Release of β -thromboglobulin during the preparation, in vitro storage and cryopreservation of platelet concentrates

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SUMMARY Beta-thromboglobulin is a platelet specific protein which is released from platelets during the platelet release reaction. The amount of β -thromboglobulin released during various conditions of in vitro storage, including cryopreservation, was compared. The results suggest the measurement of β -thromboglobulin is unlikely to be of use as a quality control assay for monitoring the in vitro viability of stored platelets.

Platelet concentrates (PCS) are now routinely stored in vitro at 22°C although there has been much controversy as to whether they should be stored at 4°C, 22°C or cryopreserved. None of these storage conditions is ideal.^{1–9}

The platelet specific proteins, β -thromboglobulin (β TG), low-affinity platelet factor 4 and platelet factor 4 (PF4), are stored in the alpha granules and are released from platelets during the platelet release reaction which normally occurs after platelet aggregation.^{10–12} β TG appears to be a proteolytic product of low-affinity platelet factor 4¹³ while PF4 is secreted as a proteoglycan PF4 complex which dissociates in plasma.¹⁴ Although PF4 and β TG are released during the platelet release reaction, β TG is more abundant and has superior stability in vivo to PF4.^{15 16} Moore *et al*¹⁴ reported that no thrombin-induced aggregation or release of β TG could be detected in preparations of washed platelets after storage for 48 h. However, β TG was detected in the supernatant plasma of PCS after 48 h which suggested that in vitro ageing was accompanied by leakage of platelet constituents. Therefore, in this study the release of platelet specific β TG into the supernatant plasma during in vitro storage of PCS at 4°C and 22°C for 48 h and in platelets that have been cryopreserved was studied in order to determine the level of release under various in vitro storage conditions.

Material and methods

PLATELET CONCENTRATE PREPARATION AND STORAGE CONDITIONS

Platelet-rich plasma (PRP) was prepared from fresh whole blood collected from random donors in citrate-phosphate-dextrose (CPD) anticoagulant within six hours of donation by centrifugation at 2900 rpm (2260 g) for 90 s at 22°C in a Damon PR 6000 centrifuge with interior wind-shielded, swing-out head (Damon IEC Ltd, Dunstable, Bedfordshire). Each PRP preparation for storage without cryopreservation was divided into two aliquots, one of which was acidified with 18 ml acid citrate dextrose (ACD; acidified preparations). PCS were prepared by centrifuging the PRP at 3500 rpm (3000 g) for 20 min at 22°C. Thirty ml of the supernatant was retained with the platelet button. The button was resuspended immediately when acidified platelets were prepared whereas non-acidified preparations were allowed to stand undisturbed at room temperature for 1–1.5 h before resuspension.¹⁷ PCS were further subdivided so that two aliquots were obtained from both the acidified and non-acidified preparations. The initial pH of the acidified preparations was 6.65 ± 0.1 and 7.25 ± 0.1 in the case of the non-acidified preparations. The platelet counts (determined using a Technicon Autocounter) were $970 \pm 240 \times 10^9$ platelets/l for acidified preparations ($n = 12$) and $990 \pm 240 \times 10^9$ platelets/l for non-acidified preparations ($n = 12$). An aliquot of each acidified and non-acidified preparation was stored at 4°C and at 22°C with rota-

tion end over end on a platelet agitator (Helmer Labs, marketed by Horwell Ltd, London) for 48 h.

Cryopreservation of platelets in 3% (wt/vol) glucose – 3% (vol/vol) glycerol and in 5% (vol/vol) DMSO was carried out as previously described.^{9,18} Acidified PCS, prepared as above, were divided into two aliquots so that each unit was cryopreserved with both cryoprotective agents.

β -THROMBOGLOBULIN ASSAY

β TG concentrations were measured using a radioimmunoassay kit (Amersham International Ltd, Amersham, Bucks).

Platelet samples (2.5 ml) were placed in the blood collection tubes provided and the tubes placed on ice for 30 min prior to centrifugation at 1500–2000 g for 30 min at 4°C. The supernatants were removed and stored in the vapour phase of liquid nitrogen. Two dilutions (in phosphate-buffered saline, PBS, pH 7.3 Dulbecco "A", Oxoid Ltd, London), of each supernatant sample were used in the assay.

The cryopreserved platelets were thawed at 37°C in a waterbath and assayed without removal of cryoprotectant.

A maximum releasable β TG value in a given PC was obtained by freezing a sample in liquid nitrogen in the absence of cryoprotective agent and thawing at 37°C. The freeze-thaw process was repeated three times before dilution of the sample 1/10 with PBS pH 7.3 containing 1% (vol/vol) Triton X-100. The sample was sonicated for 30 s in a sonicator (MSE Scientific Instruments, Crawley, West Sussex). An aliquot of the sonicated sample (2.5 ml) was processed and assayed for β TG content as described above.

To determine β TG release after thrombin-induced platelet aggregation one volume of ACD was added to eight volumes of PC and the sample centrifuged at 900 g for 15 min at room temperature in an MSE Minor Centrifuge. The button was resuspended in modified Tyrode's solution (0.15 M NaCl, 3 mM KCl, 0.1 M NaHCO₃, 0.3 mM NaH₂PO₄, 5 mM glucose pH 7.35) to give the same platelet count as that of the PC in autologous plasma. In the aggregation assays¹⁹ an amount of thrombin (human, 10 U/ml Sigma Chemical Co Ltd, Poole, Dorset) sufficient to give maximum aggregation (approximately 100 μ l/ml platelet concentrate) was added. When sedimentation of the aggregate had occurred (approximately 2 min) an aliquot of the supernatant material was processed and assayed for β TG content as described above.

Statistical analysis of the results was carried out using Student's *t* test.

Results

The results obtained for PCS stored at 4°C or 22°C for 48 h are shown in Fig. 1. Although significantly less β TG was released by the platelets during the preparation of acidified concentrates than during the preparation of concentrates which had not been acidified ($p < 0.001$), less than 10% of the maximum releasable β TG was detected during preparation of either concentrate. Platelets stored at 22°C released significantly more β TG than fresh platelets ($p < 0.001$; approximately 30% of the maximum releasable). No significant difference between platelets stored with or without acidification was apparent. There was however, significantly less β TG released from acidified platelets stored at 4°C (approximately 11% of the maximum releasable) than from those platelets stored at 4°C without

Fig. 1 Total β -thromboglobulin in platelet preparations prior to and after 48 h storage at 4°C or 22°C

acidification ($p < 0.001$; approximately 25% of the maximum releasable).

The results for cryopreserved platelets are shown in Fig. 2. The addition of either cryoprotectant to fresh platelets caused a small release of β TG. Cryopreservation in 3% glucose 3% glycerol resulted in the release of approximately 24% of the maximum releasable β TG while cryopreservation in 5% DMSO released only 12% suggesting that there was more cellular disruption during cryopreservation with 3% glucose 3% glycerol than with 5% DMSO. β TG released after cryopreservation in 5% DMSO with cooling at $1^\circ\text{C}/\text{min}$ or by placing in the vapour phase of liquid nitrogen were not significantly different. Thrombin-induced aggregation released only 45% of the maximum releasable β TG.

Fig. 2 Total β -thromboglobulin in platelet preparations prior to and after cryopreservation

Discussion

The β TG content of normal platelets has been determined by a number of authors: values of 18 and $45\text{--}113 \mu\text{g}/10^9$ platelets were reported for sonicated washed platelets²⁰ and sonicated PRP²¹ respectively while values of 54 ,²² 57.1 ± 10.6 ²³ and $23.5 \pm 0.9 \mu\text{g}/10^9$ ²⁴ platelets were reported for platelets solubilised in 1% Triton X-100. In the present study a value of $102 \pm 37 \mu\text{g}/10^9$ platelets was obtained which is higher than that obtained by others and may indicate that a more substantial disruption of platelet granules was achieved.

Snyder *et al*²⁴ determined the level of β TG release during various stages of PC preparation from whole blood collected in either CPD or CPD-adenine-1 anticoagulant. Very little release of β TG occurred until the platelet button was resuspended when a substantial increase was observed. The release of β TG was found to occur to a greater degree than the leakage of cytosolic lactate dehydrogenase.

PCS prepared as single donor PRP using the Haemonetics model 30 and ACD anticoagulant (pH 7.08) showed significantly less β TG release as compared to age-matched random donor platelets collected in CPD or CPD-adenine-1 (pH 7.17).²⁴ However, further work is required to establish whether the results are due to the anticoagulant or the centrifugation conditions and may indicate whether the centrifugation conditions used during platelet preparation significantly influence the release of β TG.

The release of three cytoplasmic enzymes (nucleoside diphosphokinase, 3-phosphoglycerate kinase and enolase) during preparation of PCS has been studied by Mourad and Pert²⁵ who found that there was a greater release of these enzymes during the preparation of concentrates from whole blood collected in CPD anticoagulant (pH 7.3) than when the anticoagulant was ACD (pH 6.9). Furthermore, reduction of the pH by addition of extra anticoagulant (pH 6.65) caused a corresponding decrease in the averaged values for the amount of the three enzymes released which is similar to the findings reported here for β TG (Fig. 1). Eriksson *et al*²⁶ reported virtually no PF4 release in PRP prepared from blood taken into CPD-PGE₁ anticoagulant while a minor release was noted in PRP prepared from blood taken into CPD anticoagulant.²⁶ These results suggest that pH and the nature of the anticoagulant used can affect the release of intracellular constituents during PC preparation. However, it has been reported that there is little difference in the *in vivo* viability of concentrates prepared under the same conditions from PRP in ACD, CPD or acidified ACD anticoagulant.²⁷ Thus, although

measurement of release of β TG and other constituents during different methods of platelet preparation may provide evidence of in vitro damage during preparation the extent of this damage is unlikely to be sufficient to affect the in vivo viability.

The results presented here for aliquots of the same PC stored at 4°C and 22°C are in agreement with the findings reported for age-matched platelets under similar storage conditions.²⁴ However, concentrates stored at 4°C for 72 h release significantly less β TG ($p < 0.05$) than concentrates stored at 22°C for 72 h.²⁴ Platelets are more metabolically active at 22°C than at 4°C which may account for some increase in release of β TG on storage at 22°C rather than at 4°C.

The results show there was significantly more release of β TG during storage of acidified platelets for 48 h at 22°C than at 4°C (Fig. 1). The latter storage condition resulted in the least release of β TG.

Measurement of PF4 release during preparation of PCS and storage at 22°C has shown that only small amounts of PF4 are released during preparation and up to 48 h storage.²⁶ The percentage leakage of the cytosolic protein lactate dehydrogenase was reported lower than the β TG release during in vitro storage.²⁴

Conclusion

The release of β TG from platelets stored at 4°C and 22°C for 48 h show no correlation with other in vitro and in vivo viability studies.^{19,3}

The results obtained here (Fig. 2) show that cryopreservation in 5% DMSO releases less β TG than cryopreservation in 3% glucose 3% glycerol and are in general agreement with those obtained previously using other in vitro assays (hypotonic shock response and serotonin uptake) which suggested that 5% DMSO is the most satisfactory cryoprotective agent.⁹ Comparison of the percentage β TG release (relative to the maximal releasable β TG) with the yields (based on platelet number⁹) obtained after cryopreservation (24% and 30% respectively for 3% glucose 3% glycerol; 12.7% and 54% for 5% DMSO with freezing at 1°C/min; 13% and 42% for 5% DMSO with freezing in the vapour phase) show that the loss of platelet numbers was much higher than the percentage β TG release. This indicates that although platelets are damaged during cryopreservation they do not release all the available β TG, presumably a substantial proportion of the α -granules remain intact.

These results suggest that while the measurement of β TG release may be of limited use for monitoring platelet preparation methods and the disruptive

effects of cryopreservation it is unlikely to be of use as a quality control assay for monitoring the in vitro viability of stored platelets.

I thank Dr ID Fraser for his support and encouragement, Dr DJ Anstee for his aid in preparing this paper, Mrs M Clarke for drawing the diagrams and Helen Lane for typing the manuscript.

References

- 1 International Forum: which are the parameters to be controlled in platelet concentrates in order that they may be offered to the medical profession as a standardised product with specific properties? *Vox Sang* 1981;**40**:115-26.
- 2 Kattlove HE. Platelet preservation—what temperature? A rationale for strategy. *Transfusion* 1974;**14**:328-30.
- 3 Slichter SJ, Harker LA. Preparation and storage of platelet concentrates II. Storage variables influencing platelet viability and function. *Br J Haematol* 1976;**34**:403-19.
- 4 Filip DJ, Aster RH. Relative haemostatic effectiveness of human platelets stored at 4°C and 22°C. *J Lab Clin Med* 1978;**91**:618-24.
- 5 Handin RI, Valeri CR. Improved viability of previously frozen platelets. *Blood* 1972;**40**:509-13.
- 6 Kim BK, Tanoue K, Baldini MG. Storage of human platelets by freezing. *Vox Sang* 1976;**30**:401-11.
- 7 Murphy S, Sayar SN, Abdou NL, Gardner FH. Platelet preservation by freezing. Use of dimethylsulphoxide as cryoprotective agent. *Transfusion* 1974;**14**:139-44.
- 8 Schiffer CA, Aisner J, Wiernik PH. Clinical experience with transfusion of cryopreserved platelets. *Br J Haematol* 1976;**34**:377-85.
- 9 Taylor M Ann. Cryopreservation of platelets: an in vitro comparison of four methods. *J Clin Pathol*, 1981;**34**:71-5.
- 10 Zahavi J, Kakkar VV. β -thromboglobulin—a specific marker of in vivo platelet release reaction. *Thromb Haemost* 1980;**44**:23-9.
- 11 Packham MA, Mustard JF. In vitro methods and the study of platelet mechanisms. *Vox Sang* 1981;**40**: suppl 1:22-35.
- 12 Kaplan K. β -thromboglobulin. In: Spaet TH, ed. *Progress in haemostasis and thrombosis*. New York: Grune and Stratton, 1980;**5**:153-78.
- 13 Niewiarowski S, Walz DA, James P, Rucinski B, Kueppers F. Identification and separation of secreted platelet proteins by isoelectric focusing. Evidence that low-affinity platelet factor 4 is converted to β -thromboglobulin by limited proteolysis. *Blood* 1980;**55**:453-6.
- 14 Moore S, Pepper DS, Cash JD. The isolation and characterisation of a platelet specific β -globulin (β -thromboglobulin) and the detection of anti-urokinase and anti-plasmin released from thrombin-aggregated washed human platelets. *Biochim Biophys Acta* 1975;**379**:360-9.
- 15 Dawes J, Smith RC, Pepper DS. The release, distribution and clearance of human β -thromboglobulin and platelet factor 4. *Thromb Res* 1978;**12**:851-61.
- 16 Kaplan KL, Nossel HL, Drillings M, Lesznik G. Radioimmunoassay of platelet factor 4 and β -thromboglobulin: development and application to studies of platelet release in relation to fibrinopeptide A generation. *Br J Haematol* 1978;**39**:129-46.
- 17 Mourad N. A simple method of obtaining platelet concentrates free of aggregates. *Transfusion* 1968;**8**:48.
- 18 Herve P, Masse M, Kieffer Y, et al. Cryopreservation des plaquettes dans l'azote liquide en presence de glycerol-glucose. Colloque de cryoimmunologie, Dijon, 1976. INSERM, **62**:163-70.

- ¹⁹ Tandy NP, Taylor MA. Platelet concentrates for transfusion: control of production and storage. *Med Lab Sci* 1980;**37**:127–36.
- ²⁰ Boughton BJ, Allington MJ, King A. Platelet and plasma β -thromboglobulin in myeloproliferative syndromes and secondary thrombocytosis. *Br J Haematol* 1978;**40**:125–32.
- ²¹ Pareti FI, Dawes J, Franchi F, Mannucci PM, Pepper DS. Content and release of β -thromboglobulin and platelet factor 4 in patients with qualitative platelet defects. *Thromb Res* 1979;**16**:537–42.
- ²² Weiss HJ, Witte LD, Kaplan KL, *et al.* Heterogeneity in storage pool deficiency: studies on granule-bound substances in 18 patients including variants deficient in α -granules, platelet factor 4, β -thromboglobulin and platelet derived growth factor. *Blood* 1979;**54**:1296–319.
- ²³ Bolton AE, Amess JAL, Lekhwani CP, Elliott P. β -thromboglobulin content of human blood platelets. *Scand J Haematol* 1980;**25**:25–9.
- ²⁴ Snyder EL, Hezzy A, Katz AJ, Bock J. Occurrence of the release reaction during preparation and storage of platelet concentrates. *Vox Sang* 1981;**41**:172–7.
- ²⁵ Mourad N, Pert JH. An enzymatic method to determine damage in human platelets. *Proc Soc Exp Biol Med* 1967;**125**:643–5.
- ²⁶ Eriksson L, Hogman CF, Busch C. Lack of conformity in the behaviour of platelets during normal storage conditions at 22°C. *Vox Sang* 1981;**40**:65–70.
- ²⁷ Slichter SJ, Harker LA. Preparation and storage of platelet concentrates 1. Factors influencing the harvest of viable platelets from whole blood. *Br J Haematol* 1976;**34**:395–402.

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