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Microfilament Reorganization in Normal and Cytochalasin B Treated Adherent Thrombocytes

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Thrombocyte adhesion following activation by a Formvar surface involves a morphologic transition resulting in a fully spread cell. Correlative SEM and whole mount TEM were used to study the cytoskeletal alterations that accompany changes in surface morphology during adhesion. Following initial adhesion, thrombocytes extend slender pseudopods containing longitudinally oriented bundles of filaments that are 13–22 nm in diameter. Concomitant with pseudopod extension, a cytoplasmic hyalomere, consisting of a dense filamentous network, extends between the pseudopods and ultimately results in a fully spread cell. Treatment of thromboyctes with cytochalasin B (10^{-5} M) caused clumping of the hyalomere filament network and retraction of the hyalomere. Examination of partially retracted cells revealed that pseudopod filament bundles were continuous with the contracting filamentous network. It is concluded that pseudopod filament bundles and cytoplasmic hyalomere filaments are interconvertible and that their organizational relationship changes in accordance with gross morphologic changes.

Key words: adhesion, microfilaments, thrombocytes, whole mount, cytochalasin B

Cell adhesion to artificial surfaces is a complex process involving surface contact, pseudopod extension, and spreading of the hyalomere. Paralleling these morphologic alterations is the reorganization of cytoskeletal elements. Light microscopy [1,2] and SEM studies [3,4] have been used to elucidate the general morphologic changes, and the stage of cytoplasmic filaments in many types of adherent cells has been studied using whole mount [4–10] and thin section TEM [11,12]. The microfilament nets and bundles observed in many cell types comprise a variety of specialized cell regions, including cytoplasmic ruffled areas, the cytocortex, and dense stress fiber bundles that extend from pseudopods to the perinuclear region [4–12]. These organized microfilament networks contain the contractile protein actin, as demonstrated in whole mount cells by immunoperoxidase [5], actin antibody immunofluorescence [13], and heavy meromyosin binding [9,14]. Although it has often been postulated that microfilaments are responsible

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for changes in cell morphology [5–9,15–17], direct evidence correlating surface morphology with the internal microfilament network is limited, with most substantiation based upon observations made following treatment with pharmacologic agents such as cytochalasin B (CB), which dramatically disrupts microfilament organization.

If cells such as blood platelets are treated with CB while in suspension, they retain the capability to adhere but fail to extend pseudopods or spread on artificial surfaces [4,18,19]. Similarly, treatment of platelets with CB after adhesion will cause retraction of the spread hyalomere and pseudopods [20,21]. Several mechanisms have been suggested to account for these CB-induced changes, including 1) CB dissociates actin from its plasma membrane binding site [22,23]; 2) CB induces contraction of microfilaments [24–26]; and 3) CB prevents actin polymerization [27–32]. Conceivably these mechanisms could act simultaneously. Since the mechanisms explaining the mode of CB action have been based primarily on in vitro biochemical studies, it is important to more clearly link the hypotheses to the actual effects at the cellular level. Direct demonstration of CB effects upon cytoskeletal organization at the cellular level has been limited; however, the recent development of whole mount TEM suggests that this technique has tremendous potential for studying the relationship between cytoskeletal and surface alterations during the normal adhering process and under conditions of pharmacologic agent treatment.

Using avian thrombocytes, cells whose primary function in hemostasis is facilitated by adhesion, we report correlative SEM/whole mount TEM studies of morphologic changes in the surface and microfilaments of adherent cells. These results are compared to the changes in microfilament organization following CB treatment.

MATERIALS AND METHODS

Cell Isolation and Adhesion

Thrombocytes (the nucleated counterpart of the mammalian platelet) were isolated from White Carneau pigeons as previously described [18]. The basic technique involved centrifugation of whole anticoagulated blood (3.8% sodium citrate 1 vol:9 vol blood) at 800g for 90 sec to obtain thrombocyte-enriched plasma. The thrombocytes were then repeatedly washed in Puck's saline-G containing 0.3% EDTA at pH 6.5 and then allowed to adhere to carbon-stabilized Formvar-coated copper grids for 40 min before being fixed for 15 min with 1% glutaraldehyde in 0.1 M cacodylate buffer containing 0.1 M surcrose. This adhesion technique is described in detail elsewhere [4,18]. Treated cells were incubated with CB (Sigma Chemical Co.) (10^{-5} M) for 30 min either before or after adhesion to the Formvar grids.

Preparation for Electron Microscopy

The whole mount technique as described by Buckley [6] and applied to platelets [4] was used for these studies. This involved, subsequent to glutaraldehyde fixation, post-fixation in 1% osmium tetroxide, rinsing with distilled water, and briefly staining with methanolic uranyl acetate. The cells on the grids were then dehydrated in a graded series of ethanol and dried from CO_2 by the critical-

point method [4,6]. Whole mount preparations for TEM were carbon coated by vacuum evaporation. On occasion, subsequent to TEM observation, the grids were coated with gold-palladium and observed by SEM. The samples were analyzed with either a Philips EM-400 (TEM) at 100 kV or a Philips SEM-501 (SEM) at 15 kV.

RESULTS

When washed thrombocytes are exposed to carbon-stabilized Formvar-coated grids, they activate and adhere. Activation first involves pseudopod extension, followed by spreading of the hyalomere between pseudopods resulting in fully spread cells [18]. With SEM used to follow the morphologic transition of adherent thrombocytes, the initial event observed was attachment to the Formvar surface (Fig. 1a). Subsequently many pseudopods were extended across the surface (Fig. 1b), and after pseudopod extension a hyalomere of cytoplasm spread between the pseudopods (Fig. 1c). The hyalomere spreading between pairs of pseudopods occurred at different rates, resulting in some regions of a cell being fully spread while other regions were just starting to spread. After 40 min of in-

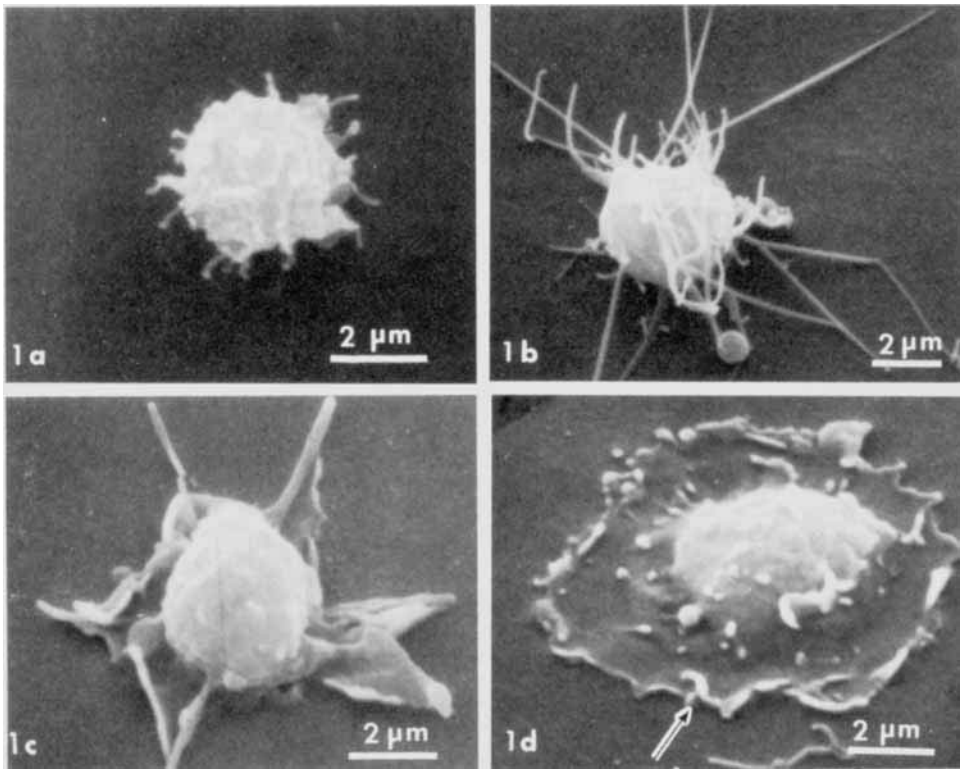


Fig. 1. SEM of thrombocytes adherent to carbon-coated Formvar grids. The initial attachment (a) is followed by pseudopod extension (b). A cytoplasmic hyalomere spreads out between pseudopods (c) resulting in a fully spread adherent thrombocyte (d) lacking pseudopods, but having several marginal membrane ruffles (arrow).

cubation most of the cells had a significant amount of pseudopod extension as compared to hyalomere spreading (Fig. 1c), indicating that hyalomere spreading is a comparatively slow process. The end product of adhesive activation was a fully spread thrombocyte lacking pseudopods but having several marginal membrane ruffles (Fig. 1d). High-magnification stereo SEM revealed that only part of individual pseudopods and hyalomere expanses were in intimate contact with the surface (Fig. 2). Typically, as shown in the stereo-pair micrographs of Figure 2, unattached distal regions of extended pseudopods and the spreading hyalomere were lifted away from the adherent surface.

When whole mount TEM is used to view cells at different stages of adhesion, the degree of involvement of cytoskeletal elements becomes apparent [4]. After initial surface contact was made, the extending pseudopod cytoplasm was composed of filament bundles anchored by a more delicate filamentous membrane (Fig. 3a). These filaments within bundles were typically 13–22 nm in diameter, whereas the fine filaments connecting them to the plasma membrane were 3–4 nm thick. As the hyalomere extended between the pseudopods, it was found to consist of a continuous filamentous network (Fig. 3b). This hyalomere organization was different from the pseudopod filament bundles in that, within the network, individual filaments were indistinguishable. Although organizationally distinct, the networks and bundles appeared to be related, for the filament bundles observed in pseudopods seemed to be continuous with the hyalomere filamentous network (Fig. 3b). The proportion of network increased as the cells became more spread, and the fully spread thrombocyte consisted of a continuous filamentous network (Fig. 4). Marginal membrane ruffles consisting of a condensation of network filaments were common in fully spread cells. Stereomicroscopy of whole mount thrombocytes revealed that these uplifted portions of the plasma membrane were involved in adhesion (Fig. 5). These areas of adhesion consisted of fine filament strands (3–4 nm in diameter) anchoring the plasma membrane to the Formvar. The various organelles of thrombocytes, including mitochondria, electron-dense serotonin storage

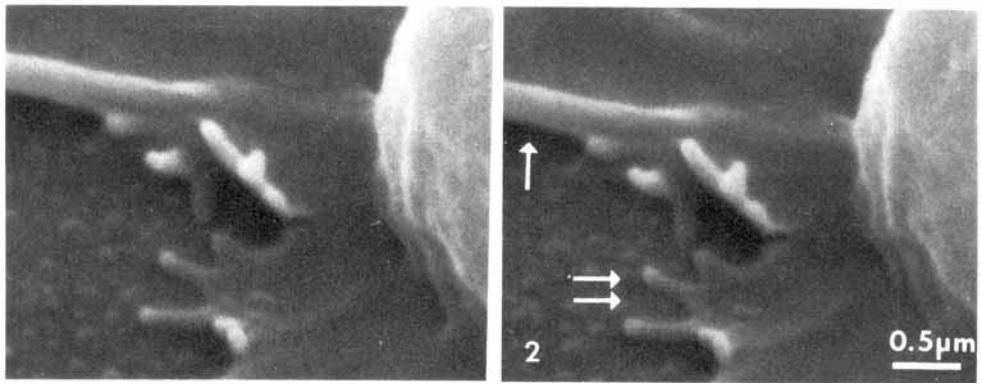


Fig. 2. SEM stereomicroscopy demonstrates that only portions of the pseudopods (arrow) or hyalomere (double arrow) are attached to the surface.

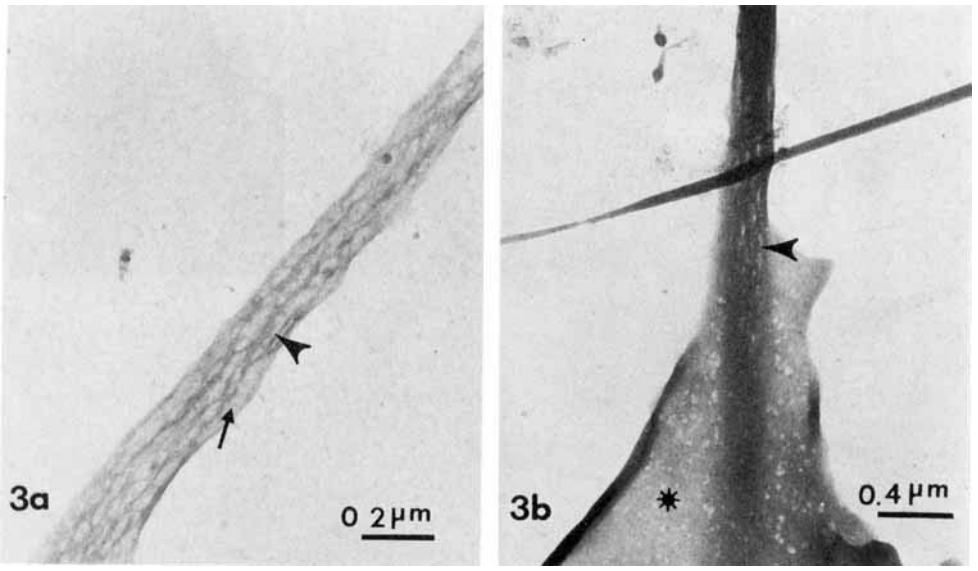


Fig. 3. Whole mount TEM of thromboyctes at various stages of adhesion revealing the ultrastructure of the filament bundles and filamentous network. (Compare to Figure 1, SEM micrographs of the correlative surface morphology.) Pseudopods (a) are composed of filament bundles (large arrow) interconnected with other bundles and the plasma membrane by fine filaments (small arrow). The spreading hyalomere (b) consists of a filamentous network (*) that is continuous with the filament bundles (arrow) of the pseudopod. The continuity is demonstrated by the gradual loss of bundle identity when going from pseudopod to hyalomere.

granules and lysosomes apparently remained in the electron-dense nuclear region, for no evidence of them was found in the peripheral hyalomere.

Treatment of thrombocytes with CB for 30 min before or after adhesion resulted in dramatic alterations in cytoplasmic organization. When thrombocytes were treated with CB prior to adhesion, they retained their adhesiveness but failed to extend pseudopods or spread (Fig. 6). Conversely, when normally adherent cells (40 min on Formvar) were subsequently treated with CB, a retraction of the hyalomere filamentous network occurred (Fig. 7). This resulted in the network being drawn into stellate clumps interconnected by 2.5–3.5 nm filament bridges (Fig. 8). These stellate clumps further condensed into dense clumps and strands (Fig. 9), which contrasted to the hyalomere filamentous network of normally adherent cells not treated with CB (Fig. 10). Unlike this effect on the hyalomere, the filament bundles in pseudopods of preadherent CB-treated cells appeared not to be affected, and the pseudopods remained extended (Fig. 7).

CB-induced clumping of the filamentous network more clearly revealed the relationship of filament bundles (pseudopods) to the network (hyalomere), for in the early stages of condensation the bundles were continuous with the network (Fig. 11). As the network condensed further the filament bundles from pseudopods were also found continuous with circumnuclear filament bundles (Fig. 12). When the filamentous network was fully retracted as a result of CB treatment, the use of stereomicroscopy

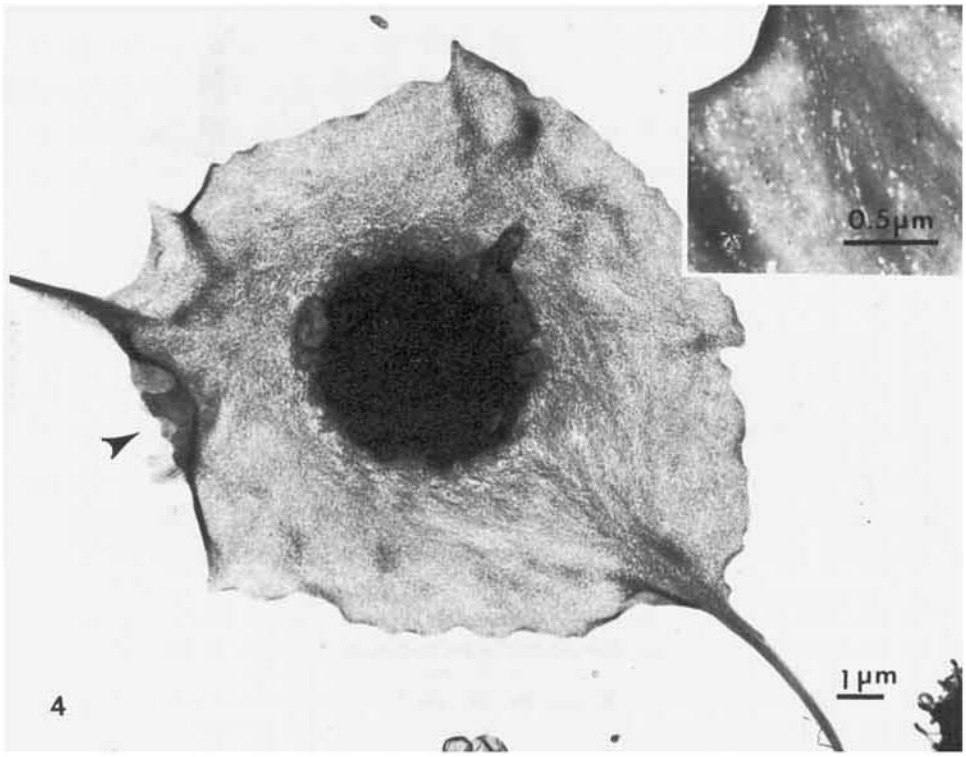


Fig. 4. Whole mount TEM of a fully spread thrombocyte consisting of a continuous filamentous network with marginal membrane ruffles (arrow). Higher magnification (inset) reveals the lack of dense filament bundles and organelles in the hyalomere.

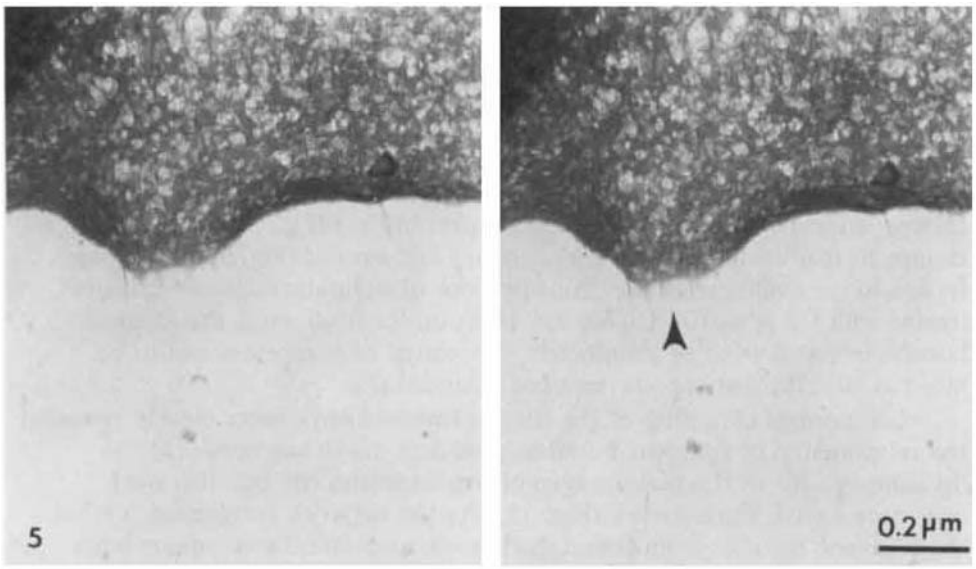


Fig. 5. Stereo whole mount TEM of an adherent region of a thrombocyte, illustrating the uplifted nature of the peripheral membrane. The elevated region is in contact with the Formvar through fine filament strands (arrow). Compare to Figure 2.

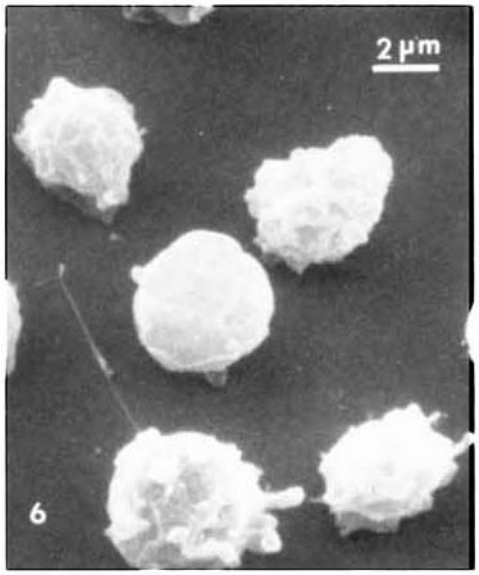


Fig. 6. SEM of thrombocytes treated with CB (10^{-5} M) before adhesion. Under these conditions thrombocytes remained adhesive but failed to extend pseudopods or spread.

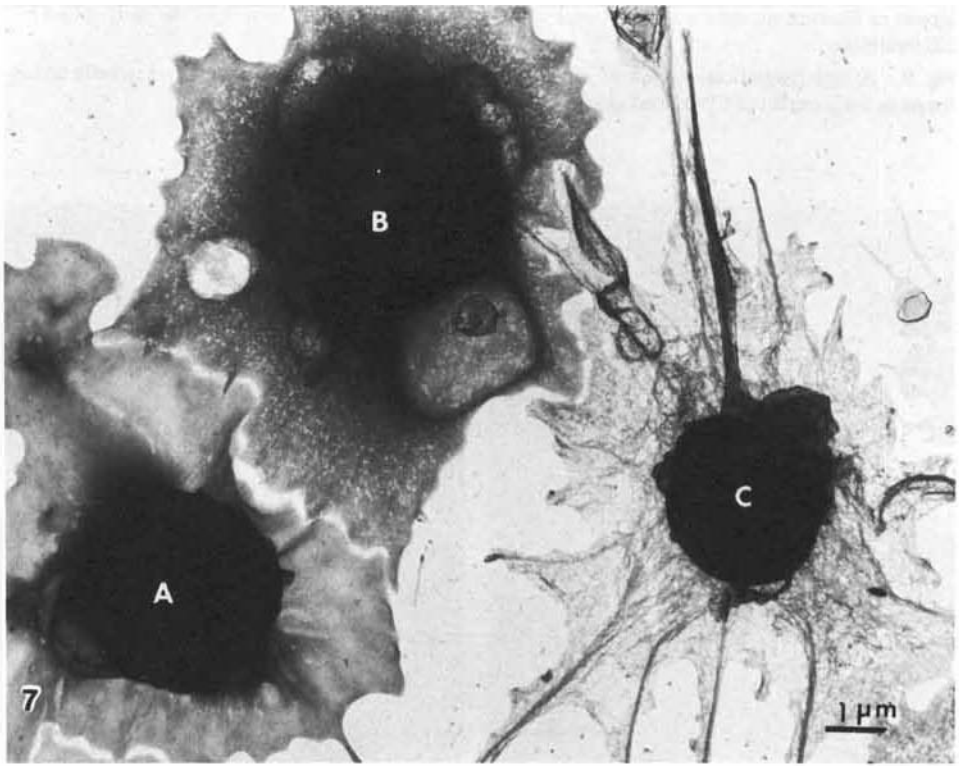
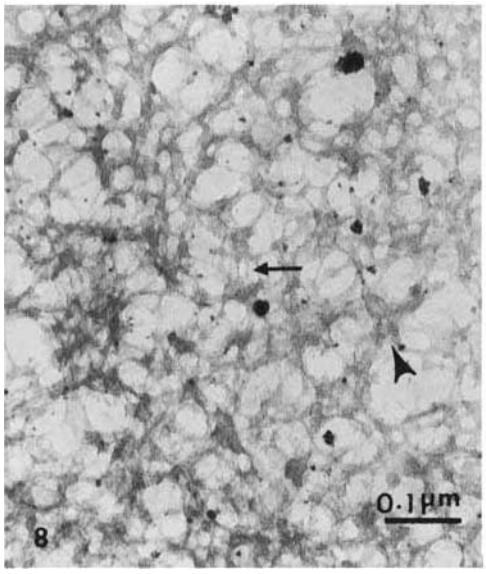


Fig. 7. CB treatment (10^{-5} M) after adhesion dramatically alters thrombocyte morphology. Cells vary in their rate of response so that all stages of CB-induced changes can be found on one grid. In this micrograph A would represent a normal cell unaffected by CB; B shows the first event in CB alteration of morphology: a subtle clumping of the continuous filamentous network. The full extent of CB action can be seen in cell C, where the continuous filamentous network has collapsed, forming dense strands and resulting in retraction of the hyalomere, but not pseudopods.



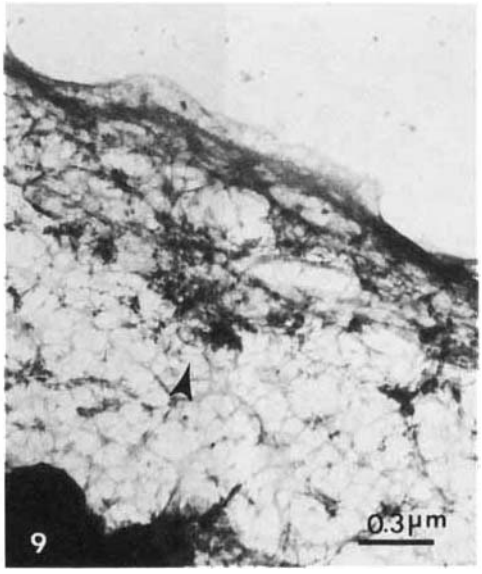
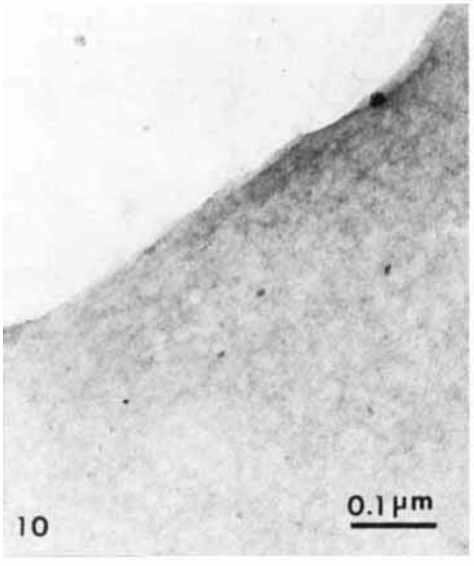


Fig 8 A high magnification region of cell B, Figure 7, demonstrating the stellate clumps (large arrow) of filamentous network connected by fine strands (small arrow) as seen in the early stages of CB treatment

Fig 9 A high magnification view of cell C, Figure 7, showing the dense clumps and strands (arrow) found in fully retracted CB-treated cells



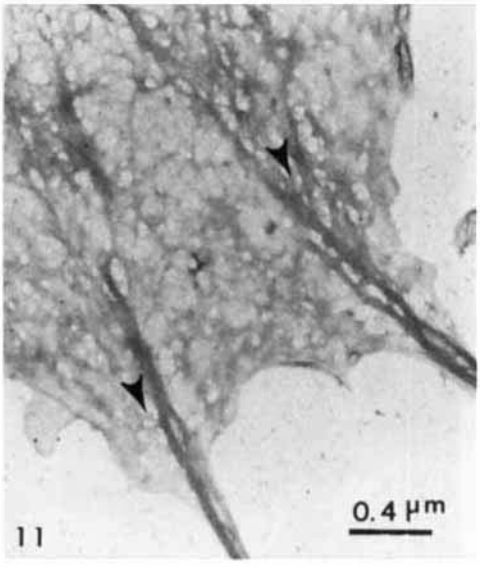


Fig 10 The filamentous network of thrombocytes that have not been treated with CB is continuous

Fig 11 In the early stages of CB-induced clumping of the filamentous network it can be seen that the filament bundles are continuous from pseudopod to perinuclear region and are interconnected with the hyalomere filaments (arrow)

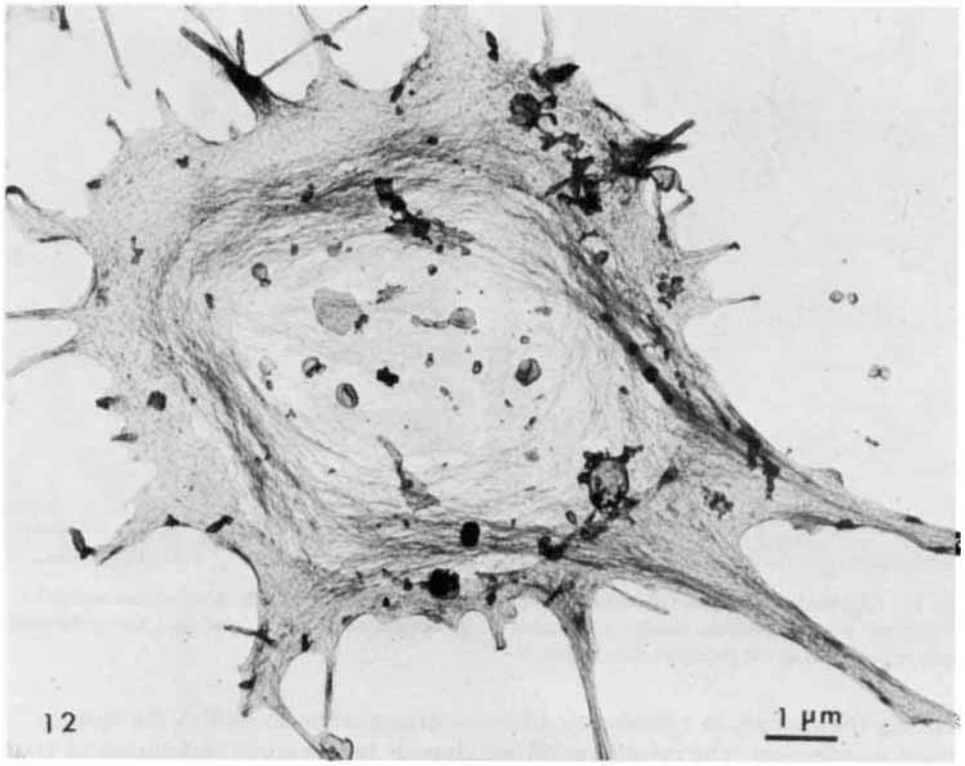


Fig. 12 A thrombocyte in which CB has caused enucleation illustrates the continuity of pseudopod filament bundles with circumnuclear filament bundles.

revealed the 3-dimensional location of filaments. With this approach it was apparent that in CB-treated cells the condensed filamentous network was ubiquitous, whereas the filament bundles were located at the bottom of the cell (near the Formvar surface). This position of filament bundles was consistent throughout the cell from pseudopods to nucleus (Fig. 13).

DISCUSSION

When avian thrombocytes are activiated by contact with a Formvar surface, they change morphology from ovoid to a pseudopod adhesive stage, then extend a cytoplasmic hyalomere resulting in fully spread cells. This sequence of morphologic changes parallels previous light microscopic [1] and SEM [3,4] studies of adherent spreading mammalian platelets. It has been suggested that microfilaments composed of actin are responsible for these morphologic changes [6,7,15–17]. Investigations in which whole mount cells have been studied using immunofluorescence of actin antibodies [13], immunoperoxidase [5], immunoferritin [33], or heavy meromyosin binding [9,14] confirm the presence of actin in the filamentous regions of spreading cells. This actin distribution appears to correlate with the ultrastructure of the cytoskeletal elements in spread cells [4–12,34]. The present observations have extended our previous studies by de-

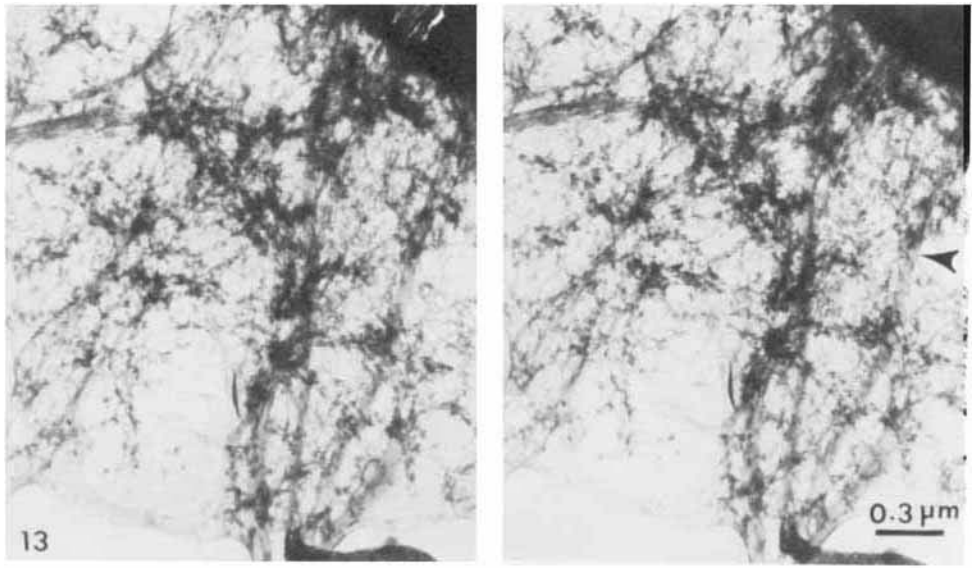


Fig. 13 Stereomicroscopy of CB-treated thrombocytes demonstrates that the filamentous network is ubiquitous, whereas filament bundles are located at the bottom of the cell (arrow) and are continuous from pseudopods to the circumnuclear region

scribing the changes in cytoplasmic filament organization in cells at the various stages of adhesion. The cytoplasm of pseudopods in this study was shown to contain filament bundles 13–22 nm in diameter, whereas the diffuse hyalomere was composed of a dense filamentous network in which individual filaments were indistinguishable when viewed by whole mount TEM. These filament bundles (pseudopods) and the filamentous network (hyalomere) appeared continuous and interconvertible. It is very likely that they represent different levels of organization of actin filaments. This observation is compatible with the suggestion that pseudopod formation, which occurs rapidly in platelets [1], would require the interconversion of filaments from nets to bundles [5].

Treatment of thrombocytes with the microfilament-disrupting agent cytochalasin B further illustrates the interrelationship of cytoplasmic filaments. Thrombocytes treated with CB before adhesion lacked the adhesion-induced morphologic alterations; CB treatment after adhesion resulted in partial reversal of the adhesion-induced changes. These observations are in agreement with the reports of others who have studied the effects of CB on mammalian platelets [20,21]. Unlike mammalian platelets, however, thrombocytes do not retract pseudopods when treated with CB. In this respect the thrombocyte is similar to HeLa and MDBK cells [25] or fibroblasts [35]. The CB-induced filament alterations in the present study were expressed as a collapse and retraction of the cytoplasmic filamentous network. Ultrastructurally, this resulted in an extracted-appearing cytoplasm which resembles that seen when adherent cells are treated with Triton X-100 [5,9,13,33].

CB-induced clumping is, as shown in this study, an orderly process involving retraction of filaments into small stellate clumps interconnected by 2.5–3.5 nm filament bridges. This retraction is followed by further condensation into

large clumps and strands. Others have suggested that CB disruption of microfilaments in adherent cells may result from a severing of actin binding to the plasma membrane [22,23]. This is consistent with our observations that subsequent to CB treatment the filamentous network retracts from the peripheral portions of the plasma membrane. Conceivably, this retraction of actin could result from the dissociation of actin from membrane acting binding protein [22,23]. If severing of the actin-to-membrane bonds does occur, clumping could result from the contraction of the actin complex to form the stellate configuration. It is noteworthy that our observations of CB-induced microfilament clumping parallels observations made by others of the cytochalasin effects on MDBK and HeLa cells [25,26,36], fibroblasts [35,36], and 3T3 cells [22].

Although severance of membrane bonds would in part explain the collapse of the peripheral hyalomere in adherent thrombocytes, the effects of CB upon actin-associated microfilament systems are complex, and the observations reported here probably result from several CB-induced microfilament changes. Recently, it has been demonstrated by several investigators that cytochalasins E, D, and B will reduce in vitro the rate of polymerization of G- to F-actin [27,28,30–32,37]. This inhibition of polymerization, which appears to involve the binding of cytochalasins to high-affinity sites on the polymerization end of nascent F-actin (27,28,30–32,37] could, with an equilibrium shift in vivo, result in net reduction of polymer length since the actin filament represents a steady state of polymerization and depolymerization. This suggestion requires experimental documentation, for Morris and Tannenbaum have shown that net reduction of polymer length by the mechanism of net depolymerization does not occur [29]. Brenner and Korn [27] have further suggested that the cytochalasin-induced steady-state change would alter not only the equilibrium between actin monomers and F-actin but also the configuration of supramolecular actin networks [27]. The observations reported in the present study are consistent with such a mechanism.

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