activation in standard, citrated platelet concentrates (PC). We have looked for and found evidence of thrombin-specific proteolysis of fibrinogen in PC plasma by measuring levels of fibrinopeptide-A with a commercially available radioimmunoassay. In addition, we have observed that stored platelets are activated and become progressively procoagulant under standard storage conditions. This observation was obtained through the use of a chromogenic platelet factor 3 assay which is described in detail (along with the results from PC samples) in the manuscript accompanying this report. The manuscript has been submitted for publication in the medical research journal Vox Sanguinis, and an abstract (copy enclosed) describing the evidence from thrombin generation in PC has been submitted for presentation at the Xth congress of the International Society on Thrombosis and Haemostasis (July, 1985). We are confident that these preliminary findings will be confirmed by the more rigorous methods we have proposed to apply in the second phase of the contract. If successful, we will have shown that a feedback loop of thrombin generation and platelet activation exists in citrated PC and that improvements in platelet storage can come from interventions that control this process.

FOREWARD

For the protection of human subjects the investigators have adhered to policies of applicable Federal Law 45CFR46. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

TABLE OF CONTENTS

TITLE		PAGE NUMBER
INTRODUCTION		1
Objective (A).	Demonstration of thrombin generation and PF3 expression by citrated platelet concentrates (PC).	1-2
Objective (B).	Investigation of the relationship between thrombin generation in stored PC.	2-3
Objective (C).	Investigation of the relationship between markers of platelet activation and markers of platelet damage in citrated PC modified by the addition of inhibitors of platelet activation.	3-4
METHODS		4-5
RESULTS		5-10
TABLE 1	FPA Levels (ng/mL) in Red Cross Platelets Concentrations	6
TABLE 2	FPA Formation in Platelet Concentrate Preparation With and Without Hirudin	7
TABLE 3	Extended Storage of PC Collected in CPDA-1 Plus Hirudin, Theophylline, and PGE-1	9
DISCUSSION		11

Annual Report
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March 25, 1985

INTRODUCTION

Our investigation into the causes of the blood platelet storage lesion has met with considerable success, especially in our major goals of documenting evidence of platelet activation and thrombin generation in standard citrated platelet concentrates. We are now in the process of strengthening and confirming these initial results with more rigorous methods. For this report, it is appropriate to describe our progress on each separate objective as below and then discuss the data and conclusions in general.

Objective (A). Demonstration of thrombin generation and PF3 expression by citrated platelet concentrates (PC).

(1). Detection of fibrinopeptide A (FPA) formed in PC during preparation and storage: Completed.

We have shown conclusively that significantly elevated levels of FPA are found in citrated PC and in the whole blood and platelet-rich plasma from which the PC is prepared. Addition of hirudin to the anticoagulant reduced the formation of FPA, demonstrating that the appearance of FPA was not primarily the result of non-specific degradation of fibrinogen. Furthermore, we found that when purified FPA was introduced into citrated PC, the FPA levels rose as expected and then fell off rapidly with an apparent half-life of 16 hrs for FPA in PC. Therefore, the findings of elevated levels of FPA in PC are at best an underestimate of the amount of thrombin activity actually present. In this regard, our FPA results become even stronger evidence that thrombin generation does occur during collection and storage of PC.

(2). Demonstration of the consumption of antithrombin activity in plasma of stored PC in conjunction with the formation of high molecular weight complexes of antithrombin III (AT-III): Nearing completion.

So far, we have obtained suggestive evidence that AT-III activity in PC plasma decreases during storage: a group of 14 citrated PC from the local American Red Cross showed a decrease of only borderline significance ($p=0.064$) from the first to the fifth day of storage. However, we could not obtain from these donors fresh blood samples which were needed to measure the true starting values of AT-III for proper evaluation of PC values of AT-III. We are in the process of finishing a study using 6 new donors where we have drawn all the necessary samples. We suspect that consumption of AT-III activity and complex formation may not be a sensitive enough marker for the purpose of demonstrating thrombin generation in citrated PC.

(3). Demonstration of the conversion of prothrombin to thrombin and plasma Factor X to Xa in PC by means of exogenous radiolabelled zymogens: Underway.

Two attempts have been made thus far to introduce radiolabelled purified prothrombin into citrated PC to directly test for generation of (radiolabelled) thrombin during storage. However, both times there was significant contamination of the prothrombin preparation which interfered with the conduct of the experiment and confounded the results. We are attempting to solve this problem by using affinity chromatography to remove certain substances (such as preformed thrombin) from the prothrombin preparation. This series of experiments, once completed, should offer direct evidence of thrombin generation in PC.

(4). Demonstration of the appearance of platelet procoagulant activity (PF3) known to support thrombin generation: Completed.

See attached manuscript. Our results show conclusively that platelets in PC become highly procoagulant during storage and thus potentially contribute to the mechanism of thrombin generation in PC.

Objective (B). Investigation of the relationship between thrombin generation and PF3 expression by modification of thrombin generation in stored PC.

(1). Effects of specific thrombin inhibitors (PPACK, DABE, hirudin) as primary anticoagulants on the relationships among thrombin generation markers, PF3 levels, platelet morphology, PF4 release, and LDH release: Nearing completion.

We have worked with PPACK only in the setting of sole agent for WB anticoagulation and platelet preservation. In this environment it performed no better than hirudin in the same setting: that is to say, not well. However, hirudin has performed well in a setting that also included citrate, PGA and theophylline (see Objective (C).(4).). We will investigate PPACK as an inexpenseive, non-antigenic substitute for hirudin in this misture.

DABE, a much higher affinity inhibitor, must be abandoned since it is no longer synthesized by our sole source and has become exorbitantly priced. Hirudin is the highest affinity inhibitor of thrombin known to man and readily available, but expensively high levels must be used to prevent thrombin's interaction with platelets (Hoffmann and Markwardt, Haemostasis. 14: 164-169, 1984). Because of these considerations, anticoagulation with hirudin alone can be done only in a small scale experiment which does not allow for all the comparisons proposed above. A more prudent approach at this juncture is to add hirudin to standard citrate anticoagulants. So far, we have shown that PC prepared in the presence of hirudin have lower FPA levels than controls, but morphology score and PF3 levels were not markedly improved. For this reason, we will include addition

of platelet activation inhibitors in further experiments to improve *in vitro* markers in PC storage (see Objective (C).(4).)

(2). Effects on the above markers of adding excess anti-thrombin III to citrated PC: Abandoned.

Because of the minimal decrease in endogenous AT-III levels noted in Objective (A).(2)., we do not believe that adding excess AT-III will be of value in limiting thrombin generation in PC.

(3). Investigation of the above markers in PC prepared from donors with severe clotting factor deficiences: Halted.

The exposure of hemophiliacs to therapeutic material possibly containing AIDS agent(s) makes the handling of blood products from these donors too hazardous for scientific research. Until such time as we are convinced that the risk of exposure for our research personnel is minimal or non-existent, we will abstain from conducting experiments with PC prepared from hemophiliac donors. Instead, we will concentrate on using specific thrombin inhibitors in PC from normal donors.

(4). Investigation of addition of low amounts of purified thrombin to PC and the consequent effects on the above markers: Underway.

We have found that addition of amounts as low as 0.02 NIH units/mL thrombin in PC can produce instantaneous changes in platelet morphology without affecting other markers and without clotting the PC plasma. Unfortunately, we found out in control studies that the stabilizers added to the thrombin preparations we used interfered with platelet metabolism, and thus these experiments must be repeated with different thrombin preparations. Our impression is that extremely low levels of thrombin can produce far-reaching effects in PC, therefore supporting our hypothesis that thrombin plays an important role in platelet storage.

(5). Demonstration of the association of thrombin formed from radiolabelled prothrombin (see (A).(3)) with the plasma membrane of stored platelets, thereby indicating a cause-and-effect relationship between thrombin generation and platelet activation in stored PC: Underway.

Progress on this objective is entirely contingent on the success of objective (A).(3). We hope to be able to count radioactivity associated with the platelet pellet in samples from PC prepared with radiolabelled prothrombin, and compare those counts to PC controls with radolabelled albumin in a "trapped volume" experiment.

Objective (C). Investigation of the relationship between markers of platelet activation and markers of platelet damage in citrated PC modified by the addition of inhibitors of platelet activation.

(1). Demonstration of the release of PF3 and platelet microvesicles into the PC supernatant plasma such as occurs with *in vitro* stimulation of platelets: Completed.

See attached manuscript. We were able to identify in citrated PC evidence of the release of a particulate fraction containing approximately half of the PF3 activity in the PC suspension. This release was not associated with apppearance of cytosolic markers such as LDH, and therefore, must be taken as evidence of platelet activation in citrated PC.

(2). Demonstration of the release of PF4 and its relationship to PF3 levels: Abandoned.

Recent evidence of the independent release of PF4 versus PF3 under in vitro conditions make this objective uninformative (Sandberg and Bode et al., Thrombosis Research, 39:63-79, 1985). The release of PF4 during PC storage has been documented by Snyder et al. (Vox Sanguinis. 41: 172-177, 1981), and all other evidence compiled to date support our conclusion that the release reaction and the expression of PF3 activity do indeed occur during the storage of citrated PC. The intricacies of the relationship between PF3 expression and the release reaction are not germane to this contract.

(3). Relationship of the above to platelet morphology changes, release of LDH, and pH measurements: Completed.

See attached manuscript. PF3 activity was expressed and released in citrated PC without concommitant release of LDH, indicating that lysis was not the primary event. Although platelet morphology score was not absolutely correlated with PF3, a general trend was quite evident in each PC examined: PF3 levels and morphology score increased monotonically with time. On the other hand, pH measurements were independent of all other markers, except that when the pH fell to 6.0 or lower, PF3 levels rose dramatically and platelet morphology became unscoreable.

(4). Modification of all the above by addition of specific platelet activation inhibitors (prostaglandin E1, theophylline, adenosine cocktail) to the anticoagulant solution in PC: Nearing completion.

As stated in objective (B).(1)., we are testing the combined effects of thrombin inhibitors and platelet activation inhibitors when added to standard citrate anticoagulants. The results so far have been extremely encouraging, in that by in vitro markers of platelet viability, we have extended the storage of PC to at least 15 days. We are now in the process of preparing many such PC to test for reproducibility of these findings. At this time, we have not found it necessary to include adenosine as part of the anticoagulant recipe; theophylline and PGE-1 seem to be sufficient to inhibit platelet activation in the presence of citrate plus hirudin.

METHODS

Measurements were made on platelet concentrates obtained from two sources:

(a). Donors were recruited locally and informed consent was

obtained in compliance with the Institutional Review Board. Platelets were separated from whole blood by differential centrifugation using a Sorvall RC-3 and standard blood bank techniques or using the IBM-2997 Blood Cell Separator.

(b). PC units (50-60 mLs.) obtained through the courtesy of the Carolinas Region American Red Cross Blood Services Center.

Platelet counts were performed on a Coulter S Plus-II and, in some cases, adjusted with platelet-poor plasma. Blood gas and pH determinations were made on an IL-713 blood gas analyzer. Platelet morphology was viewed and photographed at x1000 through Nomarski optics. Scoring was performed by at least two observers using a modification of the scoring system given by Akkerman et al. (Thrombosis and Hemostasis. 39: 146-157, 1978). Recovery from hypotonic shock was assessed according to the method of Valeri et al. (Transfusion. 14(4): 331-337, 1974). Aggregation response to single concentrations of arachidonic acid (2mg/mL) and ADP (1mmolar) agonists were measured using a Payton dual channel aggregometer. Platelet factor 3 (PF3) was measured as prothrombinase activity according to a chromogenic substrate assay described in detail in the accompanying manuscript.

Fibrinopeptide-A (FPA) measurements were performed using a competitive binding radioimmunoassay method using bentonite-extracted plasma samples according to the instructions of the reagent manufacturer (Mallinckrodt, Inc.). FPA for addition to stored PC was obtained from Sigma Chemical Co., as was Hirudin Grade IV which was used without further purification. Antithrombin-III levels were measured by Dr. Michael J. Griffith using his chromogenic assay for thrombin inhibition (Blood. 61(1): 111-120, 1983). Released lactic dehydrogenase activity (LDH) was measured in PC plasma with a chromogenic substrate assay kit from Worthington Diagnostics on a Cobas centrifugal analyzer.

RESULTS

Thrombin Generation

(a). FPA Levels: 27 PC from the local Red Cross were assayed for FPA upon receipt (Day 1) and most (N=16) were assayed again at outdate. Those assayed on Day 1 but not Day 5 were used for other purposes in other experiments. Table 1(top) shows that PC collected in CPDA-1 alone exhibited FPA levels of four to five times the upper limit of "normal" circulating levels throughout five days of storage. In order to determine if formed FPA is stable in stored PC, exogenous FPA was added to stored PC. Table 1 (bottom) shows a rapid decrease from very high levels with a half-life of 16 hours. These results suggest that FPA measurements represent a balance between formation and degradation of FPA and so underestimate the thrombin activity actually present during storage.

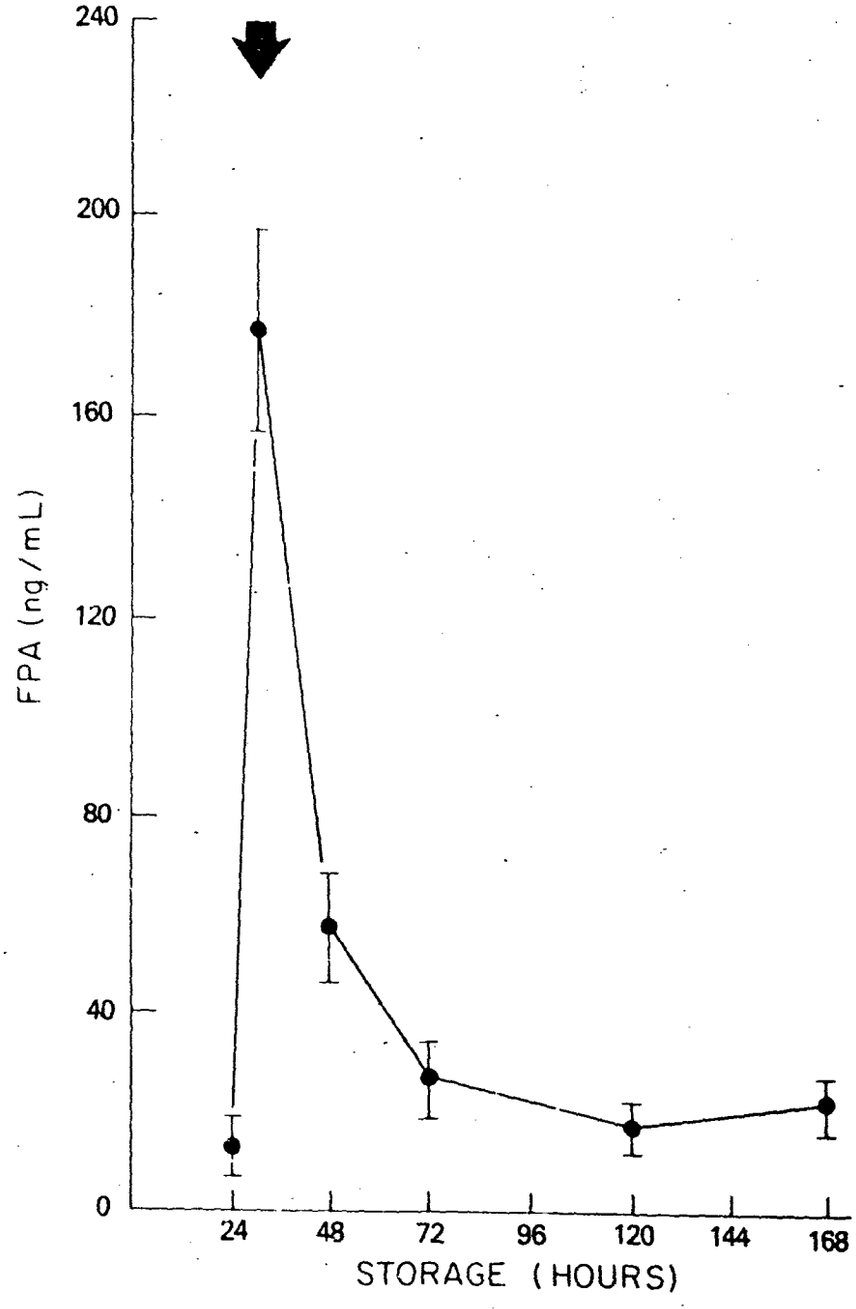


TABLE 1

FPA LEVELS (ng/mL) IN RED CROSS PLATELET CONCENTRATES

Day 1	Day 5
13.55	14.28
+/−6.34	+/−5.67
N = 27	N = 16

Normal population < 3.0 ng/mL

Purified FPA added to four 24h old CPDA-1 PC's to give approximately 200 ng/mL. Only 32% (avg.) could be detected 24h later and only 15% (avg.) after 48h. These results indicate that FPA is unstable in plasma and that the FPA levels measured in PC are an underestimate of the amount of thrombin activity actually present during storage.

(b). Hirudin Inhibition: Hirudin is a very specific, high affinity thrombin inhibitor. If the measured FPA levels are due to the action of thrombin on fibrinogen, rather than non-specific degradation, then hirudin should inhibit the formation of FPA in stored PC. Table 2 shows that hirudin partially but significantly inhibited the formation of FPA throughout the preparation of PC.

TABLE 2.

FPA FORMATION IN PLATELET CONCENTRATE PREPARATION WITH AND WITHOUT HIRUDIN

Mean FPA Levels (ng/mL)

	Whole Blood	Platelet-Rich Plasma	Platelet Concentrate
Citrate only	6.62 +/-3.32(SD)	7.93 +/-4.09(SD)	7.89 +/-3.58(SD)
Citrate + Hirudin	4.21 +/-1.86(SD)	5.83 +/-2.62(SD)	5.43 +/-2.57(SD)

Two collections of blood were made from each of eight donors (4 male, 4 female). The first collection was made into a normal CPDA-1 anticoagulant solution while the second was made into CPDA-1 and Hirudin (at a concentration of 8 units/mL in whole blood). Samples for FPA analysis were drawn at various stages of PC preparation for later analysis. A p-value < 0.001 was calculated from a paired t-test comparing the anticoagulant solutions over all donors and all samples, indicating that hirudin was associated with lower FPA levels.

(c). PF3 prothrombinase activity: Thrombin formation in PC is greatly accelerated relative to platelet-poor plasma by the formation of prothrombinase activity on the surface of platelets. Figure 1 shows that PF3 prothrombinase activity increases monotonically and irreversibly throughout seven days of storage in PC prepared and stored by a variety of means. Some PC showed PF3 activity nearly equivalent to that seen in the PF3 activity standard (frozen thawed platelets). LDH values were uniformly low, indicating that PF3 was not elevated due to lysis. Further details can be found in the accompanying manuscript.

(d). Release of Extracellular PF3 During Storage: Approximately half of the PF3 activity in each PC could be recovered from the supernatant plasma, suggesting the release of extracellular PF3 during storage. Supernatant PC plasma was chromatographed in five separate experiments on Bio-Gel A-150m, a large pore agarose gel (see Figure 3 of accompanying manuscript). In all cases, most of the PF3 activity eluted in the apparent void volume fraction along with light scattering and phosphate-

Fresh platelet concentrates were obtained from the local American Red Cross (ANRC) or were prepared as detailed below. All bags were stored with flat bed to-fro agitation at 21 C. Mean platelet counts were not different among the four types of PC.

ANRC PL-146

10 manually collected single unit concentrates in CPDA-1 in Fenwal PL-146 transfer packs.

ANRC PL-732

25 manually collected single unit concentrates in CPDA-1 in Fenwal PL-732 transfer packs.

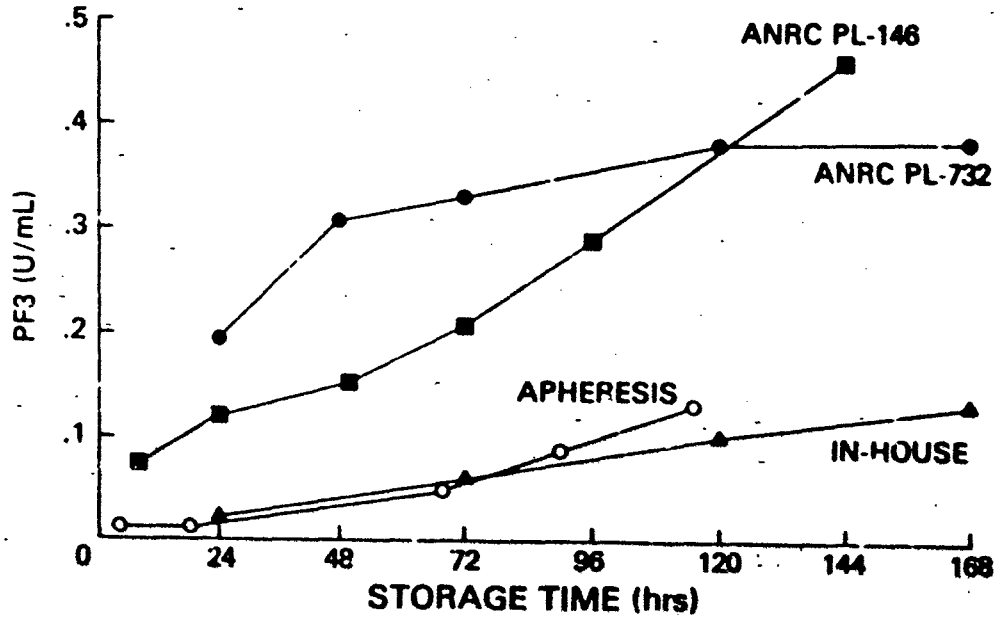
APHERESIS

8 platelet collections run on an IBM-2997 Blood Cell Separator using dual-stage channels and ACD-A anticoagulant at a 1:8 ratio with whole blood. 50 mL of each collection was stored in 300 mL Fenwal PL-732 transfer packs.

IN-HOUSE

10 platelet concentrates harvested from double-plateletpheresis using ACD-A. The first 10 mL of blood of each donation was discarded to avoid contamination of the bag with tissue factor. Platelets were stored in Fenwal PL-732 transfer packs.

FIGURE 1



containing material indicating that PF3 is related to a high molecular weight or particulate complex as has been noted by Sandberg et al. (Biochemical Journal. 203: 303-311, 1982) for collagen-activated platelets.

(e). AT-III Levels: Fourteen platelet concentrates were assayed for AT-III levels at one and five days of storage. The mean level showed a small decrease from 104.3% on day 1 to 103.1% on day five. A one-tailed, paired t-test gave a value of p=0.064, bordering on significance.

Platelet Activation and Storage

In 2 preliminary experiments with hirudin, PGE_1, and theophylline added to CPDA-1, we have been able to demonstrate by in vitro markers acceptable storage of platelets for fifteen days (see Figure 2, and Table 3). At the end of twenty days of storage, the platelets stored with these inhibitors showed substantial "storage lesion" but did not show the structural disintegration and fall in pH to <6.0 exhibited by the platelets stored in CPDA-1 only. It is generally considered that a pH of <6.0 and poor morphology score is associated with non-viable platelets which will not circulate effectively.

Table 3.

EXTENDED STORAGE OF PC COLLECTED IN CPDA-1 PLUS HIRUDIN, THEOPHYLLINE, AND PGE-1

	DAY 10		DAY 15		DAY 20	
	EXPTL	STD	EXPTL	STD	EXPTL	STD
pH	7.09	6.26	6.80	<6.00	6.27	<6.00
pCO2(mmHg)	14	15	10	5	5	4
pO2(mmHg)	142	98	130	180	180	183
MORPH.(%)	28	91	38	*	59	*
HYPOTONIC SHOCK(%)	54	36	20	0	0	0

* morphology consistent with structural disintegration
STD standard CPDA-1 anticoagulant, n=4
EXPTL experimental anticoagulant, n=2

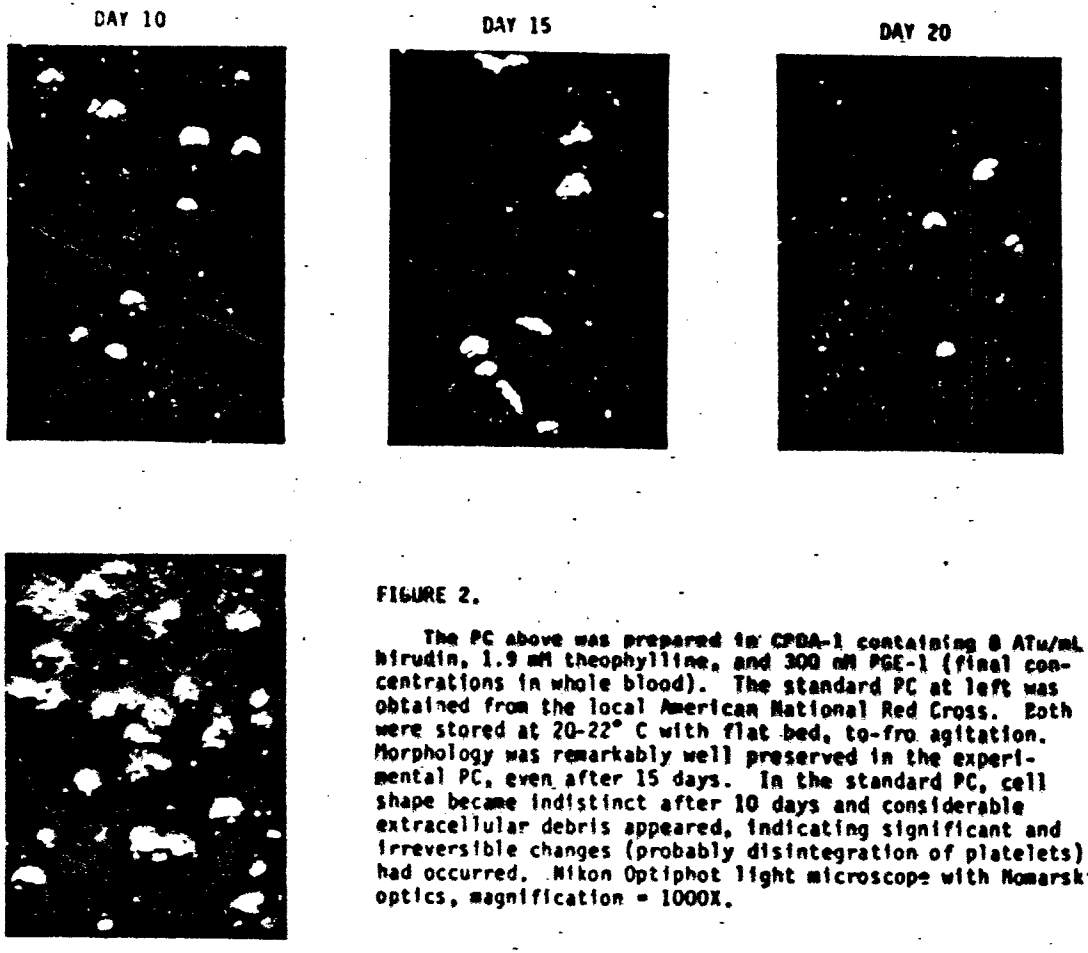


FIGURE 2.

The PC above was prepared in CPDA-1 containing 8 ATu/mL hirudin, 1.9 mM theophylline, and 300 nM PGE-1 (final concentrations in whole blood). The standard PC at left was obtained from the local American National Red Cross. Both were stored at 20-22° C with flat bed, to-fro agitation. Morphology was remarkably well preserved in the experimental PC, even after 15 days. In the standard PC, cell shape became indistinct after 10 days and considerable extracellular debris appeared, indicating significant and irreversible changes (probably disintegration of platelets) had occurred. Nikon Optiphot light microscope with Nomarski optics, magnification = 1000X.

DISCUSSION

Our data substantially support the hypothesis that thrombin is continuously and progressively formed in the preparation and storage of PC when citrate is the sole anticoagulant. The remaining issue is to find cumulative evidence of thrombin generation since FPA levels are dynamic and provide only qualitative evidence of thrombin activity. We will attempt a solution to this problem with our study of the fate of exogenously added radiolabelled prothrombin in PC. These experiments should provide direct quantitation of the extent of thrombin generation during platelet storage. Our experiments with addition of minute amounts of thrombin to fresh PC and all the accumulated evidence of platelet activation and prothrombinase expression in citrated PC have shown us that the effects of endogenously formed thrombin can not be ignored. Other platelet activation agonists such as platelet-activating factor, ADP, and shear forces, are probably present in PC, but they are generally considered as weak agonists compared to thrombin. Our conclusion is that more effective anticoagulation must be employed in the collection of blood for preparation of PC for significant improvements to take place in platelet storage.

By combining thrombin inhibition with reversible inhibition of platelet activation, we have succeeded in prolonging the shelf-life of platelets, at least by in vitro markers, to 15 days in 2 experiments. We are in the process of repeating these studies with at least 4 more donors. The combination of thrombin inhibition and inhibition of platelet activation has proved to be more fruitful than either approach alone. Perhaps this finding could explain why previous investigators have had less than promising results with addition of prostaglandins (PGE-1, PGI-2) to PC. Actually, prostaglandins by themselves provide only transitory elevations of cyclic AMP and thus only temporarily inhibit platelet activation (Mills, DCB. in Platelet Function Testing. DHEW Pub. No. (NIH) 78-1087, pp. 504-512, 1978). It seems that platelet activation and thrombin generation in PC are closely related and mutually supportive, and must be controlled simultaneously. It is beyond the scope of the present contract to test the in vivo viability of platelets stored in the presence of thrombin inhibitors and platelet activation inhibitors, but we feel that this will be an important issue in the future.

In brief, we have made progress on all major objectives in the first year. For the second year, we will focus on rigorous confirmatory evaluation of our preliminary findings in order to adequately test our hypothesis that thrombin generation and platelet activation directly contribute to the onset and severity of the platelet storage lesion. In addition to the abstract and manuscript that accompany this report, we are writing two more

manuscripts to present our results on markers of thrombin generation in PC and on the effects of our inhibitor cocktail on platelet storage. We hope that the USAMRDC finds our progress satisfactory.

Abstract form

TYPE ABSTRACT HERE/STAY WITHIN BORDER

EVIDENCE FOR THROMBIN GENERATION DURING STORAGE OF PLATELET CONCENTRATES (PC). A.P.Bode and D.T.Miller. Dept. Pathology, Univ. of North Carolina, Chapel Hill, NC 27514, USA.

Platelets stored for transfusion are usually prepared from units of citrated whole blood and concentrated into 50 mL of plasma. In this environment, ineffective anticoagulation could lead to prolonged exposure of platelets to low levels of thrombin, with resultant platelet activation. To detect thrombin generation, we measured formation of fibrinopeptide A (FPA) in 27 standard PC obtaired from the Amer. Nat. Red Cross, using a radioimmunoassay kit. We found 13.6 ng/mL ± 6.3 (SD) at 24h after collection, and 14.3 ng/mL ± 5.7 (SD) after 5d of storage. These levels were 4-5 times higher than the upper limit of circulating FPA in the normal population (3.0 ng/mL). Although it appeared that FPA levels did not increase with time, we have found that FPA is not stable in PC. When purified FPA was added to 4 fresh PC's to give approx. 200 ng/mL, only 32% could be detected 24h later, and 15% after 48h. The results suggest that the FPA levels we measured in PC were an underestimate of the amount of thrombin activity actually present during storage. Thrombin generation can be responsible for, and supported by, the expression of platelet factor 3 (PF3) by activated platelets. We have found that PF3 was expressed in significant amounts in the same PC studied above, and the PF3 levels rose monotonically during storage: (frozen-thawed platelets = 1 unit PF3/mL) mean PF3 = 0.25 u/mL at 24h, 0.28 u/mL at 72h, 0.39 u/mL at 120h. We conclude that there exists a thrombin-platelet activation feedback loop in citrated PC which may contribute to the loss of function observed in stored platelets.

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