The Effect of Cold on Platelets. I. Cold-induced Platelet Aggregation

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Low temperatures induce platelet aggregation. This effect is greatest when chilled platelet-rich plasma (PRP) is stirred while it is warming or just after it has warmed. The stirring of chilled PRP at low temperature or after prolonged warming causes little aggregation. The extent of aggregation increases as the temperature at which the PRP is chilled is lowered, and as the time of chilling is lengthened. This phenomenon resembles ADP-induced aggregation in that the shape of the aggregation curves of both are similar and both are inhibited by the same compounds. In addition, both forms of aggregation require calcium. However, cold-induced aggregation is not mediated by ADP since this compound is not found in the supernatant of chilled PRP. Nor is the effect of cold on plasma proteins responsible for cold-induced aggregation since cold causes aggregation of platelets suspended in a nonprotein medium. The mechanism of this phenomenon may be similar to ADP-induced aggregation. Its clinical significance is that it may account for the deleterious effect of cold on the preparation and preservation of platelet concentrates.

PLATELETS INCUBATED AT LOW TEMPERATURES spontaneously aggregate. Although this phenomenon was first described by Zucker and Borelli and later by Anstall and Hawkey, its mechanism remains obscure. This study was undertaken to explore the phenomenon further. Our results indicate that the platelet aggregation induced by cold in many ways resembles that induced by adenosine diphosphate, but it is not mediated by this compound.

MATERIALS AND METHODS

Platelet-rich plasma (PRP) was prepared from human 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, anticoagulated with 1/10 volume of 3.8% sodium citrate, was centrifuged for 15 min at 500 g in an International PR-2 centrifuge at room temperature, and the PRP was harvested. For most studies, the PRP from 6 to 10 specimens was pooled. This has proved to be an ideal source of PRP since we have found little variation in the response to aggregating agents among pooled specimens.

Platelet suspensions were prepared by a technique described by Bang et al. Whole blood from individual donors was centrifuged at 2500 g in an International Centrifuge, Model HN, at room temperature. The supernatant cell-free plasma was discarded. The
formed elements were resuspended in calcium free Tyrode's solution containing 0.38% sodium citrate, recentrifuged, and resuspended in fresh Tyrode's solution. This procedure was performed three times. The final centrifugation at 300 g in the International PR-2 sedimented the red cells and white cells, but left the platelets suspended. The individual specimens, containing 300,000 to 500,000 platelets/cu mm, were then harvested and pooled. The platelets in this suspension did not aggregate when 5 µM ADP was added, even if 2.5 mM calcium chloride was also added. However, if human fibrinogen was added along with the calcium, the platelets were aggregated by ADP. Aggregation did not occur in unchilled suspensions in the absence of added ADP.

All equipment for handling blood and PRP was plastic except for the aggregometer cuvettes, which were untreated glass.

Fibrinogen (Grade L, Kabi) was dissolved in saline and dialyzed against saline for 24 hr at 4°C. It was then stored at 4°C for 3 days and centrifuged in the cold at high speed to remove the cryofibrinogen. The supernatant, 95% clottable, was divided into aliquots, which were then frozen at −60°C.

Platelet Aggregation

Platelet aggregation was measured turbidometrically in an aggregometer (Chrono-Log Corp., Broomall, Pa., Model S201) attached to a Hitachi–Perkin–Elmer recorder. One-ml samples of PRP were placed in untreated glass cuvettes along with a Teflon-coated magnetized stir bar and the cuvettes were placed in the cuvette holder of the aggregometer which stirred the PRP at 1000 rpm. The holder was kept at room temperature for most of the experiments. However, to determine whether aggregation would occur at low temperatures, the temperature of the cuvette holder was lowered to 4–6°C. Water at 0–1°C was circulated through plastic tubing (2 mm I.D.) wrapped around the cuvette holder to lower its temperature to 12–15°C. This was further reduced to 4–6°C by placing dry ice against the holder. The temperature was verified by intermittent monitoring with a standard laboratory thermometer.

Spontaneous aggregation was tested after incubation of PRP at various temperatures [37°C, 23°C (room temperature), 6°C (refrigerator), and 0–1°C (melting ice)]. One-ml aliquots of PRP were pipetted into a cuvette, which was immediately placed in the aggregometer, where the PRP was stirred and aggregation was measured. When spontaneous aggregation was detected in the aggregometer, this was confirmed by either gross inspection or phase microscopy. The pH of PRP at low temperatures was around 8. That of PRP kept at room temperature or 37°C was 7.6.

Spontaneous aggregation in platelet suspensions kept at low temperatures was studied after the addition of calcium chloride, 2.5 mM, to the platelet suspension. This was placed in melting ice and, after 4 hr, 1.0 ml was pipetted into a cuvette along with 0.1 ml of fibrinogen solution (final concentration = 140 mg/100 ml). The cuvette was placed in the aggregometer at room temperature, and platelet aggregation was measured. A control suspension, kept at room temperature, was similarly studied.

Inhibitors of Platelet Aggregation

Several inhibitors of adenosine diphosphate (ADP)-induced aggregation were studied for their effect on spontaneous aggregation induced by cold. The inhibitors were incubated with PRP until ADP-induced aggregation was reduced by more than 50%. ADP-induced aggregation was measured in the aggregometer after the addition of 0.1 ml of ADP (20 µM) to 1 ml of PRP. The treated sample of PRP and a control from the same pool were then placed in melting ice and tested hourly for spontaneous aggregation. Aggregation was measured at room temperature in the aggregometer, and the degree of inhibition was calculated by the expression: % inhibition = fall in O.D. of the control minus the fall in O.D. of the test specimen, divided by the fall in O.D. of the control.

The inhibitors tested in this way were: (1) Adenosine, of which the final concentration in PRP was 30 µM. (2) 2-deoxy-D-glucose (2DG) and KCN added together; the final concentration of 2DG was 6 mM and that of KCN was either 2 mM or 5 mM. (3)
The sodium salts of p-chloromercuribenzoic acid (PCMB) and p-chloromercuribenzene sulfoic acid (PCMBS), which were added to separate samples of PRP at a final concentration of 0.002 M. At these concentrations, the pH of the PRP was not appreciably affected.

Other inhibitors which did not require prior incubation were also tested. These were: (1) Potato apyrase (Sigma, grade I); this was dissolved in saline (10 mg/ml) just prior to its use and added (1:9, v/v) to PRP, which had been anticoagulated with heparin (5 U/ml) to maintain calcium, and the PRP was then placed on melting ice and tested hourly for spontaneous aggregation along with a control specimen. (2) EDTA, at a final concentration of 0.1%. (c) Tosyl arginine methyl ester (TAME), final concentration, 0.01 M.

Release of Platelet Adenine Nucleotides and Serotonin

The release of platelet adenosine triphosphate (ATP) and ADP at low temperatures was measured after aliquots of citrated PRP were kept in melting ice for 2 and 4 hr; they were then centrifuged for 10 min at 12,000 g in a Sorvall RC-2 at 4°C. Then 0.5 ml of the supernatant platelet-poor plasma (PPP) was briefly warmed and added to 1.0 ml of freshly obtained PRP in the aggregometer. In this bioassay, aggregation indicates the presence of ADP in the supernatant.4

ADP was also measured chemically. The supernatant PPP from cold-exposed PRP was precipitated with an equal volume of a mixture of 1 part 100 μM EDTA and 9 parts 96% ethanol. The precipitated proteins were sedimented at 18,000 g in the RC-2 and the supernatant was stored at −60°C overnight. Its ATP content was measured by the induction of luminescence in a luciferin-luciferase system.5 (The measurement was kindly performed by Dr. Holm Holmsen, Temple University Medical School, Philadelphia, Pa.) The ADP in this extract was quantitatively converted to ATP by a mixture of phosphoenol pyruvate and pyruvic kinase and the total ATP was measured. The difference between the ATP content of the extract before and after the enzymatic conversion represented ADP.

Serotonin loss was measured after 10 ml of citrated PRP was incubated with 0.1 μCi of 14C Serotonin (New England Nuclear, specific activity = 10–20 mCi/mM) for 30 min at 37°C. The PRP was then placed in melting ice. At hourly intervals a sample of PRP was centrifuged in a Clay-Adams Sero-Fuge (relative centrifugal force = 1000 g) for 10 min to sediment the platelets. Then 0.1 ml of the supernatant was added to 15 ml of liquid scintillator medium and the radioactivity was measured in a Beckman 233 liquid scintillation counter.

RESULTS

Chilled platelets spontaneously aggregated only when they were warmed and stirred. When the chilled PRP was stirred while cold (that is, in an aggregometer cooled to 4-6°C), little spontaneous aggregation occurred. However, when the aggregometer was at room temperature or set at 37°C, aggregation was rapid and extensive. Maximal aggregation occurred when the chilled PRP was immediately tested in the aggregometer. When it was allowed to warm in a 37°C water bath without stirring, there was no spontaneous aggregation. Furthermore, spontaneous aggregation in the aggregometer diminished when the chilled PRP was allowed to warm for several minutes without stirring, and almost completely disappeared after 45 min of warming.

Accordingly, all our studies of cold-induced aggregation were performed by promptly placing the chilled PRP into the warm aggregometer, where it was stirred. The degree of spontaneous aggregation of chilled platelets increased as the temperature at which they were cooled decreased (Fig. 1) and as the
time of chilling increased (Fig. 2). Slight spontaneous aggregation occurred even in PRP kept at room temperature. The shape of the aggregation curve (Fig. 1) resembled that of ADP-induced aggregation and was characterized by a rapid downslope, with no lag period. When the temperature of the aggregometer was kept at 37°C, some disaggregation occurred, occasionally followed by a weak secondary wave of aggregation. This secondary wave did not occur regularly, and therefore was not studied further.

**Adenine Nucleotide and Serotonin Release**

The similarity of the shape of the aggregation curve induced by cold to that induced by ADP led us to examine the possibility that ADP was being released from chilled platelets. The following experiments excluded this.
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The supernatant PPP derived from PRP that had been chilled 4 hr in melting ice was briefly warmed and added (1/2, v/v) to fresh PRP in the aggregometer at room temperature. No aggregation was detected either turbidometrically or by phase microscopy. This suggested that neither ADP nor any other aggregating agent was appearing in the plasma.

Direct measurement of ADP and ATP in the PPP from PRP which had been chilled in melting ice for 2 and 4 hr likewise demonstrated no detectable nucleotides. The analytic method we used can detect a concentration as low as 0.25 μM.

Although ADP was not found in the plasma, the possibility still existed that it was released by the platelets and bound to their surface. To test this, heparinized PRP was incubated in melting ice in the presence of apyrase (1 mg/ml). Presumably, ADP on the surface of the platelet would be susceptible to the action of this enzyme that strongly hydrolyzes both ATP and ADP in the presence of calcium. Cold-induced aggregation was not affected by the enzyme. That apyrase was active at cold temperature was demonstrated by incubating 1 mg with 1 ml of a 50-μM solution of ADP and 0.01% calcium in melting ice. After 5 min, no ADP, as measured by its ability to aggregate fresh citrated PRP, was detectable.

As another test of platelet integrity, the platelets in PRP were labeled with 14C serotonin and placed in melting ice. After 3 hr, the radioactivity of the PPP derived from the chilled specimens was the same as the radioactivity of PRP derived from the control specimens kept at room temperature. Thus, chilling did not induce serotonin loss.

Effect of Plasma Proteins

Also explored was the possibility that the aggregating effect of cold was mediated by changes in certain plasma proteins. Since mixtures of various PRPs from various donors were used without regard to their ABO type, it was conceivable that isoantibodies were responsible for this aggregation. Therefore, PRPs from three type O donors, three type A, one type B, and a mixture of these in equal proportions were placed in melting ice for 4 hr and tested in the aggregometer. Spontaneous aggregation in the seven individual specimens was the same as that of the combined mixture and was comparable with our previous results with pooled PRP.

Since platelets contain both "I" and "i" antigens, the possibility that the small amounts of normally occurring cold agglutinins were responsible for this phenomenon was investigated. Whole blood from several donors was chilled for 15 min to adsorb the cold agglutinins onto the red cells. PRPs were prepared from these bloods in a Sorvall RC-2 at 4°C, and were harvested and incubated individually, without pooling, in melting ice. Platelet aggregation in these specimens after incubation was the same as the aggregation in specimens that had been prepared by the usual methods.

Finally, the possibility that cold caused platelet aggregation by affecting other plasma proteins was examined. Therefore, platelets were freed of plasma proteins as well as possible by washing and suspended in Tyrode's solution containing 0.38% sodium citrate and 2.5 mM calcium. One sample was incubated...
in melting ice and a second at room temperature. After 4 hr, a 1-ml aliquot from each of these specimens was removed, added to 0.1 ml of a fibrinogen solution in the cuvette, and tested for spontaneous aggregation. The platelets in the chilled specimen aggregated, but those in the room temperature specimen did not (Fig. 3). This effect was not due to release of an aggregating agent such as ADP from the washed platelets since spontaneous aggregation would not occur in unchilled suspension if they were stirred immediately after their preparation in the presence of calcium and fibrinogen. Aggregation of the chilled platelets did not occur in the absence of fibrinogen.

Effect of Inhibitors

Also studied were the effect of several known inhibitors of platelet function. Cold-induced aggregation requires calcium. When EDTA (0.1%, final con-
cen...ntation was added to the chilled PRP just before testing in the aggregometer, aggregation was almost totally blocked. Likewise, TAMe (0.01 M, final concentration), a known inhibitor of ADP-induced aggregations, had a similar instantaneous effect. Cold-induced aggregation was also inhibited when the pH of the PRP was lowered from 7.6 to 6.7 with citric acid (0.1 M) prior to chilling.

Adenosine, which inhibits ADP-induced aggregations, also inhibited cold-induced aggregation by 30–40% (Fig. 4). Cold-induced aggregation was also inhibited by sulfhydryl inhibitors. At equimolar concentrations, PCMB was more potent than PCMBS (Fig. 5). PCMB was also a more potent inhibitor of ADP-induced aggregation. Metabolic inhibitors also affected cold-induced aggregation (Fig. 6). PRP was incubated with 2 DG and KCN for 45 minutes...
at 37°C. At this time ADP-induced aggregation was inhibited by at least 75%. Then the specimens, along with a control sample were placed in melting ice. Spontaneous cold-induced aggregation was inhibited, although not to the extent that ADP-induced aggregation was inhibited.

**DISCUSSION**

Cold-induced platelet aggregation does not appear to be mediated through an effect on plasma proteins. This is in distinction to the agglutination induced by a cold-reacting protein found in the plasma of some patients with certain chronic illnesses. However, fibrinogen is required for the aggregation to occur. Platelets that were suspended in a protein-free medium and chilled would spontaneously aggregate when warmed and stirred, but only after fibrinogen was added. Fibrinogen functioned probably as a “cofactor,” since it was added after the platelet suspension was removed from the melting ice and consequently was only briefly cooled. Measurements indicated that the lowest temperature to which the fibrinogen was cooled was 5-6°C. It remained at this temperature for only a few seconds.

This requirement for fibrinogen is similar to ADP-induced aggregation which also may require this protein, or other plasma proteins as cofactors. Therefore, cold appears to effect a change in platelets that leads to their aggregation in the presence of a protein cofactor such as fibrinogen.

Cold-induced aggregation resembles ADP-induced aggregation in many other ways: (1) the shapes of the aggregation curves induced by both agents are similar; (2) both forms of aggregation required calcium; and (3) they are inhibited by acid pH, TAMe, adenosine, and sulfhydryl inhibitors, such as PCMB and PCMBS. Furthermore, both forms of aggregation are also dependent on the intactness of the platelet metabolic pathways insofar as they are inhibited by agents that block these pathways.

Finally, both ADP and cold induce similar changes in platelet morphology. They cause the platelet to transform from its normal disk shape to a sphere and induce similar increases in platelet volume. Ultrastructural studies by White have shown that ADP causes centripetal migration of subcellular platelet organelles and pseudopodia formation. These effects have also been demonstrated after prolonged platelet cooling. Notwithstanding these similarities, the cold effect does not appear to be mediated by ADP. The possibility that cold induced a leak of ADP from the platelets was excluded by biologic and chemical study of plasma derived from chilled PRP. Furthermore, the lack of inhibition of cold-induced aggregation by apyrase, a potent hydrolyzer of ADP, suggested that ADP was not present on the platelet surface. Finally, cold-exposed platelets did not leak serotonin, which is another indication of their integrity.

A more likely mechanism is that cold acts on the platelet through the same pathway as does ADP. Although this pathway has not been elucidated, Born has suggested that ADP complexes with a component of the platelet membrane in a manner similar to the action of acetylcholine on smooth muscle membrane. Nachmansohn has proposed that the effect of acetylcholine on smooth muscle cells is to cause conformational changes of specific proteins of the cell membrane.
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membrane. It is possible, therefore, that ADP likewise acts by affecting the conformation of specific proteins of the platelet membrane. Since protein conformation is affected by changes in temperature, cold may induce platelet aggregation by causing conformational changes of platelet membrane protein similar to those postulated to explain the action of ADP.

The clinical significance of cold-induced aggregation lies in the preparation and preservation of platelets for transfusion. There is more clumping in platelet concentrates prepared from chilled blood than in concentrates prepared from blood kept at room temperatures. Our observations that spontaneous aggregation of chilled platelets is greatest while they are warming, or shortly after warming, would explain this. When platelet concentrates from chilled blood are prepared by conventional methods, the platelets that are packed at the bottom of the transfer bag are warmed and then resuspended. This generally yields clumping of platelets, which is probably due to the close contact of the platelets while they are being warmed, since this period of warming is when they most readily aggregate.

Low-temperature preservation of platelets for transfusion also seems detrimental. When PRP that has been stored at low temperatures is transfused, the in vivo yield is less than that from comparable preparations stored at room temperature. It has been shown that the in vivo yield from clumped platelet preparations is less than that from unclumped preparations. Since chilled platelets aggregate when warmed, even when they are collected in ACD in plastic bags, the decreased yield and survival of chilled platelets may be due to their spontaneous aggregation rather than to decreased viability.

ACKNOWLEDGMENT

The authors wish to thank Mrs. Myrna Salomon and Miss Frances White for their technical assistance.

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