Influence of Cytochalasin B on the Shape Change Induced in Platelets by Cold

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The present investigation has employed transmission and scanning electron microscopy to examine the influence of cytochalasin B (CB) on the shape change and pseudopod formation that develop when discoid blood platelets are exposed to low temperature. CB in two concentrations protected platelets from the physical alterations induced by cold. The greater of the two amounts of drug was more effective, suggesting a dose dependence. Microtubules were absent from untreated control platelets chilled for brief periods of time but remained in approximately 20% of CB-treated cells exposed to low temperature for an hour. The findings support the concept that CB preserves discoid shape by stabilizing structural elements of the cytoskeletal support system in blood platelets.

Blood platelets have a characteristic disclike form in the circulation and in platelet-rich plasma maintained at 37°C in vitro. When platelets are stimulated by aggregating agents or exposed to low temperature, they lose their discoid form and become irregularly spherical with multiple pseudopods. Although the changes in surface contour induced by cold and aggregating agents are nearly identical, the internal response of the cells is quite different. The shape change induced by aggregating agents is associated with the movement of organelles to platelet centers where they are encircled by the microtubules and cytoplasmic microfilaments. Organelles remain randomly dispersed despite loss of discoid form in chilled platelets, and microtubules disappear completely. The dynamic alterations caused by aggregating agents have been related to the platelet contractile response, whereas those stimulated by chilling are considered secondary to the loss of circumferential microtubules.

Recently, a new group of chemical substances, the cytochalasins, have been introduced that reputedly interfere with contractile activities in many cell types by dispersing or preventing formation of contractile microfilaments. Evaluation of the effects of cytochalasin B (CB) on platelet function, however, did not support this hypothesis of the drug’s action. CB stabilized the discoid form of platelets, inhibited pseudopod formation, and altered internal transformation in drug-treated platelets exposed to aggregating agents, but it did not appear to disperse, prevent formation, or inhibit...
contraction of microfilaments. The present investigation has examined the influence of CB on the physical alterations induced in chilled platelets, a phenomenon that is not considered to be contractile in nature.

**MATERIALS AND METHODS**

The techniques used in this laboratory to obtain blood from normal donors in 3.8% trisodium citrate anticoagulant, to separate platelet-rich plasma (C-PRP), and to prepare samples of control and experimental platelets for study in the electron microscope were described in detail in recent reports.7,9,17,18 Cytochalasin B (CB) was dissolved in ethanol at 4 mg/ml and was diluted in Hank's buffered salt solution (HBSS) to concentrations of 250 μg/ml and 100 μg/ml.16,19 Prostaglandin E1 (PGE1) (generously supplied by Dr. J. E. Pike of the Upjohn Co., Kalamazoo, Mich.) was also dissolved in ethanol and diluted with HBSS to a concentration of 0.5 μg/ml.

Samples of C-PRP, 0.9 ml in volume, were combined with 0.1 ml of HBSS, 0.1 ml of the PGE1 solution, or 0.1 ml of one of the two concentrations of CB. Final concentrations were 0.05 μg of PGE1/ml of C-PRP, and either 25 or 10 μg of CB per ml of C-PRP. The control and experimental samples, after combination with test agents, were maintained at 37°C for 15 min. Then the samples were chilled to 4°C for either 30 or 60 min. After incubation at low temperature, the samples were fixed in the manner used routinely in this laboratory, except that the glutaraldehyde solutions were chilled to 4°C before use.9 The doubly fixed platelets were dehydrated in a series of alcohols and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and were examined in a Philips 200 transmission electron microscope (TEM).

The fixation of samples for scanning electron microscopy differed from our usual schedule. After the first step of fixation for 15 min in an equal volume of chilled 0.1% glutaraldehyde in White’s saline, pH 7.3, the cells were diluted with 5 ml of cold 3% glutaraldehyde in the same buffer. The platelet samples were maintained in suspension in the fixative for 2 hr at 4°C. At this time the suspensions were centrifuged and washed, first in White’s saline, then three times in distilled water. The washed cells were resuspended in buffered 1% osmic acid for 1½ hr and then were sedimented and rinsed three times in distilled water. Dehydration was carried out in the usual manner, except that the cells were resuspended after each exchange of alcohol. Drops of the 95% ethanol suspension were spread on polished aluminum stubs, dried in air, coated with a 50 Å layer of gold in a vacuum evaporator, and examined in a Cambridge Stereoscan scanning electron microscope (SEM).

The experiments described above were done for TEM and SEM at the same time on three separate occasions. A few additional experiments were carried out to assess specific questions, and the samples were prepared for TEM only. Samples of C-PRP were chilled to 4°C for 15 min and were then combined with a final concentration of 10 or 25 μg/ml of CB. Incubation was continued for another 30 min at low temperature. At that time some control and CB-treated samples were fixed in the cold, and others were restored to the 37°C water bath. Rewarmed control and CB-treated platelets were fixed at 10, 20, 30, and 60 min after returning to 37°C.

**RESULTS**

**Transmission Electron Microscopy**

Thin sections of platelets from samples of C-PRP, separated from blood at room temperature and maintained at 37°C before fixation, reveal the characteristic discoid form of the unaltered cells (Fig. 1). The surface membrane is slightly irregular, and small pseudopodial extentions are apparent on some platelets, but the majority of the cells retain a lentiform configuration. In cross sections the circumferential bundle of microtubules is visible as a small group
cytochalasin B

Fig. 1. Platelets from sample of citrate platelet-rich plasma (C-PRP) fixed at 37°C. Discoid shape of cells is preserved. Circumferential microtubules (T) supporting the asymmetric form are evident as small groups of circular profiles at polar ends of cross-sectioned platelets. Granules (G), dense bodies (DB), mitochondria (M), and the open canalicular system of channels (CS) are randomly dispersed in cytoplasm of cells. ×14,500.

of hollow circular profiles at the polar ends of each cell. Granules, dense bodies, mitochondria, and glycogen, in clumps or single particles, are distributed randomly in the coarse matrix of the cytoplasm.

The effects of chilling to 4°C on the fine structure of platelets was discussed in detail previously.9 Platelets lose their discoid form and become extremely irregular (Fig. 2). Within 10 min most of the chilled cells are relatively spherical, and channels of the open canalicular system appear dilated. After 15 min at 4°C pseudopod extension is prominent. Alterations in surface contour evident at 15 min remain relatively static throughout 1 hr of incubation. The organelles inside the chilled platelets are dispersed and often appear more separated from each other than in cells maintained at 37°C. Microtubules disappear from platelets 5–15 min after exposure to 4°C.

The effects of CB on platelets incubated with the drug at 37°C for up to 60 min at concentration ranging to 30 μg/ml of C-PRP were evaluated in a previous study.16 A high concentration of CB combined with platelets for
Fig. 2. Platelets from sample of C-PRP chilled to 4°C for 1 hr and fixed at that temperature. Cells have lost their discoid form and become irregular. Multiple pseudopods (Ps) extend from the irregular surfaces and lie between the sectioned platelets. Organelles inside platelets remain randomly dispersed, and elements of open canalicular system (CS) appear dilated. Microtubules have disappeared completely. × 15,500.

60 min caused slight dilation of the open canalicular system and changes in the cytoplasmic matrix, but did not influence the discoid shape of the cells.16-19

Thin sections of platelets chilled to 4°C for 30 or 60 min after combining with CB at a concentration of 10 μg/ml were slightly irregular in contour and had a few short pseudopods extending from their surfaces (Fig. 3). The plethora of pseudopods encountered in thin sections of control samples, however, were absent in the preparations preincubated with CB. The alterations usually caused by chilling were even less apparent in platelet samples combined with 25 μg/ml of CB before exposure to cold (Fig. 4). After 60 min of incubation at 4°C, most platelets retained a discoid appearance, and pseudopods were rarely encountered. Elements of the open canalicular system were more dilated than in unchilled control platelets, but this effect was also apparent in CB-treated cells after incubation at 37°C. Microtubules were evident in 10%-30% of the CB-treated cells chilled to 4°C for 60 min. In many cells the tubular elements were found in the position usually occupied by the circumferential bundle of microtubules.
Fig. 3. Platelets incubated with cytochalasin B at concentration of 10 μg/ml of C-PRP prior to chilling and fixed after 1 hr at 4°C. Cells are somewhat irregular, and channels of open canalicular system (CS) appear swollen. However, the degree of shape change and pseudopod (Ps) formation are markedly reduced when compared to untreated sample in Fig. 2. Circumferential microtubules (T) can be identified in some of the drug-treated cells. × 16,000.

Scanning Electron Microscopy

The effects of CB on preservation of discoid shape after exposing platelets to low temperature were dramatically demonstrated by scanning electron microscopy (Fig. 5). Platelets in samples of C-PRP without CB developed long pseudopodial extensions and, after 30–60-min incubation in the cold, were usually clumped together (Fig. 5B).

Platelets combined with 10 μg/ml of CB were relatively discoid in shape and had short, blunted pseudopods (Fig. 5C). The cells were frequently found clumped together, but the size of the aggregates were smaller than in the samples of C-PRP that had not been exposed to CB before chilling. There were no differences in the samples chilled for 30 or 60 min.

CB at 25 μg/ml of C-PRP almost completely prevented the cold-induced changes in platelets (Fig. 5D). The cells remained relatively discoid in form, and pseudopods were not evident. They were less regular in appearance than control platelets fixed at 37°C without CB (Fig. 5A), but this may have been due to effects of the drug on elements of the open canalicular system. Aggre-
Fig. 4. Platelets incubated with 25 μg CB/ml C-PRP before chilling to 4°C for 1 hr. Although elements of the open canalicular system are dilated, platelets retain their discoid form. Pseudopodial extensions are very short when present and occur on very few cells. Circumferential bands of microtubules (T) are evident in approximately one-fourth of platelets. X 17,700.

gates of three or four platelets were found in the chilled samples containing 25 μg/ml of CB, but the cells more commonly remained separated. Findings at 60 min were identical to those made at 30 min.

Prostaglandin E₁ did not affect the shape change or pseudopod formation of platelets examined at 30 and 60 min after chilling to 4°C. The agent does appear to influence the shape change during the first few minutes after chilling, but this influence is not apparent on longer incubation. However, the tendency of platelets to aggregate spontaneously at low temperature was inhibited by PGE₁, as others have noted previously.²⁰

Addition of CB at any concentration had no apparent effect on the shape change that had already developed in platelets chilled to 4°C for 15 min before being exposed to the agent. Examination of thin sections revealed changes identical to those found in control samples of C-PRP chilled for similar periods of time. CB had no influence on the rate of recovery of discoid form or assembly of microtubules in platelets warmed to 37°C, after being chilled to 4°C and combined with CB after shape change had occurred. The last finding was discussed briefly in a previous publication.¹⁶
Fig. 5. Platelets in this composite follow same sequence as in the four previous illustrations but have been studied by scanning rather than transmission electron microscopy. Untreated platelets fixed at 37°C in (A) retain typical discoid form. Surfaces appear smooth, except for dimples that probably represent connections between channels of open canalicular system (CS) and cell wall. Untreated platelets in (B) were chilled to 4°C for 1 hr. Cells tend to form aggregates and appear as clumps rather than isolated cells. Pseudopod (Ps) formation is extensive, and may be an important factor in the aggregation evident in chilled samples. Platelets in (C) were incubated with CB (10 μg/ml of C-PRP) prior to chilling at 4°C for 1 hr. Clumps of cells in chilled samples treated with CB are smaller than in control preparations. Pseudopod (Ps) formation is less extensive, and those that have developed are short. Platelets in (D) were exposed to CB at final concentration of 25 μg/ml of C-PRP and then cooled to 4°C for 1 hr. Cells have retained their discoid form. Multiple dimples on cell surface may represent communications of open canalicular system (CS) with outer wall. (A) × 8600; (B) × 6200; (C) × 5000; (D) × 7300.
DISCUSSION

Results of the present investigation indicate that cytochalasin B inhibits the shape change and pseudopod formation that develop in platelets on exposure to low temperature. The higher of the two concentrations of CB employed was more effective, suggesting that the protective influence of the drug on platelet discoid shape was dose dependent. CB had no effect on the altered morphology of platelets that had been chilled for 15 min before exposure to the drug; their irregular appearance remained unchanged during the subsequent incubation and fixation at low temperature. Yet, the drug did not stabilize the altered form of chilled cells, for when platelets treated with CB in the cold were warmed to 37°C, they recovered their discoid shape as rapidly as control cells. Therefore, in these experiments CB specifically protected unaltered discoid platelets combined with the drug at 37°C from the effects induced by low temperature but did not stabilize pseudopodial extensions or the irregular form of chilled platelets from the influence of warming.

Preservation of circumferential microtubules in 10%-30% of CB-treated platelets incubated for 1 hr at 4°C was unexpected. Tubules were completely absent in control platelets exposed to similar conditions simultaneously. The sensitivity of platelet microtubules to low temperature has been pointed out previously, and Behnke concluded from his studies that platelets chilled for 10 min are completely devoid of microtubules. Therefore, even though the effect was not uniform, the protective influence of CB on microtubules in a significant number of chilled platelets was impressive. CB is the first chemical agent, with the possible exception of urea, to inhibit disassembly of cold labile platelet microtubules. *

There are significant differences in the response of platelets to chilling and aggregating agents. The shape change and pseudopod formation are similar, but chilling does not stimulate the internal transformation that results in the central clumping of platelet organelles, nor does it trigger degranulation. Furthermore, the activation of platelets by aggregating agents that leads to shape change cannot be identical to the effect of chilling on platelet contour and pseudopod formation, despite the physical resemblance. As a result, the response of platelets to chilling provided a useful opportunity to test the influence of CB on shape per se, apart from membrane activation, metabolic response, and other features of the platelet reaction to aggregating agents. The ability of CB to inhibit the effects of cold on platelet discoid shape suggests that the drug acts specifically on structures involved in maintaining asymmetric form. This possibility is supported by the observation that other chemicals, such as prostaglandin E and aspirin, that inhibit the platelet response to aggregating agents have little or no effect on the shape change induced by cold.

*Studies completed since submission of this manuscript indicate that dibuteryl cyclic adenosine monophosphate (C-AMP) with PGE, also protect platelet microtubules from the effects of chilling.
Microfilaments are present in the pseudopods of chilled platelets.\textsuperscript{9-11} If the microfilaments in platelets exposed to cold are actinlike\textsuperscript{25,26} and CB inhibits their assembly, then the influence of the drug on the shape change induced by low temperature would be compatible with the mechanism of action proposed by Wessells et al.\textsuperscript{15} However, several investigations have demonstrated that assembly of actin filaments is temperature dependent.\textsuperscript{27} Under appropriate conditions, in vitro F-actin will dissociate into the globular form when chilled and will reassemble into filaments when warmed to 37°C. A similar sensitivity of contractile microfilaments to low temperature has been reported in several cell types.\textsuperscript{28} On the basis of these observations, the possibility that chilling causes assembly of actinlike contractile microfilaments in blood platelets seems remote.

The presence of microtubules and absence of pseudopods in a significant number of CB-treated platelets after chilling suggests another mechanism for the protective influence of the agent. Previous studies have demonstrated that platelets contain a system of structural fibers, the submembrane filaments,\textsuperscript{29,30} that are closely associated with the cell wall. Submembrane filaments appear to interact with the cytoskeletal support system maintaining platelet discoid shape and extend the supportive influence of the circumferential band of microtubules to the surface membrane. They may also act as structural filaments stabilizing the rigid pseudopods extending from chilled platelets. The similarity of submembrane filaments and the subfilaments that make up microtubules has been pointed out previously.\textsuperscript{17,29-31} It is possible that CB protects platelets from cold-induced shape change and pseudopod formation by stabilizing structural filaments, rather than by preventing assembly or dispersing contractile protein filaments.\textsuperscript{14,15} This proposed mechanism of drug action could explain the presence of microtubules and the virtual absence of pseudopods in chilled, CB-treated platelets and avoid the problem created by assigning the effect of CB specifically to contractile microfilaments that depolymerize in the cold.\textsuperscript{27,28}

In conclusion, the results of this study and previous investigations\textsuperscript{16,19} indicate that CB stabilizes the discoid form of blood platelets and inhibits their response to agents and conditions that stimulate shape change, pseudopod formation, and internal transformation. Although CB may influence contractile filaments in some cell systems\textsuperscript{14,15} and the transport of glucose and glucosamine in others,\textsuperscript{32-34} it is doubtful that these mechanisms of drug action are involved in the protection of platelets from alterations produced by chilling. The findings raise the possibility that CB may have a direct effect on structural elements maintaining platelet discoid shape.

REFERENCES

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