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 (βTG), 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 βTG appears to be a proteolytic product of low-affinity platelet factor 4 while PF4 is secreted as a proteoglycan PF4 complex which dissociates in plasma.13 Although PF4 and βTG are released during the platelet release reaction, βTG is more abundant and has superior stability in vivo to PF4.12,13 Moore et al14 reported that no thrombin-induced aggregation or release of βTG could be detected in preparations of washed platelets after storage for 48 h. However, βTG 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 βTG 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 ± 240 × 10^10 platelets/l for acidified preparations (n = 12) and 990 ± 240 × 10^10 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.\(^*\) Acidified PCS, prepared as above, were divided into two aliquots so that each unit was cryopreserved with both cryoprotective agents.

**β-TROMBOGLOBULIN ASSAY**

βTG 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 βTG 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 βTG content as described above.

To determine βTG 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 NaHCO\(_3\), 0.3 mM NaH\(_2\)PO\(_4\), 5 mM glucose pH 7.35) to give the same platelet count as that of the PC in autologous plasma. In the aggregation assays an amount of thrombin (human, 10 U/ml Sigma Chemical Co Ltd, Poole, Dorset) sufficient to give maximum aggregation (approximately 100 μl/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 βTG 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 βTG 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 βTG was detected during preparation of either concentrate. Platelets stored at 22°C released significantly more βTG 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 βTG released from acidified platelets stored at 4°C (approximately 11% of the maximum releasable) than from those platelets stored at 4°C without...
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The results for cryopreserved platelets are shown in Fig. 2. The addition of either cryoprotectant to fresh platelets caused a small release of βTG. Cryopreservation in 3% glucose 3% glycerol resulted in the release of approximately 24% of the maximum releasable βTG 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. βTG released after cryopreservation in 5% DMSO with cooling at 1°C/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 βTG.

Discussion

The βTG content of normal platelets has been determined by a number of authors: values of 18 and 45–113 μg/10^9 platelets were reported for sonicated washed platelets and sonicated PRP respectively while values of 54, 57·1 ± 10·6 and 23·5 ± 0·9 μg/10^9 platelets were reported for platelets solubilised in 1% Triton X-100. In the present study a value of 102 ± 37 μg/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 determined the level of βTG release during various stages of PC preparation from whole blood collected in either CPD or CPD-adenine-1 anticoagulant. Very little release of βTG occurred until the platelet button was resuspended when a substantial increase was observed. The release of βTG 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 βTG release as compared to age-matched random donor platelets collected in CPD or CPD-adenine-1 (pH 7·17). 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 βTG.

The release of three cytoplasmic enzymes (nucleoside diphosphokinase, 3-phosphoglycerate kinase and enolase) during preparation of PCS has been studied by Mourad and Pert 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 βTG (Fig. 1). Eriksson et al reported virtually no PF4 release in PRP prepared from blood taken into CPD-PGE, anticoagulant while a minor release was noted in PRP prepared from blood taken into CPD anticoagulant. 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. Thus, although
measurement of release of βTG 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. However, concentrates stored at 4°C for 72 h release significantly less βTG (p < 0.05) than concentrates stored at 22°C for 72 h. Platelets are more metabolically active at 22°C than at 4°C which may account for some increase in release of βTG on storage at 22°C rather than at 4°C.

The results show there was significantly more release of βTG 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 βTG.

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. The percentage leakage of the cytosolic protein lactate dehydrogenase was reported lower than the βTG release during in vitro storage.

**Conclusion**

The release of βTG from platelets stored at 4°C and 22°C for 48 h show no correlation with other in vitro and in vivo viability studies.

The results obtained here (Fig. 2) show that cryopreservation in 5% DMSO releases less βTG 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. Comparison of the percentage βTG release (relative to the maximal releasable βTG) with the yields (based on platelet number) 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 βTG release. This indicates that although platelets are damaged during cryopreservation they do not release all the available βTG, presumably a substantial proportion of the α-granules remain intact.

These results suggest that while the measurement of βTG 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.

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**References**

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