American Journal of Pathology, Vol. 152, No. 2, February 1998

Microtubule Coils *versus* the Surface Membrane Cytoskeleton in Maintenance and Restoration of Platelet Discoid Shape

James G. White and Gundu H. R. Rao

From the Departments of Laboratory Medicine and Pathology, and Pediatrics, University of Minnesota Medical School, Minneapolis, Minnesota

The discoid form of blood platelets is important to their function in hemostasis. Recent studies have suggested that the spectrin-rich surface membrane cytoskeleton and the cytoplasmic, actin-rich cytoskeleton are responsible for discoid shape, shape change, and recovery after activation or chilling. Earlier studies had suggested that circumferential coils of microtubules supported the disc shape of resting platelets and that their repositioning or reassembly restored disc shape after exposure to low temperature. The present study has used the chilling-rewarming model, together with microtubule stabilizing (taxol) and disassembling (vincristine) agents to retest the relative importance of the surface membrane cytoskeleton and circumferential microtubules in platelet discoid shape and its restoration. Washed platelet samples were rested at 37°C and chilled to 4°C; chilled and rewarmed to 37°C for 60 minutes; or chilled, rewarmed, and exposed to the same cycle in the presence or absence of vincristine or taxol and fixed for study by disseminated interference phase contrast microscopy and electron microscopy. Rhodamine-phalloidin and flow cytometry were used to measure changes in actin filament assembly. Chilling caused loss of disc shape, pseudopod extension, disassembly of microtubule coils, and assembly of new actin filaments. Rewarming resulted in restoration of disc shape, pseudopod retraction, disassembly of new actin filaments, and reassembly of circumferential microtubule coils. Vincristine converted discoid platelets to rounded cells that extended pseudopods when chilled and retracted them when rewarmed, leaving spheres that could undergo the same sequence of changes when chilled and rewarmed again. Taxol prevented cold-induced disassembly of microtubules and limited pseudopod formation. Rewarming caused retraction of pseudopods on taxol-treated, discoid cells. Cytochalasin B, an agent that blocks new actin filament assembly, alone or in combination with taxol, inhibited the cold-induced shape change but not dilation of the open canalicular system. Rewarming eliminated open canalicular system dilation and restored lentiform appearance. The results indicate that microtubule coils are the major structural elements responsible for disc shape and its restoration after submaximal stimulation or rewarming of chilled platelets. *(Am J Pathol 1998, 152:597–609)*

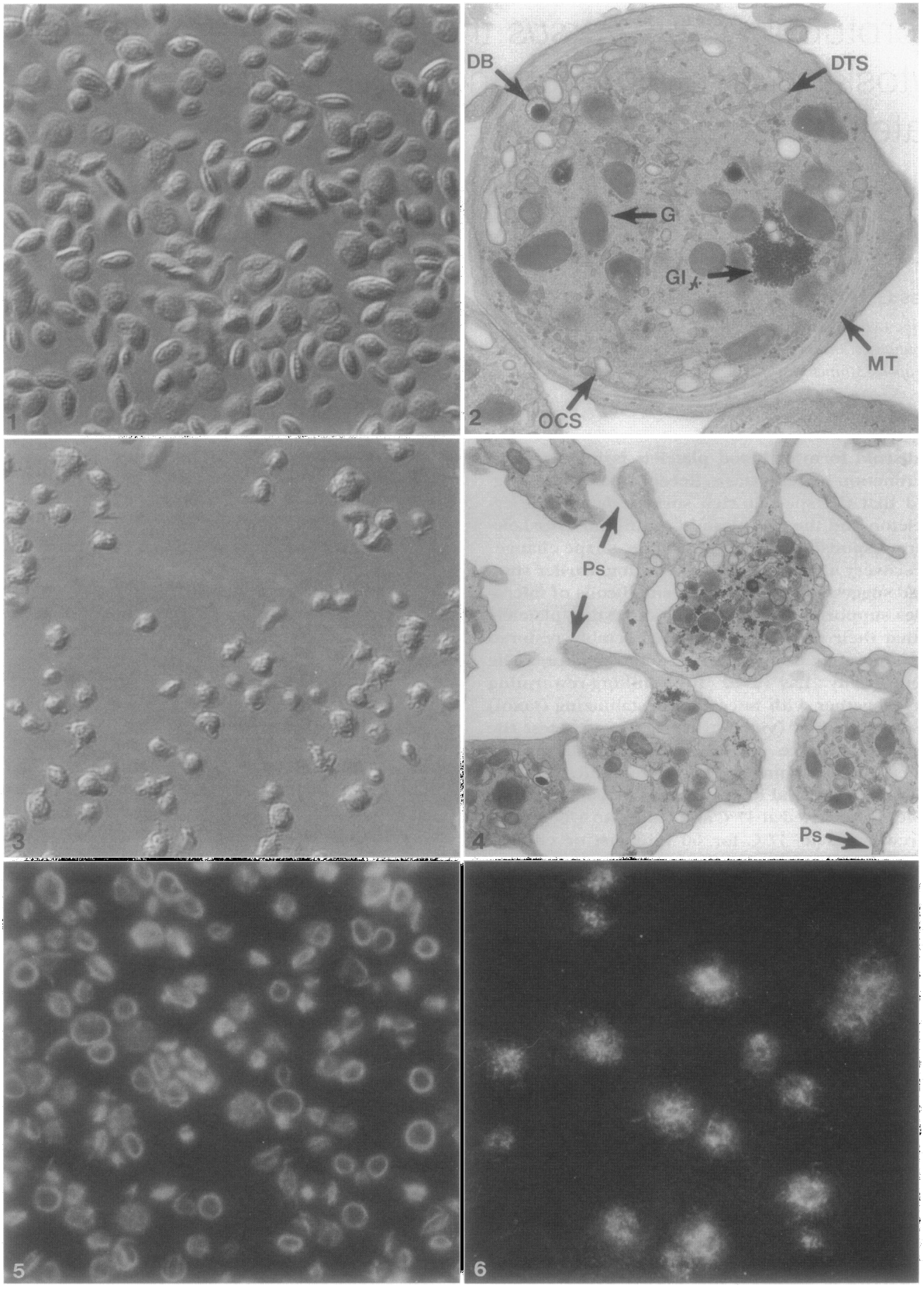
The discoid shape of blood platelets is critical for their function in hemostasis.[1] Therefore, it is important to determine the basis for their lens-like form. Recent investigations[2,3] have indicated that the spectrin-reinforced surface membrane cytoskeleton and the actin-rich cytoplasmic cytoskeleton are responsible for maintaining discoid form, for shape change caused by surface or suspension activation, and for recovery when stimulation is incomplete.

This concept has led to a new proposal to explain the mechanism involved in the shape changes induced in platelets by chilling and rewarming.[4] Exposure to low temperature caused severing of actin filaments associated with the surface membrane cytoskeleton. Cold also caused an elevation of cytoplasmic calcium, activation of gelsolin, uncapping and severing of established cytoplasmic actin filaments, formation of multiple nucleation sites, and rapid assembly of new actin filaments. The cooled platelets lost their discoid form, became swollen in appearance, and extended filopodia and lamellipodia. Rewarming to 24 to 37°C did not restore the irregular, cold platelets to their resting, lens-like form. Rather, the new actin filament bundles formed in the cold underwent reorganization resulting in elimination of pseudopodia and formation of irreversible spheres.

As attractive as the new concepts are, they ignore a component of cytoskeletal structure, the circumferential coil of microtubule, that early studies suggested was important in platelet discoid form, shape change, and recovery.[5–7] The present study has attempted to determine the relative importance of the surface membrane cytoskeleton and the circumferential coil of microtubules in supporting platelet disc shape and restoration of ellip-

Accepted for publication November 15, 1997.

Address reprint requests to Dr. James G. White, Departments of Laboratory Medicine, Pathology, and Pediatrics, UHMC Box 490, University of Minnesota Medical School, 420 Delaware Street SE, Minneapolis, MN 55455.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tical form after chilling and rewarming. Results indicate that the circumferential coil of microtubules, not the surface membrane cytoskeleton, is the major structural support for platelet discoid shape.

Materials and Methods

Platelet Preparation

Blood for this study was obtained from paid normal human donors after informed consent. The blood was mixed immediately with citrate/citric acid dextrose in a ratio of 9 parts to 1 part anticoagulant. Methods for preparing the citrate anticoagulant and suspensions of washed platelets resuspended in Hanks' balanced salt solution have been described in detail previously.[8,9] The washed cell suspensions were maintained in a constant temperature water bath at 37°C for at least 30 minutes before use.

Chilled Platelets

Effects of cold were studied by chilling samples of platelets in a slurry of ice and water and then maintaining the cells in a cold room (2 to 4°C) for 30 minutes. Platelets were rewarmed by transfer to the constant temperature water bath at 37°C or to room temperature at 24°C. Rechilling and rewarming cycles were accomplished in the same manner.

Changes in Actin Filament Assembly

The state of actin filament polymerization in platelets was measured using rhodamine bound to phalloidin as described by Oda et al[10] and by Winokur and Hartwig.[4] Samples of washed platelets were incubated at 37°C; 37°C followed by 4°C; 37°C followed by 4°C and then 37°C; 37°C, 4°C, 37°C, and then 4°C; or 37°C, 4°C, 37°C, 4°C, and then 37°C. The samples were incubated at each temperature for 30 minutes. Each time point was repeated in six different experiments, except the last interval, which was done just three times. Actin polymerization or depolymerization was stopped by adding warm or cold 1% paraformaldehyde, depending on whether the cells were at 4°C or 37°C. Platelets were permeabilized with 0.1% Triton X-100 for 10 minutes and stained with rhodamine phalloidin to demonstrate filamentous actin. A resting sample at 37°C and a sample exposed to 0.2 U/ml thrombin for 5 minutes were included as controls in each experiment. For purposes of calculation the percentage of assembled actin filaments in resting platelets was set at 40% and the percentage in thrombin-activated platelets at 80%.[11] Mean fluorescence was determined by flow cytometry using a Becton Dickinson (Mountain View, CA) FACScan after forward and side-scatter gating. Ten thousand platelets per sample were tested using a 575-nm bandpass filter. Assembly of actin in temperature-affected platelets was calculated using mean fluorescence relative to resting and thrombin-activated platelets.

Effects of Microtubule Disassembling Agents

Vincristine, colchicine, and nocodazole were tested to select an agent that would completely remove circumferential coils of microtubules without causing morphological injury to the platelet.[12] Each agent was combined with samples of washed platelets at concentrations of 10^{-6}, 10^{-5}, and 10^{-4} for 30 minutes at 37°C. The cells were then fixed in an equal volume of 0.1% glutaraldehyde in White's saline and processed for electron microscopy.

Samples of platelets at 37°C were combined with 10^{-4} mol/L vincristine for 15 minutes and fixed or, after 15 minutes, chilled to 2 to 4°C for 30 minutes and then fixed. Additional platelet samples were chilled to 2 to 4°C for 25 minutes, combined with chilled vincristine at 10^{-4} mol/L for 10 to 15 minutes, and then rewarmed to 37°C for 1 hour before fixation. Some of the latter samples were chilled a second time to 2 to 4°C and fixed after a 30-minute exposure to low temperature or rewarmed again for 1 hour and then fixed.

Effects of a Microtubule Stabilizing Agent

Taxol, an agent extracted from *Taxus brevifolia*,[13] stabilizes microtubules in a variety of cell types,[14,15] including platelets.[16,17] When combined with platelets at concen-

Figure 1. Control washed platelets fixed and mounted as described in the text and photographed in the DIC microscope under a 100× oil immersion objective. Some platelets are attached to the glass whereas others are floating. The attached cells are circular in form and contain numerous dots, which are α-granules. Floating cells are tipped at various angles, including 90°, showing flattened surfaces of the discoid cells. Even though some of the floating platelets are slightly out of focus due to movement, all are discoid in appearance. Magnification, ×780.

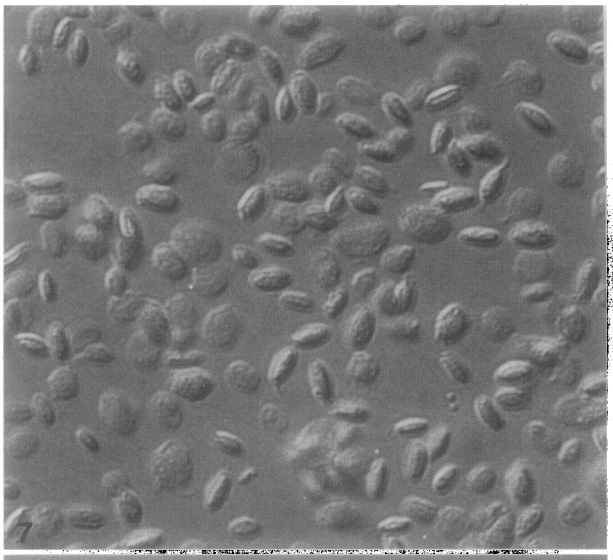
Figure 2. Thin section of discoid platelet from sample maintained at 37°C for 30 minutes. A circumferential coil of microtubules (MT) just under the surface membrane appears to support the lens-like form. Organelles, including numerous α-granules (G), occasional dense bodies (DB), mitochondria, and masses of glycogen (Gly) are randomly dispersed in the cytoplasm. Elements of the OCS and dense tubular system (DTS) are also present. Magnification, ×19,000.

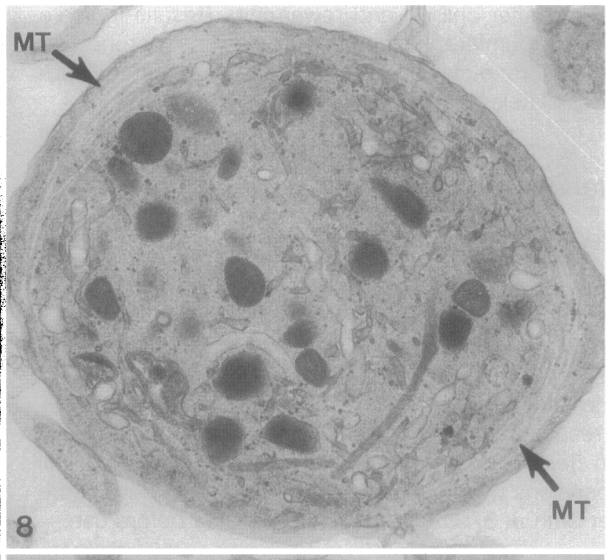
Figure 3. Platelets chilled to 4°C for 30 minutes, fixed, and prepared for DIC microscopy as described. The cells were photographed and printed at the same magnification as the platelets in Figure 1. Platelet bodies appear smaller because of disc-to-sphere conversion in the cold and extension of surface membrane to cover pseudopods. Magnification, ×780.

Figure 4. Thin section of platelet sample chilled to 4°C for 30 minutes. The cells are irregular in form and have extended multiple pseudopods (Ps). Organelles remain randomly dispersed. Magnification, ×10,500.

Figure 5. Indirect immunofluorescence of B-tubulin in control human platelets prepared at room temperature as described in the text. Microtubule coils appear as fluorescent circles in nearly every platelet. Magnification, ×780.

Figure 6. Platelets from a sample of washed cells chilled to 4°C for 30 minutes before mounting, fixation, and staining for B-tubulin. Fluorescently labeled tubulin is diffusely distributed in the cytoplasm of the chilled cells. Microtubule coils are absent. Magnification, ×780.

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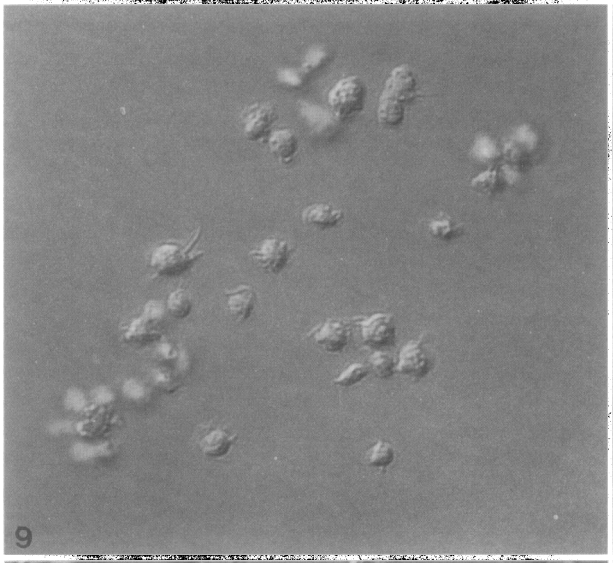

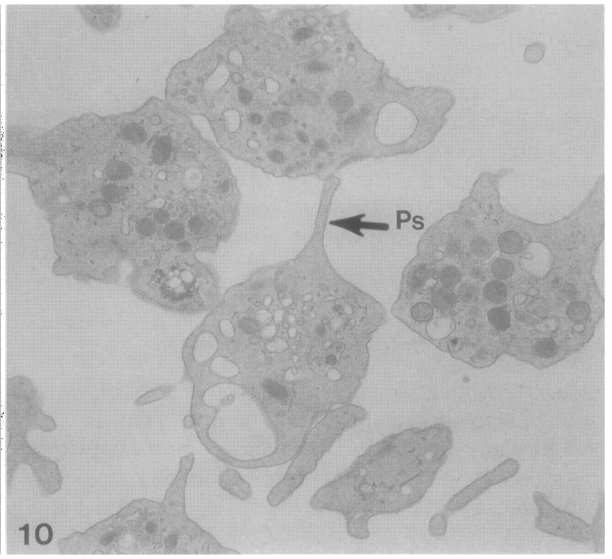
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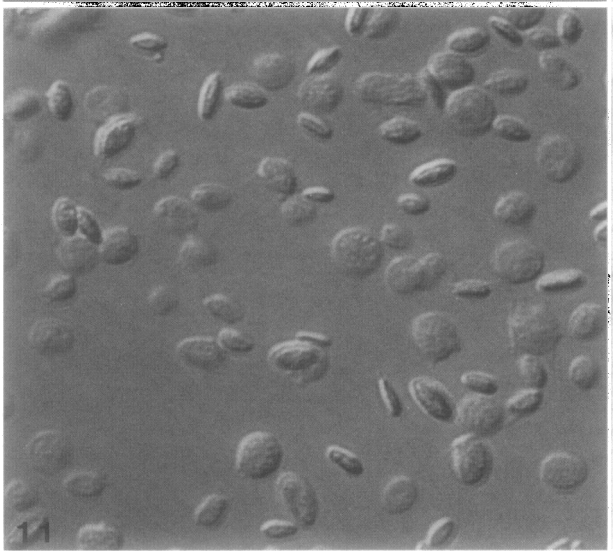

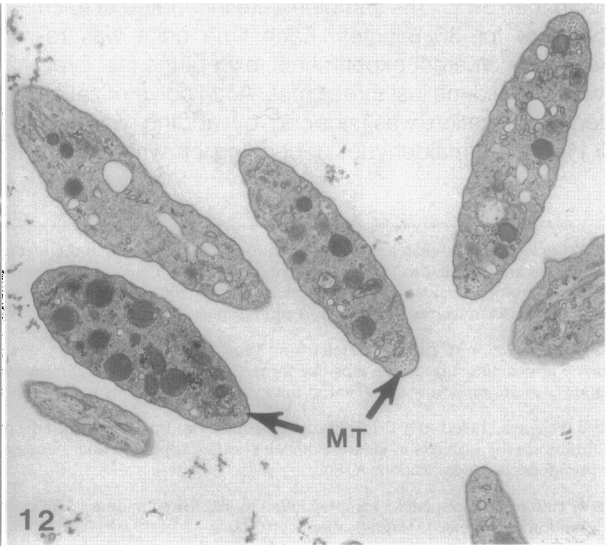
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trations of 10^{-4} or 10^{-5} mol/L, it prevents the cold-induced disassembly of circumferential coils of microtubules.[18] In the present study, samples of washed platelets were combined with taxol at 10^{-4} or 10^{-5} mol/L for 15 minutes and then chilled to 4°C for 30 minutes or chilled for 30 minutes and rewarmed to 37°C for 60 minutes. The samples were then combined with an equal volume of cold or warm 0.1% glutaraldehyde in White's saline and processed for electron microscopy as described below.

Inhibition of Actin Filament Formation

Cytochalasin B, an agent that inhibits new actin filament assembly,[19] was shown earlier to significantly inhibit cold-induced shape change.[20] In the present study, washed platelets were combined with 10^{-5} mol/L taxol and 10^{-5} mol/L cytochalasin B for 30 minutes and then chilled to 4°C for 30 minutes or chilled to 4°C for 30 minutes and rewarmed to 37°C for 60 minutes. Samples were either fixed for study in the electron microscope or for fluorescence microscopy as described below.

Preparation for Electron Microscopy

Platelets were fixed for study in the electron microscope by methods described previously from our laboratory.[21,22] Fixation was accomplished by combining the sample with an equal volume of 0.1% glutaraldehyde in White's saline (a 10% solution of a 1:1 mixture of 1) 2.4 mmol/L NaCl, 0.1 mmol/L KCl, 46 mmol/L $MgSO_4$, 64 mmol/L Ca $(NO_3)_2$, and 4 H_2O and 2) 0.13 mmol/L $NaHCO_3$, 8.4 mmol/L NaH_2PO_4, and 0.1 g/L phenol red, pH 7.4). After 15 minutes, the samples were centrifuged to pellets, and the supernatant fixative was removed and replaced with 3% glutaraldehyde in the same buffer. The samples resuspended in the second aldehyde fixative were maintained at 4°C for 30 minutes and then sedimented to pellets. Supernatant was removed and replaced with 1% osmic acid in distilled water containing 1.5% potassium ferrocyanide for 1 hour at 4°C. All samples were dehydrated in a graded series of alcohol and embedded in Epon 812. Thin sections cut from the plastic blocks on an ultramicrotome were examined unstained or after staining with uranyl acetate and lead citrate to enhance contrast. Examination was carried out in a Philips (Mahwah, NJ) 301 electron microscope. The entire experimental sequence has been repeated on three separate occasions on blood from different donors.

Preparation for Differential Interference Phase Contrast Microscopy

Platelets fixed as described above for study in the electron microscope were also evaluated by differential interference phase contrast (DIC) microscopy. Drops of fixed cells from each experiment were placed on glass slides and allowed to settle for 1 hour. The slides were then rinsed twice with phosphate-buffered saline to remove unattached cells, shaken to remove excess fluid, covered with a drop of 3% glutaraldehyde in Hanks' balanced salt solution and mounted under coverslips. Slides were examined in a Zeiss Axiophot microscope equipped for DIC under a 100× oil immersion objective. Photographs of the resting or activated platelets were taken at an exposure time of 4 seconds. All 35-mm negatives were printed and enlarged at the same magnification settings on the photo enlarger. At least 10 photographs were taken on two slides prepared from each experiment.

Preparation for Immunofluorescence Microscopy

Control platelets and cells chilled to 4°C for 30 minutes were prepared as described in detail earlier for study by immunofluorescence techniques.[26,27] Briefly, 20 μl of washed platelets were placed on polylysine-coated glass slides for 10 minutes at 24°C and then fixed in methanol followed by acetone at −10°C. Similar volumes of platelets chilled to 4°C for 30 minutes were deposited on polylysine-coated glass slides placed in a petri dish floating on an ice-water bath in the cold room held at 2 to 4°C for 10 minutes before fixation in methanol acetone. The slides were washed and stained with a monoclonal antibody to tubulin (Amersham, Arlington Heights, IL) followed by a fluorescein-conjugated anti-immunoglobulin antibody (Cappel, West Chester, Pa). After washing and mounting under coverslips, the samples were examined by phase-fluorescence in a Zeiss photomicroscope

Figure 7. Platelets chilled to 4°C for 30 minutes and then rewarmed to 37°C for 1 hour, fixed, and examined by DIC microscopy. The cells have retracted the pseudopods extended in the cold and recovered their lens-like appearance. They appear identical to the control platelets in Figure 1. Magnification, ×780.

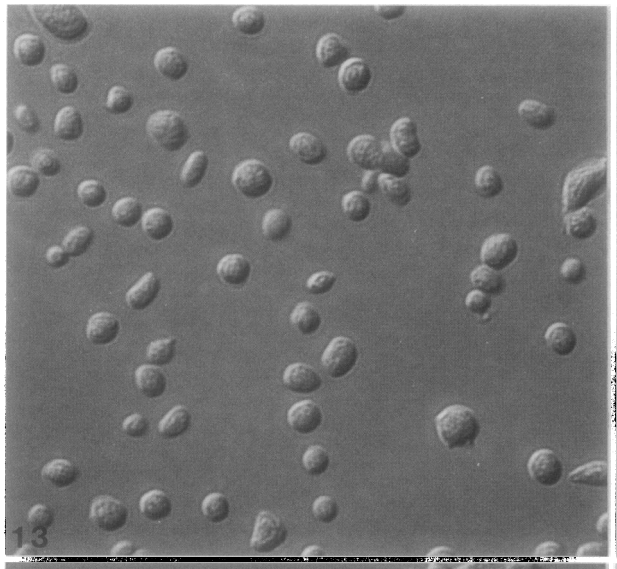
Figure 8. This section of rewarmed platelet. The circumferential coil of microtubules (MT) has completely reassembled and lies just under the cell membrane along its greatest circumference. Magnification, ×21,000.

Figure 9. Platelets chilled to 4°C for 30 minutes, rewarmed to 37°C for 1 hour, and then chilled again to 4°C for 30 minutes, fixed, and examined by DIC microscopy. The platelets have lost their discoid shape again, become relatively spherical in form, and have extended pseudopods. They appear identical to the cells in Figure 3. Magnification, ×780.

Figure 10. Warm, chilled, rewarmed platelets chilled again to 2 to 4°C for 30 minutes. Their appearance is identical to the cells in Figure 4. Microtubule coils have disassembled completely, the cells are irregular, and multiple pseudopods have reformed. Magnification, ×10,500.

Figure 11. Platelets chilled for 30 minutes, rewarmed for 1 hour, chilled again to 4°C, and rewarmed a second time to 37°C for 1 hour, fixed, and prepared for evaluation by DIC microscopy. The cells have retracted the pseudopods caused by exposure to the cold and recovered their discoid form. The cells appear identical to the platelets in Figures 1 and 5. Magnification, ×780.

Figure 12. Warm, chilled, rewarmed, and chilled platelets rewarmed a second time. Pseudopods have been eliminated, the cells have recovered discoid form, and circumferential coils of microtubules (MT) have reformed. Magnification, ×10,500.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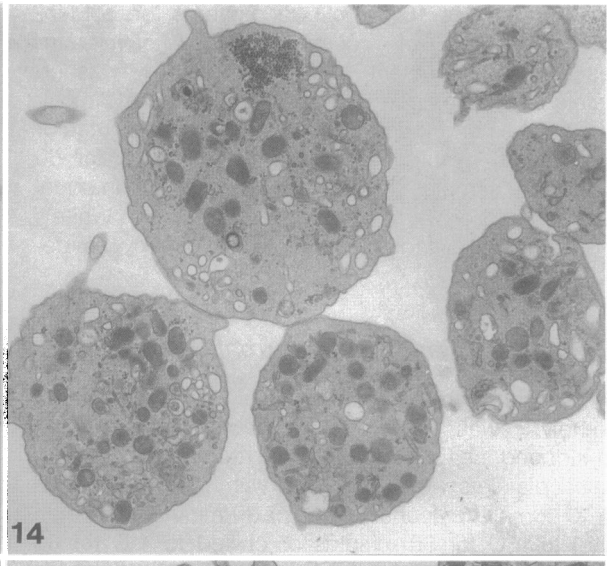
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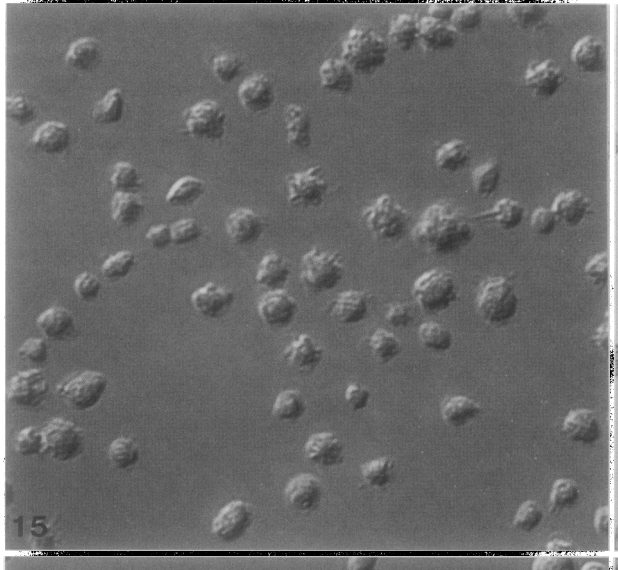
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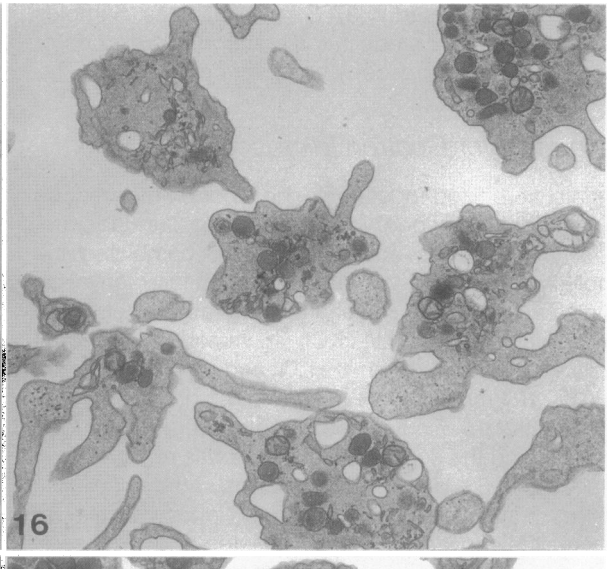
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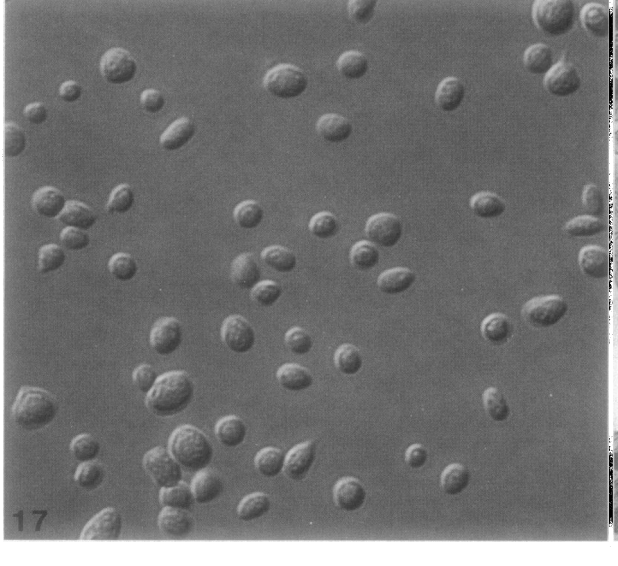
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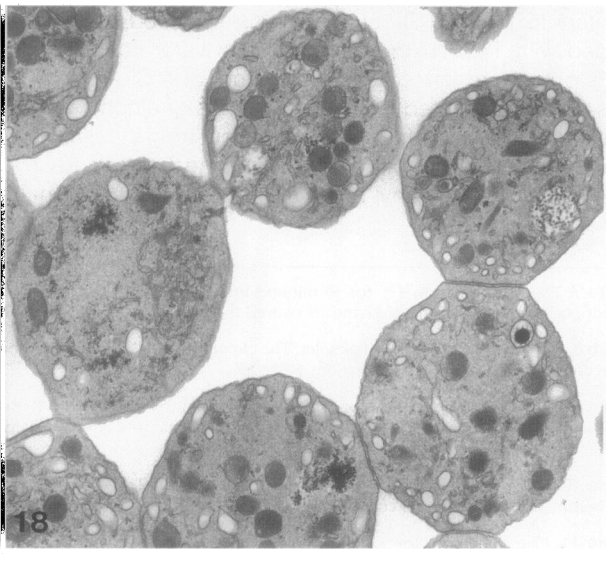
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(Oberkochen, Germany) equipped with an ultraviolet power source and appropriate barrier filters.

Results

Control Resting Platelets at 37°C

The morphology of resting platelets maintained at 37°C for 30 minutes has been reported in detail[21–23] (Figure 1). Their discoid form is supported by a circumferential microtubule coil lying just below the plasma membrane in the equatorial plane (Figure 2). α-Granules, dense bodies, mitochondria, masses of glycogen particles, and elements of the surface-connected open canalicular system (OCS) and dense tubular system are randomly dispersed in the cell matrix.

Chilled Platelets at 4°C

Platelets maintained in the cold for 30 minutes lose their discoid form and become roughly spherical with many surface extensions[24,25] (Figure 3). Microtubule coils are completely disassembled. Examination of random photographs from three experiments revealed a complete absence of microtubules in over 100 platelets (Figure 4). Organelles remained randomly dispersed in the cytoplasmic matrix of cold cells. Examination of control and chilled platelets fixed in methanol and acetone and subsequently stained with anti-tubulin antibody and fluorescein-coupled anti-immunoglobulin antibody revealed microtubule coils in nearly all cells prepared at room temperature (Figure 5) and their complete absence from platelets chilled for 30 minutes (Figure 6).

Rewarmed Platelets at 37°C

Placement of platelets chilled for 30 minutes in a constant temperature water bath at 37°C for 1 hour restored the characteristic discoid morphology of resting platelets to most of the cells (Figure 7). Random photomicrographs taken on thin sections of platelets from three experiments revealed recovery by 84 of 100 platelets. Twelve were spindle shaped, whereas four cells were severely damaged. Microtubules had reassembled and occupied their usual position under the cell membrane in the equatorial plane (Figure 8), whereas in spindle-shaped platelets, microtubule bundles were parallel to the long axis. Spherical platelets with normal morphology but absent microtubule coils were not observed.

Rechilled Platelets at 4°C

Chilling a second time resulted in complete disassembly of microtubules in all platelets in three experiments (Figure 9). Platelets were irregular in form with multiple pseudopods and randomly dispersed internal organelles (Figure 10). They did not differ in appearance from platelets chilled just once.

Second-Time Rewarmed Platelets at 37°C

Most platelets that had been chilled, rewarmed, chilled again, and rewarmed a second time were as discoid in appearance as control platelets not exposed to cold (Figure 11). Circumferential coils of microtubules were reassembled and re-established in their usual positions (Figure 12). Organelles were randomly dispersed in the cytoplasm of twice-chilled, twice-rewarmed platelets. Of 100 platelets, 18 were spindle shaped with microtubule bundles in the long axis. Only 5 of over 100 platelets photographed in thin sections from three separate experiments revealed irreversible damage, loss of membrane integrity, and absence of microtubule coils. Spherical platelets with normal morphology were not found in these studies.

Effects of Microtubule Dissolving Agents

The influence of the three microtubule disassembling agents on platelet microtubules and morphology was variable. Colchicine, even at 10^{-4} mol/L, failed to remove microtubule coils from all platelets. Remnants of microtubules or microtubule coils were present in 45 of 100 cells. Platelets treated with the high concentration of colchicine were often irregular, channels of the open canalicular system were frequently dilated, and many platelets appeared damaged. Nocodazol at the highest concentration tested (10^{-4} mol/L) also failed to completely remove microtubule coils but did not appear to cause morphological injury as observed with colchicine. Thin sections revealed residual microtubules in 7 of 100 platelets treated with 10^{-4} mol/L nocodazole. Vincristine at 10^{-5}

Figure 13. Platelets exposed to 10^{-4} mol/L vincristine for 15 minutes, fixed, and examined by DIC microscopy. The cells have lost their discoid shape and become spherical in form. Magnification, ×780.

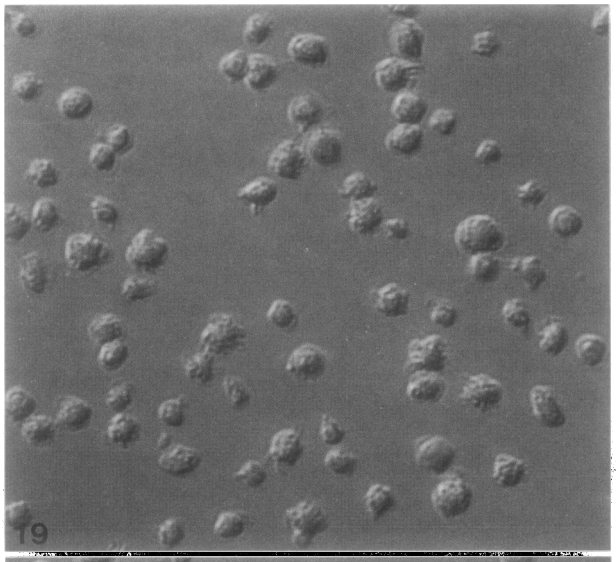
Figure 14. Platelets incubated with 10^{-4} mol/L vincristine for 15 minutes before fixation. Microtubule coils have disassembled completely. The cells have lost their discoid shape and become spherical. Magnification, ×16,000.

Figure 15. Vincristine (10^{-4} mol/L) platelets exposed to 4°C for 30 minutes, fixed, and prepared for study by DIC microscopy. Vincristine-treated platelets develop pseudopods similar to those formed by untreated platelets on incubation at 4°C. As a result they are essentially identical in appearance to untreated platelets after exposure to cold as in Figure 3. Magnification, ×780.

Figure 16. Platelets combined with 10^{-4} mol/L vincristine at 37°C for 15 minutes and then chilled to 4°C for 30 minutes. The cells have become irregular with multiple pseudopods and are indistinguishable from the cells in Figures 3 and 7.

Figure 17. Platelets treated with vincristine (10^{-4} mol/L) chilled to 4°C for 30 minutes and then rewarmed to 37°C for 1 hour. The cells have retracted the pseudopods formed in the cold and recovered the spherical form they assumed after exposure to vincristine and before chilling. Magnification, ×780.

Figure 18. Chilled, vincristine-treated platelets rewarmed to 37°C for 60 minutes. Pseudopods have disappeared completely. The cells are almost perfect spheres. Microtubule coils have not reassembled. Magnification, ×13,000.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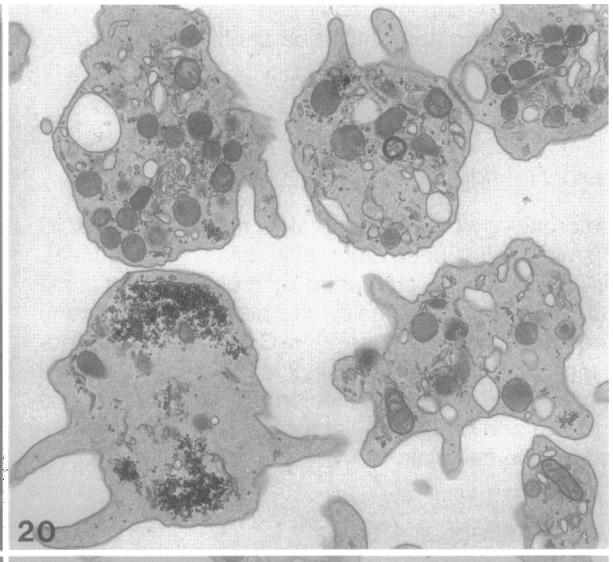
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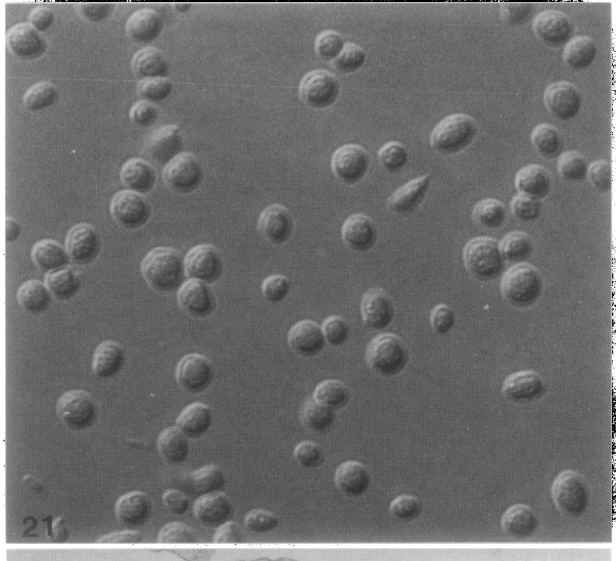
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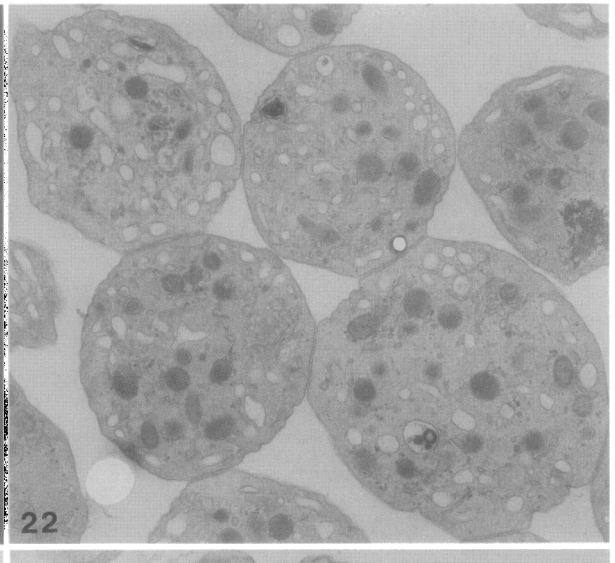
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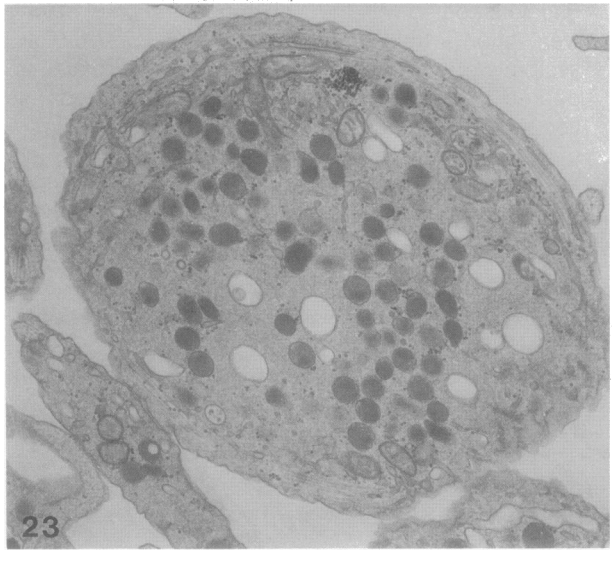
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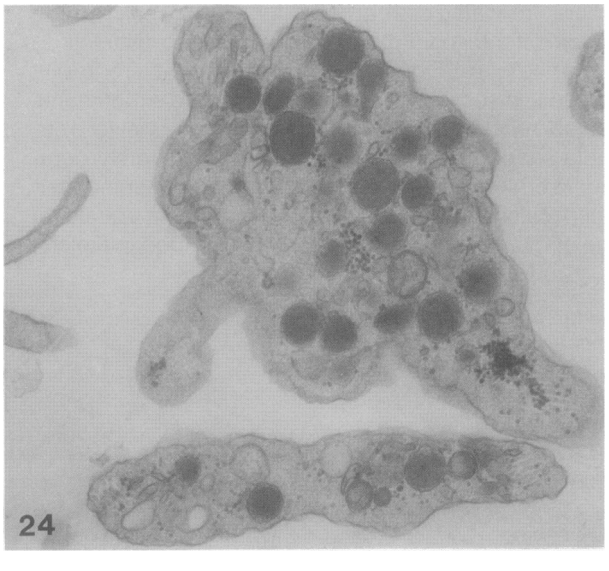
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mol/L revealed only 1 platelet in over 100 randomly photographed cells with a microtubule coil. At a concentration of 10^{-4} mol/L, vincristine removed circumferential coils from all platelets without causing signs of morphological injury. Therefore, we chose to use 10^{-4} mol/L vincristine in the present study to be certain that all microtubules in treated cells had disassembled.

Incubation of platelets treated with 10^{-4} mol/L vincristine at 37°C for 15 minutes, followed by fixation, revealed disappearance of circumferential coils of microtubules and conversion of the discoid cells to spheres (Figures 13 and 14). Exposure of spherical, vincristine-treated warm platelets to low temperatures (2 to 4°C) for 30 minutes resulted in conversion to irregular forms with multiple pseudopods (Figures 15 and 16). Their appearance was indistinguishable from chilled platelets not treated with vincristine.

Addition of 10^{-4} mol/L vincristine to already chilled platelets followed by rewarming to 37°C for 1 hour caused disappearance of pseudopods and conversion to spherical forms (Figures 17 and 18). Chilling the rewarmed spheres to 2 to 4°C again for 30 minutes resulted in redevelopment of irregular forms with pseudopods (Figures 19 and 20), and rewarming a second time caused conversion to spheres (Figures 21 and 22). Microtubules and microtubule coils were absent from all vincristine-treated cells, and organelles remained randomly dispersed.

Effects of a Microtubule Stabilizing Agent

Platelets combined with either 10^{-4} or 10^{-5} mol/L taxol at room temperature or 37°C for 15 minutes were identical in appearance to untreated control platelets (Figure 23). Taxol-treated platelets retained their circumferential coils of microtubules after chilling to 2 to 4°C for 30 minutes.[18] Most of the cells were discoid in appearance, but many had one or two pseudopodial extensions (Figures 24 and 25). When taxol-treated platelets were rewarmed to 37°C, pseudopods disappeared, and the cells were restored to their resting, discoid shape (Figure 26).

Microtubule Stabilization and Inhibition of Actin Filament Assembly

The resting, discoid shape of platelets was well preserved after incubation with cytochalasin B and taxol for 30 minutes at 37°C or room temperature. Cytoplasmic matrix appeared more grainy than in untreated cells, but organelles were unaffected and remained randomly dispersed (Figure 27). Discoid form was preserved in most of the cytochalasin B taxol-treated platelets after incubation at 4°C for 30 minutes, and circumferential coils of microtubules remained intact (Figures 28 and 29). Exposed surfaces of the chilled cells, however, were more irregular than those of unchilled platelets due to occasional short pseudopods and frequent dilatation of OCS channels. Rewarming to 37°C for 1 hour eliminated the OCS dilatation caused by cold and restored the smooth surface appearance to the discoid platelets (Figure 30).

Influence of Temperature on Platelet Actin-Filament Assembly

Phalloidin binds exclusively to filamentous actin in platelets.[10] Approximately 40% of the actin in resting platelets is assembled into filaments, whereas 60% is in the molecular form.[11] Therefore, the fluorescence intensity of rhodamine-phalloidin in resting platelets was assumed to represent 40% assembled actin. Thrombin causes rapid assembly of new actin filaments from molecular actin until approximately 80% is in the polymerized form. Thus, the fluorescence intensity of rhodamine-phalloidin in thrombin-activated platelets was assumed to represent 80% filamentous actin. Chilling caused a significant increase in fluorescence intensity indicating an increase in the percentage of actin in filamentous form in cold platelets (Figure 31). Rewarming caused the newly assembled actin filaments in chilled platelets to disassemble as reported previously by Pribluda and Rotman.[24] Subsequent chilling, rewarming, and chilling produced results essentially identical to the initial chilling and rewarming. Actin filaments assemble in the cold and disassemble when platelets are rewarmed (Figure 31).

Discussion

Results of the present study have confirmed an important role for the circumferential coil of microtubules in establishing and supporting the discoid shape of resting platelets and restoring their lens-like appearance after partial activation or after rewarming of chilled platelets.[5–7,24,25] It was common laboratory practice up until 1960 to prepare

Figure 19. Platelets chilled, rewarmed, and treated with vincristine (10^{-4} mol/L) and chilled a second time to 4°C for 30 minutes. Cell bodies remain spherical but have developed pseudopodial extensions. They resemble platelets in Figures 3, 7, and 13. Magnification, ×780.

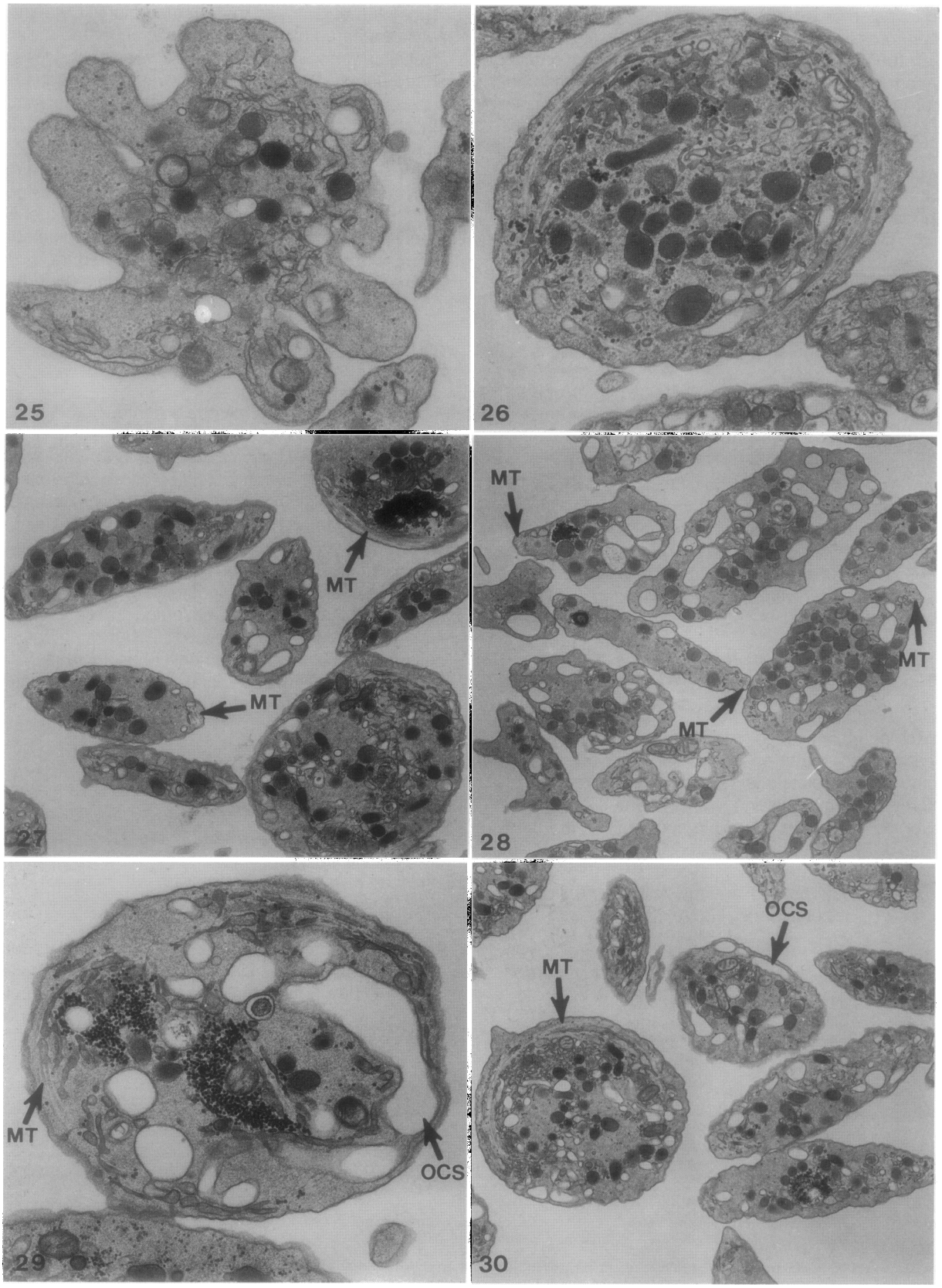
Figure 20. Vincristine-treated warm platelets, chilled, rewarmed, and then chilled again. The spherical platelets have developed multiple pseudopods. Magnification, ×13,000.

Figure 21. Platelets treated with vincristine (10^{-4} mol/L) chilled twice for 30 minutes and rewarmed twice. Pseudopods formed during the second exposure to low temperature have retracted, and the cells have recovered their spherical form. Magnification ×780.

Figure 22. Vincristine-treated warm platelets chilled, rewarmed, chilled again, and rewarmed a second time. Pseudopods have been eliminated, and the spherical form has been restored. Microtubules are not evident in the spherical platelets. Magnification, ×15,000.

Figure 23. Platelet sample incubated with 10^{-5} mol/L taxol for 15 minutes before fixation. Discoid form is well preserved. Magnification, ×15,000.

Figure 24. Platelet sample treated with 10^{-4} mol/L taxol for 15 minutes and then chilled to 2°C for 30 minutes. Most cells retain their discoid form supported by circumferential coils of microtubules. Magnification, ×19,000.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TEMPERATURE CYCLING	PERCENT F-ACTIN*	ESTIMATED PERCENT INCREASE
Resting	40.0	
Resting - 4°C	48.8 ± 2.0	22.0
4°C-37°C	39.0 ± 1.8	-
4°C-37°C-4°C	46.7 ± 2.4	19.7
4°C-37°C-4°C-37°C	40.2 ± 2.7	-
4°C-37°C-4°C-37°C-4°C	48.0 ± 3.6	19.4

*Mean and the standard error (n = 6)

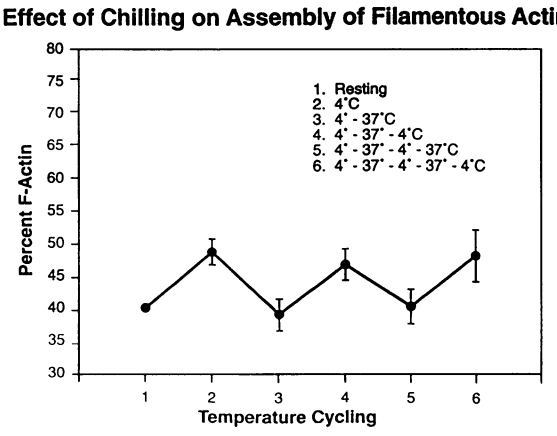

Figure 31. Results of six experiments measuring the state of filamentous actin in control and chilled platelets. Chilling increases the content of filamentous actin in platelets, whereas rewarming reduces it to resting platelet levels. The tabular results are presented in graphic form in the chart.

platelet-rich plasma and washed platelets in the cold to prevent activation before use in experimental studies and to keep the cells chilled during fixation in osmic acid. Introduction of glutaraldehyde as the initial fixative and recognition that room temperature or 37°C were more appropriate for platelet studies led to discovery of the circumferential coil of microtubules.[5–7]

The observation that conditions that preserved the microtubule coils also resulted in preservation of platelet discoid form led to the concept that the two phenomena were associated.[6,7] Microtubules were located just under the surface membrane along the greatest circumference of the disc when the cell was sectioned in the equatorial plane.[23,29] Platelet activation in suspension or on surfaces resulted in constriction of microtubule coils into tight rings around centrally concentrated organelles, freeing peripheral areas of the cytoplasm for shape change and pseudopod extension.[21,22] Platelets that had undergone shape change, but not irreversible transformation, could recover discoid form with restoration of the circumferential coils of microtubules to resting positions under the cell walls.[30]

Investigations with microtubule disassembling and stabilizing agents also supported the concept that microtubule coils maintained discoid shape. Chemical removal of the coils by colchicine, vincristine, or other vinca alkaloids converted the discoid cells to rounded, irregular forms.[12] Taxol stabilizes microtubules and promotes their assembly.[13] When taxol-treated platelets were chilled to 2 to 4°C, most of the cells remained relatively discoid.[18] Pseudopods were less frequent and short compared with those on untreated platelets. In the present study, rewarming the chilled, taxol-treated platelets resulted in disappearance of pseudopods and restoration of discoid form.

Chilling and rewarming experiments with untreated platelets were particularly supportive of the idea that microtubule coils were responsible for platelet discoid shape.[24,25] Exposure to low temperature caused microtubules to disassemble and platelets to change shape. The disc-like cells became relatively spherical in appearance with multiple pseudopods. When the chilled platelets were rewarmed, the circumferential microtubules reassembled in most cells, and the platelets reassumed their discoid form without pseudopods.[31,32] The several lines of evidence gave credence to the concept that microtubule coils and platelet disc shape are directly related.

Bovine platelets differ from human cells in this regard. They also contain a circumferential coil of microtubules, but in bovine platelets it does not appear to serve the same function that it does in human cells.[33] Bovine platelets must be exposed to colchicine or vinca alkaloids for at least 1 hour longer than human cells to disassemble the microtubule rings. Chilling requires up to 2 hours to dissolve bovine platelet microtubules, and their removal does not cause loss of discoid shape.[34] Even after disappearance of the rings in bovine platelets the cells remain discoid and just as resistant to aspiration into a micropipette as untreated, control platelets.[34]

Figure 25. Platelet combined with 10^{-5} mol/L taxol for 15 minutes and then chilled to 2°C for 30 minutes. The circumferential coil of microtubules has not been disassembled by cold. It retains its usual position under the cell membrane. Pseudopods on taxol-treated platelets are short and less common than on chilled platelets not treated with taxol. Magnification, ×19,000.

Figure 26 Platelet from sample of platelets treated with 10^{-4} mol/L taxol for 15 minutes, chilled to 2°C for 30 minutes, and then rewarmed to 37°C for 60 minutes. The cell is discoid in form, and pseudopods are absent. Magnification, ×19,000.

Figure 27. Platelets combined with 10^{-5} mol/L taxol and 10^{-5} mol/L cytochalicin B for 30 minutes at 37°C before fixation. Discoid shape and circumferential coils of microtubules (MT) are well preserved. Magnification ×13,000.

Figure 28. Platelets treated with taxol (10^{-5} mol/L) and cytochalicin B (10^{-5} mol/L) chilled to 4°C for 30 minutes. The cells are relatively discoid in appearance and contain circumferential coils of microtubules (MT). Surface irregularity is due to dilation of OCS channels. Magnification, ×13,000.

Figure 29. Higher-magnification image of a platelet treated with 10^{-5} mol/L taxol and 10^{-5} mol/L cytochalicin B before chilling to 4°C for 30 minutes. The discoid shape and circumferential coil of microtubules are well maintained, but some surface irregularity is due to dilatation of OCS channels. Magnification, ×27,500.

Figure 30. Platelets treated with taxol and cytochalicin before chilling to 4°C for 30 minutes and then rewarmed to 37°C for 1 hour before fixation. The cells are discoid and contain circumferential coils of microtubules (MT). External surfaces are smooth and OCS channels are no longer dilated. Magnification, ×13,000.

The basis for these differences in human and bovine platelets appears due to variations in their intrinsic anatomy and functional expression.[35] A peripheral arrangement of assembled actin filaments plays a role in supporting the discoid shape of bovine platelets. As a result, both disassembly of microtubules by colchicine and prevention or reversal of actin filament assembly by cytochalasin B were required to cause bovine platelets to lose their discoid form.[33]

The recent study emphasizing the role of new actin filament assembly in cold-induced platelet distortion[4] stems from investigations suggesting a major role for the surface membrane cytoskeleton and associated proteins, such as spectrin, rather than the circumferential coil of microtubules, in supporting the discoid form of resting platelets.[2,3] As chilling causes calcium flux, new actin filament assembly and shape change, phenomena also observed when platelets are activated by potent agonists without disassembly of microtubules,[3] it seemed reasonable to focus on new actin filament assembly,[4,10,20,28,36] rather than disappearance of microtubules, as a basis for cold-induced platelet distortion.

However, the concepts that disassembly of microtubule coils or assembly of actin filaments cause the shape change induced by chilling are not mutually exclusive. Both phenomena appear to contribute significantly. The real difference appears to be in their relative contributions to the shape of resting and rewarmed platelets. That question seemed to be resolved by early studies showing that reassembly of circumferential coils of microtubules on rewarming chilled platelets was associated with recovery of discoid shape.[20,25,31,32]

The present study has confirmed the early findings and added the observation that a second cycle of chilling and rewarming results in reassembly of the circumferential coil of microtubules and restoration of lens-like form. The role of microtubules in the recovery was confirmed by adding vincristine to platelets before or after chilling, thereby blocking reassembly of microtubule coils during rewarming.[12] The vincristine-treated platelets became spheres devoid of pseudopodial extensions on exposure to 37°C. These findings support the concept that microtubule coils are the major support mechanism for the disc-like form of resting platelets and primarily responsible for return of the resting discoid form when chilled platelets are rewarmed.

The experiments with vincristine also confirm earlier reports[10,20,28,36] suggesting that assembly of actin filaments in chilled platelets is primarily responsible for cold-induced pseudopod extension. Treatment of platelets with vincristine at 37°C converted the discs to irregular, rounded cells. Chilling vincristine-treated platelets resulted in their transformation to irregular forms with multiple pseudopods. Exposure of untreated chilled platelets to vincristine in the cold had no effect on their irregular form with surface extensions. However, when the chilled, vincristine-treated platelets were rewarmed, their pseudopods disappeared and the cells were converted to spherical forms as indicated above. Chilling these platelets a second time transformed them to irregular forms with many pseudopods, and another rewarming restored the spherical form without pseudopods. Thus, actin filament assembly is responsible for extension of pseudopods in the cold and disassembly of actin for recovery of smooth-surfaced, vincristine-treated spheres on rewarming.

These studies suggested that the best way to preserve platelet discoid shape in the cold was to stabilize microtubule coils and inhibit new actin filament assembly by incubating platelets with both taxol and cytochalicin B before chilling. Platelets treated with the two agents retained circumferential coils of microtubules and remained relatively discoid at low temperature. However, cytochalicin B appeared to cause channels of the OCS to dilate in the cold, resulting in irregularity of the exposed surface. Rewarming eliminated the OCS swelling and completely restored the resting, discoid appearance. The mechanism of cytochalicin-induced dilation of OCS channels in the cold is unknown.

Winokur and Hartwig[4] found in their study that rewarming chilled platelets to temperatures between 24 and 37°C resulted in disappearance of pseudopods and formation of irreversible spheres. It was suggested that the actin filament coils that cause pseudopod extension in chilled cells undergo reorganization that eliminates pseudopods and transforms irregular platelets into irreversibly spherical cells. However, the state of actin filament assembly was apparently not measured in the rewarmed, irreversible spheres by Winokur and Hartwig.[4]

In contrast, earlier studies of temperature-induced activation had demonstrated that 60% of newly formed actin filaments in chilled platelets disassembled when the cells were rewarmed.[28] We have repeated the Pribluda and Rotman study using rhodamine-phalloidin to measure the state of actin filament assembly in warm and cold platelets[4,10]. Our results are similar to theirs. Chilling causes significant assembly of new actin filaments that disassemble when the cells are rewarmed. The actin filament assembly-disassembly cycle was repeated when the platelets were chilled and rewarmed a second time. Thus, actin filament assembly in chilled cells causes pseudopod formation, and loss of the circumferential coils of microtubules contributes to cold-induced disc-sphere transformation. Rewarming causes the actin filaments to disassemble and pseudopods to disappear, whereas reassembly of the circumferential coil of microtubules restores lentiform appearance to most cells.

In summary, twice-chilled and -rewarmed platelets in this study were discs, not irreversible spheres. Their ability and that of vincristine-treated cells to undergo further shape change and recovery indicated that rewarmed discs or vincristine-treated spheres are not irreversible. Actin filament assembly in the cold appears primarily responsible for pseudopod formation and disassembly for their disappearance on rewarming. The presence of circumferential microtubule coils appears primarily responsible for the discoid shape of resting and rewarmed platelets, whereas the state of actin filament assembly is primarily involved in pseudopod extension and retraction and less essential than microtubule coils for maintaining discoid shape in human platelets.

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