The Effect of Cold on Platelets. II. Platelet Function After Short-term Storage at Cold Temperatures

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Citrated platelet-rich plasma (PRP) was kept at cold temperatures or room temperature. After 4 hr or more at these temperatures, the PRPs were warmed 1 hr at 37°C. This prevents the spontaneous aggregation seen in chilled PRP that is stirred immediately after warming. Platelet aggregation in response to connective tissue (CT), epinephrine, and adenosine diphosphate (ADP) was considerably greater in the PRPs originally kept at cold temperatures. In addition, chilling would restore the aggregation of platelets whose function had deteriorated due to prolonged storage at warm temperatures. Neither ADP-induced refractoriness, serotonin uptake, or CT-induced serotonin release was affected by cold. Retention in glass bead columns was greater in platelets that had been chilled than in platelets kept at room temperature or 37°C. Thus, the storage of platelets at cold temperatures leads to changes that improve platelet aggregation but may also increase platelet adhesion, which would account for the decreased in vivo survival of platelets preserved for transfusion at cold temperatures.

In a previous study, we indicated that cold temperature induces platelet aggregation. This phenomenon is responsible for the clumping that occurs in platelet concentrates prepared in the cold. Since clumping decreases their in vivo recovery, we concluded that the preparation of platelet concentrates under such conditions is probably detrimental.

However, it is not clear what temperature is best for the preservation of platelets for transfusion. Traditionally, platelets prepared for transfusion have been stored at cold temperatures. However, recent studies have indicated that the preservation of platelets at cold temperatures, either as platelet-rich plasma or as concentrates, shortens their in vivo survival. Thus, in many institutions, platelet concentrates are stored at room temperature, and this is the recommendation of the American Association of Blood Banks (Manual of Blood Component Preparation, 1969.).

In addition, it is not clear what temperature of storage best preserves platelet function. Accordingly, this study was undertaken to determine which temperature of storage is better for preservation of in vitro platelet function, and whether storage at cold temperature causes a change in platelets that might effect their ability to circulate in vivo. Our results indicate that (1) platelet function, as measured by aggregation in response to ADP, connective...
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tissue, and epinephrine, is better preserved at cold temperatures; and (2) cold induces a change in platelets that leads to their increased retention in glass bead columns.

MATERIALS AND METHODS

Platelet-rich plasma (PRP) was prepared from small aliquots of blood obtained in separate plastic tubes from random blood donors at the end of a routine blood donation at the New York Blood Center. The blood was anticoagulated with one-tenth volume of 3.8% sodium citrate and was centrifuged for 15 min at 150 g in an International PR-2 centrifuge at room temperature. The PRPs from several donors, irrespective of blood type, were harvested and pooled.

Platelet aggregating agents were as follows:
(1) Connective tissue (CT) was prepared from human omental fat by a modification of the method of Zucker and Borelli. The final product was diluted in imidazole-buffered saline (IBS, pH 7.3) to the lowest concentration that would cause brisk platelet aggregation.
(2) Adenosine diphosphate (ADP, Sigma) was dissolved in IBS (pH 6.8) and stored at -60°C. This was thawed before use and diluted to the appropriate concentration in IBS (pH 7.3).
(3) Epinephrine (Sigma) was dissolved in IBS (pH 7.3) with 10 mM ascorbic acid to retard oxidation and was stored at -60°C. This was thawed before use and diluted to the appropriate concentration in IBS (pH 7.3).

All equipment used to handle PRP was plastic, except for aggregometer cuvettes that were untreated glass.

Platelet Aggregation

Platelet aggregation was measured turbidimetrically in an aggregometer (Chrono-Log, Broomall, Pa.) attached to a Hitachi-Perkin-Elmer recorder. One milliliter samples of PRP were placed in untreated glass cuvettes, along with a Teflon-coated, magnetized stir bar, and the cuvettes were placed in the aggregometer in which the PRP was kept at 37°C and stirred at 1000 rpm. Aggregating agents were added in 0.1 ml volumes. In all studies, the presence of aggregation was confirmed by observation with phase microscopy.

Reversal of Spontaneous Cold-induced Aggregation

Spontaneous cold-induced aggregation was studied after keeping PRP in melting ice (0-1°C) for 2-4 hr. A 1.0 ml aliquot was then placed in the aggregometer without warming, and spontaneous aggregation was promptly measured. The effect of warming on reversing the spontaneous aggregation of chilled PRP was tested by incubating aliquots of the chilled PRP in a 37°C water bath, without agitation, and measuring the spontaneous aggregation of this warming PRP after various intervals.

Preservation of Platelet Aggregation

The ability of cold to preserve platelet aggregation was examined. Aliquots of PRP were kept in melting ice, in a refrigerator (6°C), or at room temperature (RT, 23°C). PRPs kept at these various temperatures for periods ranging from 4 to 20 hr were placed in a 37°C water bath for 1 hr, to reduce spontaneous aggregation in the chilled samples. The platelets were then tested for their ability to aggregate in response to ADP, epinephrine, and connective tissue. To determine if deterioration in platelet function was due to the lack of metabolic substrates, adenosine (Sigma, 100 μM final concentration) or dextrose (300 mg/100 ml final concentration) was added separately to PRP in some experiments.
**Inhibition of Platelet Aggregation**

The response of PRP to aggregating agents was inhibited to about 20% of control by two methods. The first method was to incubate PRP at 37°C for 4 hr. In the second method, 2-deoxyglucose (Sigma, 0.006 M) and KCN (0.002 M) were added to PRP, and the mixture was incubated at 37°C for 45 min.1

**ADP-induced Refractoriness**

The effect of cold on the ability of platelets to develop refractoriness to ADP after prior ADP exposure was studied. Aliquots of PRP were kept either in melting ice or at RT 3 hr and were warmed 1 hr in a 37°C water bath. At this time, ADP (10 μM final concentration) was rapidly pipetted into the PRP without stirring, and the mixtures were kept at 37°C. At frequent intervals, 1.0 ml aliquots of these mixtures were pipetted into an aggregometer cuvette containing the stir bar. One-tenth milliliter ADP (2 μM final concentration in the PRP) was added; the cuvette was promptly placed in the aggregometer, and platelet aggregation was measured.

**Platelet Retention**

Platelet retention was measured in 30-cm lengths of Tygon tubing (internal diameter, ¼ inch) filled with 3 g of glass beads (Superbrite, type 070, 3M Co., St. Paul, Minn.). A nylon filter and 20-gauge disposable needle were fixed at the distal end. Five milliliters of PRP in a plastic syringe were pumped through the tubing at room temperature with a Harvard pump at the rate of 1.0 ml/min. Four successive 1 ml aliquots from the effluent were collected separately. Platelets in the last two aliquots and in a sample that had not passed through the column were counted with a Coulter Counter, Model B. Platelet retention was calculated from an average of the last two aliquots.

The effect of cold on platelet retention was studied by keeping the PRP in a plastic tube at 0-1°C, 6°C, 23°C, or 37°C for 4 hr, placing it in a 37°C water bath for 1 hr, aspirating it into a plastic syringe, and then promptly passing it through the glass bead-filled tubing that was at room temperature. In some studies, the final incubation at 37°C was continued overnight (19 hr).

**Serotonin Uptake**

Serotonin uptake of chilled platelets was measured after keeping the PRP in melting ice for 4 hr and then at 37°C for 1 hr. 14C-Serotonin (Amersham Searle, 30 mCi/mM), dissolved in ethanol at a concentration of 8 μCi/ml, was added to this PRP (final concentration, 0.016 μCi/ml); the mixture was incubated at 37°C for 30 min, and the platelets were then sedimented at room temperature in a Clay Adams Sero-fuge. One-tenth milliliter of the supernatant was combined with liquid scintillator (4 ml absolute alcohol and 10 ml toluene containing 4 g/liter PPO-(2,5-diphenyloxazole) and 50 mg/liter POPOP-(1,4-bis [2,5-phenyloxazolyl] benzene), and the radioactivity was measured in a Beckmann 233 liquid scintillation counter. A control sample of PRP from the same pool, kept at room temperature for 4 hr, was tested similarly.

**CT-induced Serotonin Release**

CT-induced serotonin release was measured as follows: fresh PRP was labeled with 14C-serotonin for 30 min at 37°C, was thereafter kept in melting ice or at room temperature for 4 hr, and was then warmed for 1 hr at 37°C. Saline or various dilutions of the stock CT in saline was added; the PRP was stirred for 5 min, at which time CT-induced aggregation was complete. The platelets were sedimented in a Clay-Adams Sero-fuge, and the radioactivity of the PRP and the supernatant PPP (platelet-poor plasma) was measured. The per cent release was calculated from the expression (cpm PPP/cpm PRP) CT minus (cpm PPP/cpm PRP) saline.
Fig. 1. Progressive loss of spontaneous platelet aggregation in PRP is correlated with interval of incubation at 37°C. Chilled PRP was brought to 37°C without disturbance; then aliquots were tested in aggregometer and change in optical density (OD) was measured. OD is expressed as chart units. One chart unit represents a change of 1%/a (2.3 mm) on charting paper. Representative experiment.

RESULTS

Reversal of Cold-induced Aggregation

As reported earlier,¹ the platelets of chilled PRP spontaneously aggregated during simultaneous warming and stirring. When, however, the PRP was warmed to 37°C without disturbance before being placed in the aggregometer, the extent of aggregation progressively decreased as the duration of incubation at 37°C increased (Fig. 1). Maximum decrease of spontaneous aggregation occurred around 60 min. Even then, some spontaneous aggregation was evident (5–7 OD units of increase in light transmission on the aggregometer tracing, where 90 OD units represent complete aggregation). When the chilled PRP was warmed to and kept at room temperature, the decline in spontaneous aggregation with progressive time of incubation was slower.

Platelet Aggregation

The function of platelets kept at cold temperatures was compared to that of platelets kept at room temperature. The chilled platelets aggregated better in response to ADP, epinephrine, and connective tissue (Fig. 2). Clearly, the aggregation response to epinephrine and connective tissue was much better preserved in the platelets that had been chilled. This response was about 80% of that seen in freshly prepared specimens of PRP. PRP that had been kept at refrigerator temperature (6°C) similarly maintained its ability to respond to aggregating agents. When PRP was kept overnight in ice or at room temperature, its response to aggregating agents was markedly diminished. However, the chilled PRP still responded better than that kept overnight at room temperature. Neither adenosine nor dextrose added to the PRP kept at room temperature, either 4 hr or overnight, improved its responsiveness to aggregating agents.

The effect of chilling appeared to be a “rejuvenating” one. PRP was kept at 37°C for 4 hr, at which time the response to aggregating agents had decreased. The PRP was then placed on ice for 2 hr. It was then warmed to and kept
Fig. 2. Effect of cold on platelet aggregation induced by ADP (2 μM), connective tissue, and epinephrine (10⁻⁵ M). In each frame, curves on the left represent PRP kept in melting ice 4 hr and warmed 1 hr at 37°C. Curves on the right represent PRP kept at RT 4 hr and warmed 1 hr at 37°C. Representative experiment.

at 37°C for 1 hr and was retested with the same aggregating agents. The response of the PRP to ADP, epinephrine, and connective tissue was significantly improved (Fig. 3). However, when the ability of platelets to respond to aggregating agents was inhibited by KCN and 2-deoxyglucose, subsequent chilling did not improve aggregation (i.e., aggregation was still inhibited 80% as compared to control).

Serotonin Uptake and Release

Although chilling strikingly preserved CT-induced platelet aggregation, serotonin release was relatively unaffected (Table 1). The CT-induced release from platelets kept at room temperature was 35%, as compared with 41% for platelets kept in melting ice. Serotonin uptake was unaffected by prior chilling; it was 93%–95% in all experiments.

Development of Refractoriness

One specific feature of the ADP-induced aggregation of platelets that had been chilled and then rewarmed is that they disaggregate less than platelets that had been kept at room temperature. It has been suggested that disaggregation is due to the cells becoming refractory to ADP. Accordingly, this aspect was explored. Our results indicate that platelets that had been chilled and then warmed to 37°C became essentially as refractory to ADP as platelets kept initially at room temperature and then warmed to 37°C (Fig. 4). This temperature (37°C) was selected because refractoriness to ADP develops more rapidly at this temperature than at lower temperatures.

Platelet Retention

Platelet retention by glass beads was increased by cold temperature (Fig. 5). The least retention was seen in the PRP that was kept at 37°C, and the greatest retention was observed in the PRP that was kept in melting ice (Fig. 5). This increased retention is not reversible, for even when the chilled PRP was warmed for 19 hr at 37°C the degree of retention was not diminished.
Fig. 3. Restorative effect of chilling on warmth-induced platelet deterioration. Curve on the right represents PRP kept at 37°C for 3 1/2 hr and tested with ADP (2 μM), connective tissue, and epinephrine (10^-5 M). In each frame, curves on the left represent the same PRP, tested after chilling and rewarming. Representative experiment.

Morphology

The morphology of chilled platelets was examined by phase microscopy. Chilled platelets appeared spherical and formed pseudopodia. After 2 hr of chilling, they did not resume their normal disc shape, even when rewarmed and kept at 37°C for 20 hr. Shorter periods of chilling were not tested.

The spherical shape was also evident in the aggregometer tracings. In these tracings, the normal oscillations of the PRP kept at 37°C, which is due to their disc shape,11 were not seen in the chilled and rewarmed PRPs.

DISCUSSION

Clearly, cold temperatures profoundly affect platelets. The most obvious effect is morphologic. Cold causes platelets to sphere and to lose their microtubular system.12-17 This is in accord with the very early observations of Aynaud,12 who reported that prolonged (4 hr) chilling (ice bath) of platelets caused them to become contracted, rounded, granular, with irregular edges, alterations that are not equally intense and that are less intense with shorter intervals of chilling. The changes are partly reversed when platelets are chilled for less than 1 hr and then restored to 37°C.12,13,16-17 After 24 hr of chilling, the changes are essentially irreversible,16 with microtubular reassembly becoming sporadic on rewarming. In our study, even 2 hr of chilling appeared to cause shape changes that were irreversible no matter how long the platelets were subsequently rewarmed.

Of interest is the effect of cold in preserving platelet aggregating ability in vitro. Similar observations have been published by Shively et al.18 who

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<th>Experiment</th>
<th>Room Temperature (%)</th>
<th>Melting Ice (%)</th>
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<td>35</td>
<td>41</td>
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<td>2</td>
<td>35.5</td>
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Room temperature, 23°C; melting ice, 0-1°C.

Table 1. Connective Tissue-induced Release of 14C-Serotonin in Platelet-rich Plasma
measured platelet aggregation in rotating glass flasks. The reason platelet aggregating ability deteriorates in vitro is unknown. However, the loss of this function of stored platelets is apparently reversed in vivo after transfusion, since the transfused platelets supported normal in vitro aggregation after their in vivo circulation. Part of the effect of cold might be to prevent this deterioration.

Our observation, however, that the subsequent chilling of platelets whose aggregating ability had deteriorated at $37^\circ$C restored response to aggregating agents, suggests another mechanism.

This other mechanism is probably similar to the change caused by cold, which induces spontaneous aggregation. The latter is not merely a general increase in membrane "stickiness," since prior chilling should have improved the response to aggregating agents when platelet metabolism was blocked by KCN and 2-deoxyglucose. This was not the case. Thus, cold potentiates the effect of aggregating agents, but in so doing it is necessary that platelet metabolism be intact.

CT- and epinephrine-induced aggregation were more enhanced by prior

Fig. 4. Development of refractoriness to ADP. PRP was kept on ice (straight line) or at room temperature (dashed line) for 3 hr, and both subsequently were kept at $37^\circ$C for 1 hr. ADP (10 $\mu$M) was added to each, and aliquots were tested periodically. Each point represents aggregation in response to more ADP (2 $\mu$M). Incubation time indicates period after addition of 10 $\mu$M ADP. Representative experiment.

Fig. 5. Per cent of PRP platelets retained in glass bead column after incubation at the temperatures noted. Bars represent values for the last 2 ml of effluent with 1 SD.
chilling than was ADP-induced aggregation. Since all CT-induced aggregation and the secondary wave of epinephrine-induced aggregation depend on the release of platelet ADP, we were surprised to find that chilling did not significantly enhance \(^{14}\)C-serotonin release, which parallels ADP release. It is unlikely that the effect of cold is due to preservation of the release reaction.

The reason for the better response to epinephrine and CT vis-a-vis ADP may relate to our findings that chilled platelets do not disaggregate as readily as unchilled platelets. Total aggregation probably represents an equilibrium between aggregation and disaggregation. A decrease in disaggregation would probably be reflected as an augmented total aggregation. Decreased disaggregation would most apparently increase total aggregation in situations where aggregation rates are slow, such as with epinephrine- and CT-induced aggregation. Decreased aggregation would be least apparent in situations where aggregation rates are rapid, such as ADP-induced aggregation. We would postulate that the major effect of cold in increasing aggregation is to prevent disaggregation.

It has been shown that platelets exposed to ADP without stirring become refractory to the aggregating effects of additional ADP. It has been postulated that this refractoriness is responsible for the disaggregation that occurs when platelets are aggregated with low concentrations of aggregating agents. However, our results indicate that chilled platelets develop this refractoriness as readily as do nonchilled platelets. Consequently, the decreased disaggregation we have observed is not due to an inability to develop refractoriness to ADP.

Our results with platelet retention are similar to those of Hellem and Stormorken, who found that retention in glass bead columns increased as platelet storage temperature decreased. This occurred within a few hours of storage. Even though we warmed the PRP to 37°C to prevent spontaneous aggregation, which these investigators did not do, increased retention was observed in PRP previously stored at cold temperatures as compared to PRP stored at warmer temperatures. Moreover, we found greater retention of PRP stored at 37°C or room temperature than did Hellem and Stormorken. This may be due to our use of Tygon tubing, which in itself is said to increase retention.

These findings have important implications for platelet preparation and preservation for transfusion. Because of the spontaneous aggregation of cold platelets followed by rewarming and stirring, the preparation of platelet concentrates at cold temperature would lead to difficulty in subsequent resuspension. However, preservation at cold temperature may not necessarily be harmful, as had been previously reported, if the platelet concentrates are carefully warmed before infusion to inhibit spontaneous aggregation during infusion. Although the decreased ability of platelets to disaggregate and their increased retention in glass bead columns may reflect changes that compromise in vivo survival, the preservation, or actual enhancement in some functions, of platelets stored at cold temperature may justify their use. On the face of it, it would seem disadvantageous to use platelets stored at cold
temperature as prophylaxis due to their shortened in vivo survival, but because of their better preserved function they may be useful in patients who are actively bleeding because of thrombocytopenia.

REFERENCES

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