

# Cytoskeletal Distribution and Function during the Maturation and Enucleation of Mammalian Erythroblasts

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**Abstract.** We have used murine splenic erythroblasts infected with the anemia-inducing strain of Friend virus (FVA cells), as an in vitro model to study cytoskeletal elements during erythroid maturation and enucleation. FVA cells are capable of enucleating in suspension culture in vitro, indicating that associations with an extracellular matrix or accessory cells are not required for enucleation to occur. The morphology of FVA cells undergoing enucleation is nearly identical to erythroblasts enucleating in vivo. The nucleus is segregated to one side of the cell and then appears to be pinched off resulting in an extruded nucleus and reticulocyte. The extruded nucleus is surrounded by an intact plasma membrane and has little cytoplasm associated with it. Newly formed reticulocytes have an irregular shape, are vacuolated and contain all cytoplasmic organelles. The spatial distribution of several cytoskeletal proteins was examined during the maturation process. Spectrin was found associated with the plasma membrane of FVA cells at all stages of maturation but was segregated entirely to the incipient reticulocyte during enucleation. Microtubules formed

cages around nuclei in immature FVA cells and were found primarily in the incipient reticulocyte in cells undergoing enucleation. Reticulocytes occasionally contained microtubules, but a generalized diffuse distribution of tubulin was more common. Vimentin could not be detected at any time in FVA cell maturation. Filamentous actin (F-actin) had a patchy distribution at the cell surface in the most immature erythroblasts, but F-actin bundles could be detected as the cells matured. F-actin was found concentrated between the extruding nucleus and incipient reticulocyte in enucleating erythroblasts. Newly formed reticulocytes exhibited punctate actin fluorescence whereas extruded nuclei lacked F-actin. Addition of colchicine, vinblastine, or taxol to cultures of FVA cells did not affect enucleation. In contrast, cytochalasin D caused a complete inhibition of enucleation that could be reversed by washing out the cytochalasin D. These results demonstrate that F-actin plays a role in enucleation while the complete absence of microtubules or excessive numbers of polymerized microtubules do not affect enucleation.

THE circulating erythrocytes of adult mammals differ from those of other vertebrate species in that they are anucleate. Nuclei are shed from nucleated precursors in the bone marrow during the terminal stages of erythroid maturation to yield reticulocytes that then give rise to mature circulating erythrocytes. In addition to being anucleate, mature mammalian erythrocytes differ from nucleated erythrocytes of other vertebrates in their shape and in the composition of their cytoskeleton. Mammalian erythrocytes are biconcave disks, while the nucleated erythrocytes of other vertebrates are typically elliptical and biconvex. Intermediate filaments and microtubules are not present in mature mammalian erythrocytes, whereas erythrocytes of nonmammalian vertebrates have microtubules arranged in a subplasmalemmal ring in the equatorial plane of the cells (the so-called "marginal band" of microtubules) (2), and, at least in avian erythrocytes, intermediate filaments of the vimentin type link the nucleus to the membrane cytoskeleton (6).

Previous studies of cytoskeletal organization in mam-

malian erythroblasts have been inhibited by the lack of a model system in which large numbers of erythroblasts could be isolated and manipulated for experimental purposes. We have developed an experimental system in which a large, relatively homogeneous population of erythroblasts at the pronormoblast stage can be isolated from the spleens of mice infected with the anemia-inducing strain of Friend virus (FVA cells).<sup>1</sup> Our previous work has demonstrated that, when these cells are cultured in the presence of physiological concentrations of erythropoietin (EP), they undergo a sequence of differentiation events in vitro that are very similar to those seen in erythroblasts in vivo. These events include: a progressive decrease in cell size, disappearance of nucleoli, condensation of nuclei, accumulation of hemoglobin,

1. *Abbreviations used in this paper:* EP, erythropoietin; F-actin, filamentous actin; FVA cells, murine splenic erythroblasts infected with the anemia-inducing strain of Friend virus; MEL, murine erythroleukemia; PHEM buffer, 60 mM 1,4 Pipes, 24 mM Hepes, 10 mM EGTA, 2 mM MgCl<sub>2</sub>, 0.5 mM PMSF (pH 6.9).

and the eventual extrusion of nuclei to yield reticulocytes (11). In the present study, we have concentrated on the enucleation process in FVA cells. Previous morphological studies of enucleation in mammalian erythroid precursors have suggested that the process of enucleation is similar to that of cytokinesis in that a cytoplasmic constriction resembling a cleavage furrow forms between the extruding nucleus and incipient reticulocyte (3, 19, 24–26). Skutelsky and Danon (26) have presented evidence that microtubules are important in the correct positioning of the constriction between the incipient reticulocyte and extruding nucleus. Others (20) have found that cytochalasin B can inhibit the burst of enucleation that occurs when murine splenic erythroblasts are incubated for short periods *in vitro*, indicating that filamentous actin (F-actin) plays a role in the process. However, changes in actin filament distribution that occurred before enucleation were not determined; actin was not localized in enucleating cells; and the roles of other cytoskeletal elements in enucleation were not assessed. Using FVA cells as a model, we have been able to determine the distribution of cytoskeletal elements during maturation through the use of electron and immunofluorescence microscopy and to determine the effects of various cytoskeletal modifiers on enucleation.

## Materials and Methods

### Materials

Pure human recombinant EP was obtained from AMGEN (Thousand Oaks, CA). Cytochalasin D, and colchicine were obtained from Sigma Chemical Co. (St. Louis, MO). Vinblastine sulfate was obtained from Eli Lilly Co. (Indianapolis, IN). Rhodamine-conjugated phalloidin was obtained from Molecular Probes, Inc. (Eugene, OR). Antiserum to sea urchin tubulin was obtained from Polysciences Inc. (Warrington, PA). Antiserum to murine fibroblast vimentin (1, 13) was the gift of Dr. Bonnie Asch (Roswell Park Memorial Institute, Buffalo, NY). Antiserum to human erythrocyte spectrin (32) was the gift of Dr. Heinz Furthmayr (Yale University, New Haven, CT). Taxol was obtained from Dr. M. Suffness at the Natural Products Branch of the National Cancer Institute.

### Cell Culture

Erythroblasts infected with the anemia-inducing strain of FVA cells were isolated and cultured as previously described (9, 21). Briefly, CD<sub>2</sub>F<sub>1</sub> mice were infected with FVA cells. 2 wk later, their spleens had enlarged ~10-fold, with erythroid precursors constituting >95% of the nucleated cells. The immature erythroblasts from these spleens were separated from splenic erythrocytes and more mature erythroblasts by velocity sedimentation at unit gravity. The immature erythroblasts were cultured with 0.2 U of EP/ml in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY) containing 30% FBS, 0.1% BSA and 0.1 mM  $\alpha$ -thioglycerol.

### Electron Microscopy

Cells were cultured with EP for varying periods of time from 2 h to 48 h. The cells were collected by centrifugation at 500 g for 5 min, washed with Ca<sup>2+</sup>-Mg<sup>2+</sup>-free Dulbecco's PBS at 37°C and fixed in 3.0% glutaraldehyde,

0.1% tannic acid, 0.1 M sodium cacodylate (pH 7.4). Cells were washed with 0.1 M sodium cacodylate (pH 7.4), pelleted, and gently resuspended in melted 2.0% agar in glass distilled water at 42°C. The agar was allowed to solidify, cut into 1 mm<sup>3</sup> blocks, rinsed with 0.1 M sodium cacodylate and postfixed with 0.5% osmium tetroxide/0.1 M sodium cacodylate (pH 6.0) on ice for 1.5 h. The blocks were rinsed once in 0.1 M sodium cacodylate (pH 7.4), once in distilled water, and stained en bloc with 2.0% aqueous uranyl acetate. Blocks were then dehydrated in acetone and embedded in low viscosity resin (28). Thin sections were stained with 1.5% uranyl acetate in 50% methanol and lead citrate (30) and viewed on an electron microscope (JEM 100S; JEOL USA, Cranford, NJ) at 80 kV.

### Immunofluorescence

FVA cells were collected from culture by centrifugation and resuspended in prewarmed PBS and allowed to adhere to alcian blue-coated coverslips for 15 min at 37°C. Coverslips were dipped once in PBS to remove nonadherent cells and then fixed in 2.0% paraformaldehyde in PBS for 10 min at 37°C. Coverslips were washed extensively in PBS, and the cells were then permeabilized by immersion in 0.5% Triton X-100 in PBS for 5 min. Incubations with antibodies or rhodamine-conjugated phalloidin were performed, and coverslips were observed on a microscope (Labphot; Nikon Inc., Garden City, NY) equipped with epifluorescence optics. Photomicrographs were taken on Plus-X film (Eastman Kodak Co., Rochester, NY) and developed in Diafine.

### Gel Electrophoresis and Western Blotting

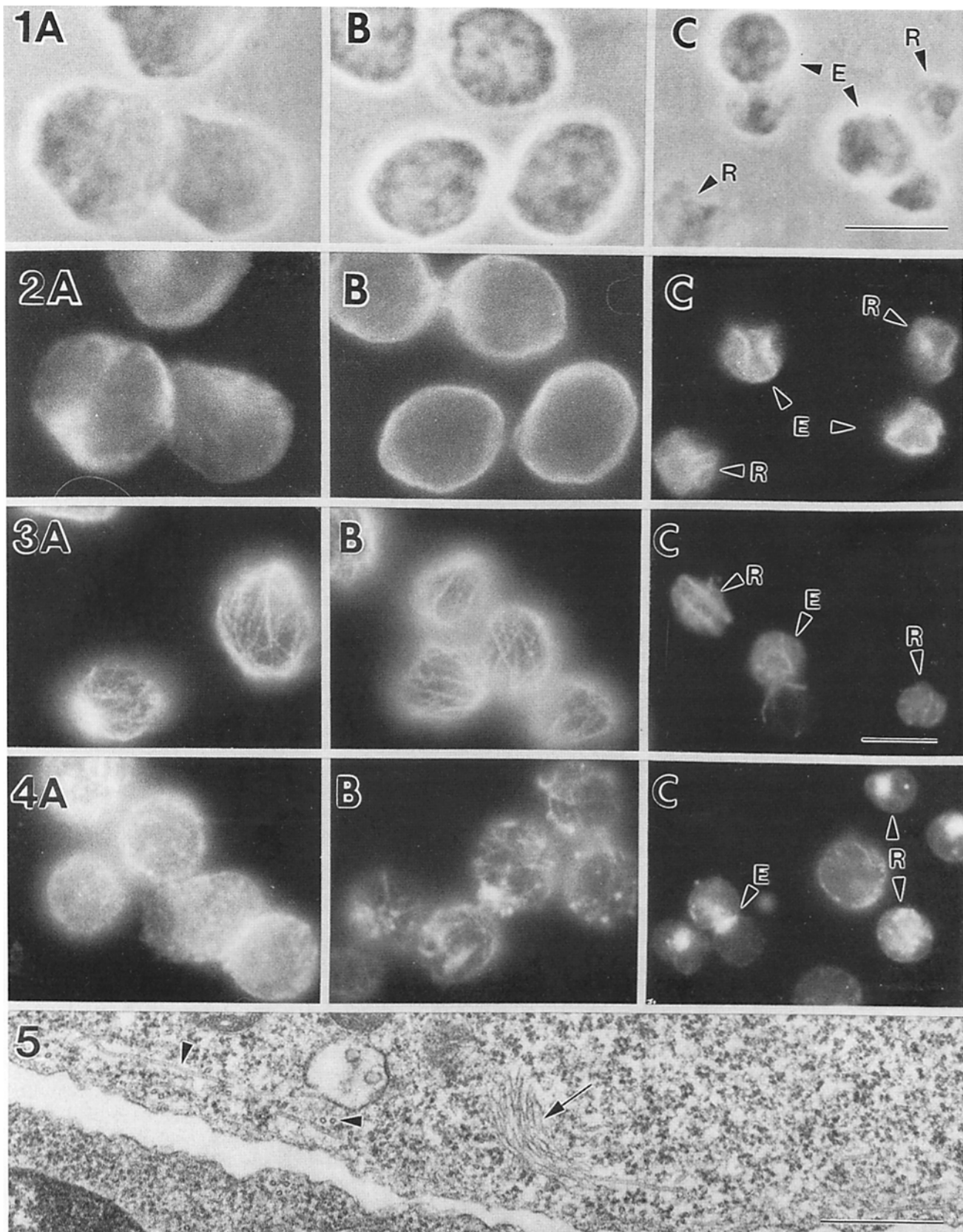
Cytoskeletons were prepared from FVA cells after 2, 24, and 45 h of culture by first washing cells in 60 mM 1,4 Pipes, 24 mM Hepes, 10 mM EGTA, 2 mM MgCl<sub>2</sub>, 0.5 mM PMSF (pH 6.9) (PHEM buffer) (22) followed by lysis in PHEM buffer + 0.15% Triton X-100 for 1 min. Cytoskeletons were then pelleted at 500 g for 5 min. After washing in PHEM buffer, cytoskeletal pellets and soluble fractions were placed in sample buffer and separated by SDS-PAGE as described by Laemmli (12). For Western blot analysis, proteins were electrophoretically transferred to nitrocellulose (29). Vimentin antibody was applied and the blots washed (13). Secondary antibodies conjugated to horseradish peroxidase were detected using 4-chloro-1-naphthol and hydrogen peroxide.

### Cytoskeletal Modifiers and Evaluation of Enucleation

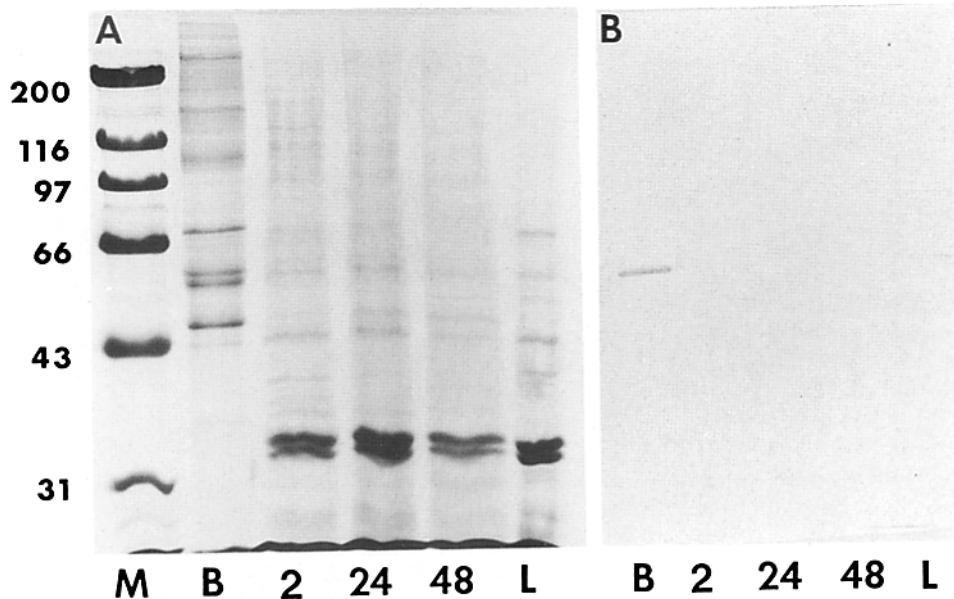
Stock solutions of taxol and cytochalasin D were made in DMSO and added to cultures to give a final concentration of 5  $\mu$ M and 5  $\mu$ g/ml, respectively. Colchicine and vinblastine sulfate stock solutions were each made in PBS and added to cultures to give a final concentration of 5  $\mu$ g/ml. Control cultures received only DMSO or PBS. FVA cells were removed after 2, 24, 32, 36, 40, 44, and 48 h of culture in the presence of EP, with modifiers being added at 30 h of culture. Cells were collected by making cytocentrifuge preparations and stained with 3,3'-dimethoxybenzidine to detect hemoglobin and counter stained with hematoxylin to detect nuclei. 300 nuclei were counted on each slide using a 100 $\times$  objective and placed into one of three categories: free nucleus, nucleus in an enucleating cell, or nucleus in a cell not enucleating. The number of reticulocytes seen in the same number of high power fields of view were counted as well. Triplicate cultures were used at each time point. Slides were coded and counted in a double blind manner.

Possible toxic effects of the cytoskeletal modifiers on FVA cells were determined by measuring heme synthesis in cultures containing each of the modifiers and control cultures containing only vehicle. As a measure of heme synthesis, the incorporation of <sup>59</sup>Fe into protoporphyrin to yield heme was determined as previously described (8). Triplicate analyses were performed at 36, 42, and 48 h of culture.

**Figure 1.** Phase-contrast microscopy of FVA cell maturation. *A*, FVA cells after 2 h of culture. *B*, FVA cells after 24 h of culture. *C*, FVA cells after 45 h of culture. *E*, enucleating cells with a pronounced constriction between the smooth, round extruding nucleus, and the irregularly shaped incipient reticulocyte, and *R*, free reticulocytes. Bar, 10  $\mu$ m. **Figure 2.** Localization of spectrin in the FVA cells illustrated in Fig. 1 (*A–C*). *A*, FVA cells after 2 h of culture. *B*, FVA cells after 24 h of culture. *C*, FVA cells after 45 h of culture. By comparing Fig. 2 *C* with Fig. 1 *C*, it can be seen that the membrane associated with the extruding nucleus lacks spectrin. **Figure 3.** Localization of microtubules in FVA cells. *A*, FVA cells after 2 h of culture. *B*, FVA cells after 24 h of culture. *C*, FVA cells at 45 h of culture. Bar, 10  $\mu$ m. **Figure 4.** Localization of F-actin in FVA cells using rhodamine-conjugated phalloidin. *A*, FVA cells after 2 h of culture. *B*, FVA



cells after 24 h of culture. What appear to be numerous bundles of F-actin are present in the cytoplasm. C, FVA cells after 45 h of culture. **Figure 5.** Electron micrograph of the peripheral cytoplasm of an FVA cell after 24 h of culture in the presence of EP. Arrowheads indicate longitudinal and cross sectioned profiles of microtubules that are found just beneath the plasma membrane. The arrow points to a small bundle of thin filaments. Bundles such as these may represent the filamentous structures stained by rhodamine-conjugated phalloidin (See Fig. 4 B). Bar, 0.5  $\mu$ M.



**Figure 6.** Demonstration that vimentin is undetectable in the cytoskeleton of FVA cells. *A*, Coomassie blue-stained gel containing molecular weight markers of the weights indicated (lane *M*); a preparation enriched in intermediate filaments isolated from mouse brain (lane *B*); FVA cell triton-insoluble cytoskeletons made at 2, 24, and 48 h of culture (lanes 2, 24, and 48) and triton-insoluble cytoskeletons made from mouse splenic lymphocytes (*L*). *B*, Western blot of an identical gel reacted with a murine vimentin antiserum. Lanes are labeled as in *A*.

## Results

### Morphology of Enucleating FVA cells

Fig. 1 (*A–C*) are phase-contrast micrographs of FVA cells at the times of culture indicated. After 45 h of culture in the presence of EP (Fig. 1 *C*) large numbers of hemoglobinized reticulocytes, enucleating cells and extruded nuclei (not shown) can be seen. Enucleating cells typically possess a pronounced constriction between the incipient reticulocyte and the extruding nucleus (Fig. 1 *C*). The incipient reticulocyte contains all cellular organelles except the nucleus, and the extruding nucleus is surrounded by a plasma membrane (see electron micrograph in Fig. 7; reference 11). The constriction between the incipient reticulocyte and the extruding nucleus suggests that the nucleus is actively squeezed out of the cell. This process is similar to that seen in uninfected enucleating erythroblasts (3, 19, 24–26) indicating the enucleation process in FVA cells *in vitro* is similar to that occurring in uninfected erythroblasts *in vivo*.

### Fluorescent Localization of Cytoskeletal Elements in FVA Cells

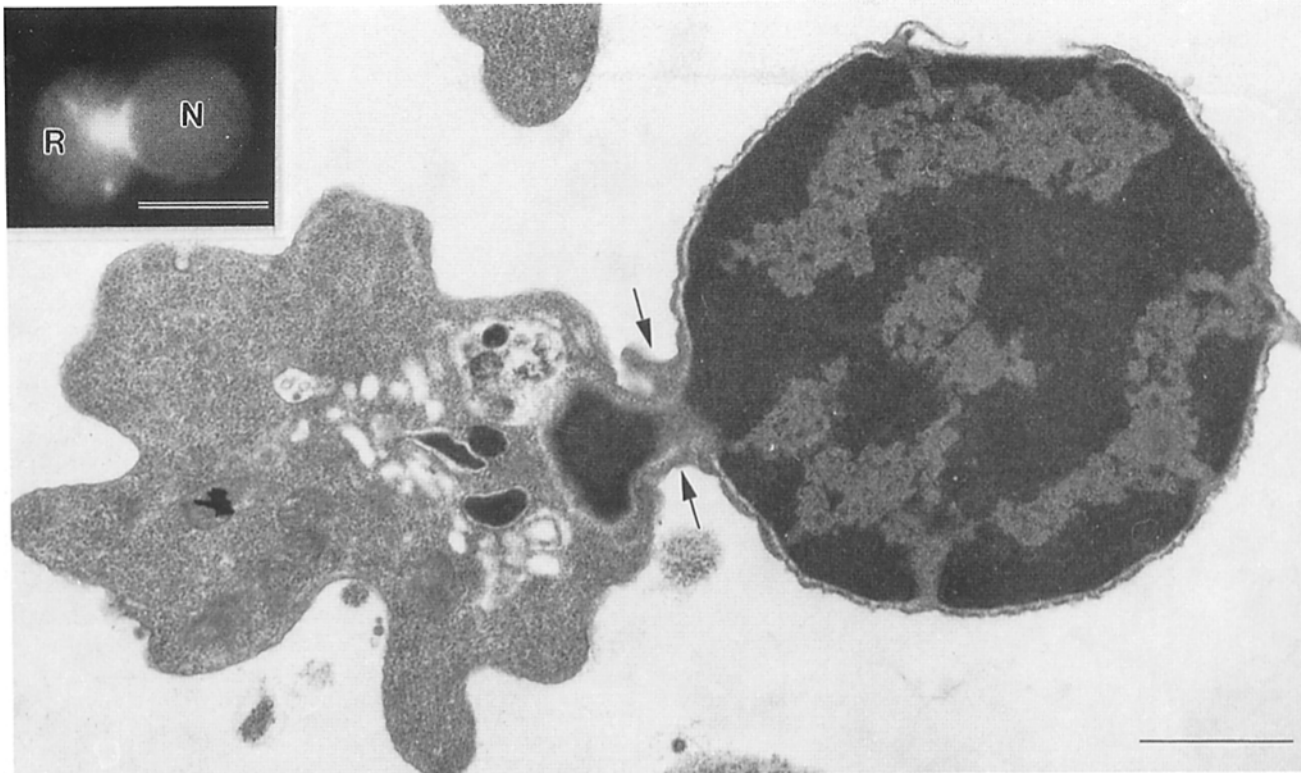
Fig. 2 is a series of fluorescence micrographs showing the distribution of spectrin during the maturation of the same FVA cells illustrated in Fig. 1. Early cells (Fig. 2 *A*) exhibit a diffuse cytoplasmic fluorescence with the spectrin antibody, but some cells have a distinct concentration of spectrin at the plasma membrane. As the cells mature through the first 24 h of culture, the diffuse cytoplasmic staining remains and nearly all cells acquire the distinct staining associated with the plasma membrane (Fig. 2 *B*). In enucleating cells (Fig. 2 *C*), spectrin fluorescence is associated entirely with the incipient reticulocyte. Reticulocyte plasma membranes react strongly with the spectrin antiserum (Fig. 2 *C*), while plasma membranes surrounding extruded nuclei do not react with the antiserum (not shown).

Microtubule distribution during the course of *in vitro* maturation of FVA cells is illustrated in Fig. 3. Throughout the first 24 h of culture, microtubules form a radial array just

under the plasma membrane and appear to form a basket around the nucleus (Fig. 3, *A* and *B*). This distribution was confirmed by EM (Fig. 5). No structure resembling a marginal band of microtubules was seen in any cells. As FVA cells neared the stage of enucleation (Fig. 3 *C*), the microtubule arrays became less organized around the nucleus. In cells that were in the process of enucleation (Fig. 3 *C*), microtubules were found almost entirely associated with the incipient reticulocyte. Reticulocytes could be seen to contain microtubules, but they also exhibited a generalized diffuse antitubulin fluorescence. Some cells appeared to enucleate without polymerized tubules being present at all.

Vimentin filaments could not be seen in FVA cells when a vimentin specific antiserum was used for immunofluorescence even though vimentin filaments could be detected in murine lymphocytes fixed and processed for immunofluorescence at the same time as the FVA cells. However, FVA cells did exhibit a slight, generalized, diffuse cytoplasmic fluorescence when stained with the vimentin antiserum (not shown). To investigate further whether or not vimentin was present in FVA cells, cytoskeletons of FVA cells and murine splenocytes and a preparation enriched in intermediate filaments from mouse brain were subjected to SDS-PAGE, transferred to nitrocellulose and reacted with the vimentin antiserum. As shown in Fig. 6, a band migrating at  $\sim 57$  kD reacted with both the brain intermediate filament preparation and the splenocyte cytoskeletal preparation. However, no vimentin could be detected in FVA cell cytoskeletons prepared at 2, 24, or 48 h of culture. Vimentin was also undetectable in the Triton-soluble fractions of FVA cells at each of these time points by gel-blot analysis (not shown).

Fig. 4 illustrates the distribution of F-actin during the course of FVA cell maturation through the use of rhodamine-conjugated phalloidin. In early cells (Fig. 4 *A*), there is diffuse staining in the cytoplasm with a distinct punctate pattern at the cell surface. After the cells have been in suspension culture for 24 h, actin filament bundles can be seen in the cytoplasm (Fig. 4 *B* and Fig. 5). By 45 h of culture, the distribution of F-actin is markedly different (Fig. 4 *C*). Cells in the process of enucleation are often seen to have an in-



**Figure 7.** Composite of electron and fluorescence micrographs demonstrating the position of the F-actin aggregate in enucleating cells. The electron micrograph demonstrates the ultrastructural morphology of an enucleating FVA cell. The arrows indicate the pronounced constriction between the extruding nucleus and incipient reticulocyte. Bar, 1  $\mu\text{m}$ . Inset: photomicrograph of a cell with a similar morphology stained with rhodamine-conjugated phalloidin demonstrating an enrichment of F-actin in the constriction region. *R*, incipient reticulocyte; *N*, extruding nucleus. Bar, 5  $\mu\text{M}$ .

tensely staining aggregate of F-actin between the extruding nucleus and incipient reticulocyte, whereas reticulocytes have one or more intensely fluorescent patches in their cytoplasm. Cells not in the process of enucleation maintain an F-actin distribution similar to that of earlier stage cells. Fig. 7 is a composite electron/fluorescence micrograph of two cells with similar morphology showing the location of F-actin fluorescence in relation to the ultrastructurally defined constriction between the extruding nucleus and incipient reticulocyte. Such micrographs suggest that F-actin is actively involved in the extrusion of the nucleus in FVA cells.

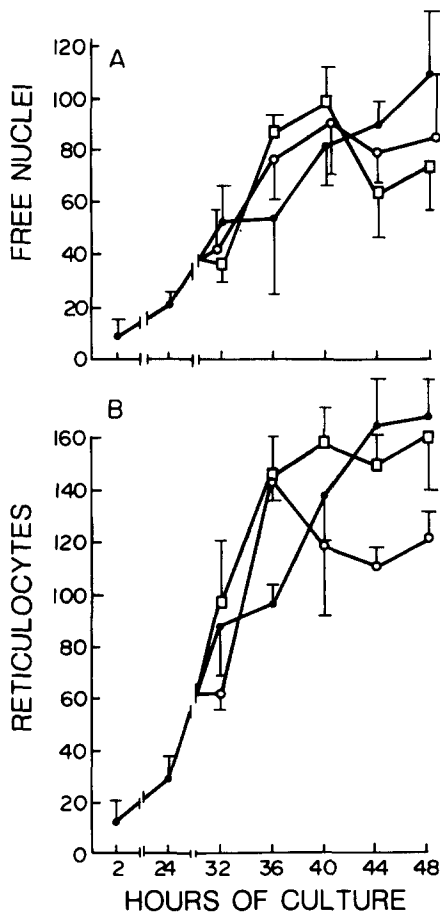
#### **Effects of Cytoskeletal Modifiers on Enucleation**

Since microtubules and F-actin were the only filamentous elements found in FVA cells and since F-actin was observed to aggregate in the region between the extruding nucleus and incipient reticulocyte during enucleation, the effects on enucleation resulting from perturbing these filamentous systems were examined.

Fig. 8 illustrates the time course of the appearance of free nuclei and reticulocytes in control FVA cell cultures and in FVA cell cultures containing 5  $\mu\text{g/ml}$  colchicine or 5  $\mu\text{g/ml}$  vinblastine sulfate. Polymerized microtubules were absent from colchicine- or vinblastine-treated FVA cells by 2 h after addition as determined by immunofluorescence microscopy (Fig. 9). As can be seen from Fig. 8, the loss of microtubules did not interfere with the ability of FVA cells to enucleate or

cause a greater than normal number of cells to enucleate. In contrast to colchicine and vinblastine, taxol is an agent which induces an excessive polymerization of microtubules. Fig. 10 illustrates the effects of 5  $\mu\text{M}$  taxol on FVA cell enucleation as opposed to control cultures that received only the DMSO carrier. Free nuclei appeared in taxol-treated cultures at a comparable rate and in nearly identical numbers as those in control cultures (Fig. 10 *A*). Reticulocytes appeared in taxol-treated cultures in nearly identical numbers as in controls at the earlier times after addition of taxol, but reticulocyte number decreased at later time points (Fig. 10 *B*). Taxol induced the appearance of large bundles of microtubules in FVA cells that persisted throughout the period of culture (Fig. 9 *C*). Excess microtubules were seen in enucleating cells and in reticulocytes as well (not shown). The results obtained with colchicine, vinblastine, and taxol suggest that microtubules are not required for erythroblast enucleation and that the loss of polymerized microtubules does not induce enucleation.

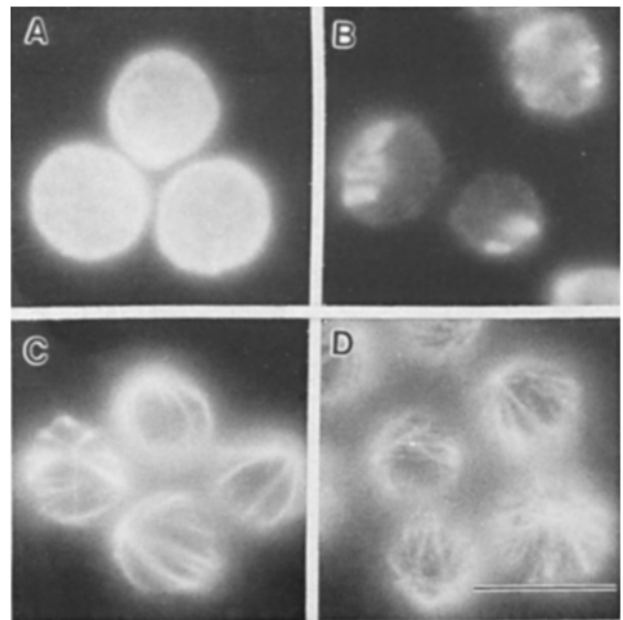
The presence of large bundles of microtubules in taxol-treated reticulocytes is in contrast to the limited number of polymerized microtubules that were seen in control cultures. The microtubules observed in taxol-treated reticulocytes may have had a destabilizing effect on the reticulocytes and therefore caused the decrease in their numbers at later times of culture. A slight decrease in the number of reticulocytes compared to controls was also seen in vinblastine treated cultures (Fig. 8 *B*). Vinblastine treated reticulocytes contained tubulin paracrystals (not shown), indicating that abnormal



**Figure 8.** Effects of colchicine and vinblastine on enucleation. After 30 h of culture in the presence of EP, 5  $\mu\text{g}/\text{ml}$  of either colchicine ( $\square$ ) or vinblastine ( $\circ$ ) was added to FVA cells. Control cultures ( $\bullet$ ) received only saline. The accumulation of free nuclei (*A*) and reticulocytes (*B*) resulting from treatment with each are shown. Data points represent mean  $\pm$  1 standard deviation of triplicate measurements.

cytoskeletal inclusions may have a destabilizing effect on newly formed reticulocytes.

The effect of 5  $\mu\text{M}$  cytochalasin D on enucleation is also demonstrated in Fig. 10. Cytochalasin D caused an immediate and complete blockage of the appearance of free nuclei and reticulocytes in FVA cell cultures. Cytochalasin D treatment induced the appearance of abnormal F-actin aggregates in FVA cells as revealed by staining with rhodamine-conjugated phalloidin (Fig. 11, *A* and *B*). In addition to inhibiting enucleation, cytochalasin D inhibited cytokinesis in FVA cells as indicated by the frequent multinucleated cells seen in cytocentrifuged preparations made at later times of culture (Fig. 11, *C* and *D*). To test the reversibility of the effects of cytochalasin D, FVA cells were cultured as above with the exception that cytochalasin D was added at 34 h. At 40 h of culture, FVA cells were collected by centrifugation, washed twice with medium lacking cytochalasin D and then replated at the same density. One half of the cultures that had previously been exposed to 5  $\mu\text{g}/\text{ml}$  cytochalasin D had cytochalasin D added back at the same concentration. The other half had only DMSO carrier added back. Control cultures were treated in the same manner with the exception that



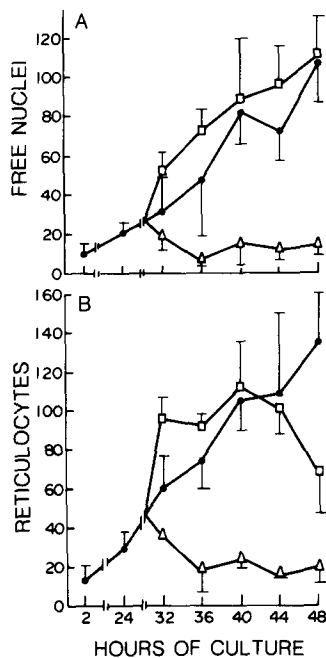
**Figure 9.** Effect of colchicine, vinblastine, and taxol on microtubules in FVA cells. *A*, FVA cell incubated for 24 h in the presence of 5  $\mu\text{g}/\text{ml}$  colchicine. Tubulin fluorescence is diffuse throughout the cytoplasm. *B*, FVA cell incubated for 2 h in the presence of 5  $\mu\text{M}$  vinblastine. Cytoplasmic inclusions of tubulin paracrystals are present. *C*, FVA cell incubated for 2 h in the presence of 5  $\mu\text{M}$  taxol. Larger than normal microtubule bundles are visible throughout the cytoplasm. *D*, Control FVA cell illustrating normal microtubule distribution.

DMSO carrier was added at 34 h and added back at 40 h. Triplicate samples were cytocentrifuged and analyzed for enucleation at 36, 40, 44, and 48 h of culture as described in Materials and Methods. The reversibility of the cytochalasin D-induced blockage of enucleation is illustrated in Fig. 12. Control cultures exhibited the typical increases in both free nuclei and reticulocytes throughout the course of the experiment. Cultures that received cytochalasin D at 34 h did not have an increased number of free nuclei or reticulocytes at 40 h, indicating enucleation was inhibited during that time. Cultures that had cytochalasin D washed out and then had cytochalasin D added back continued to exhibit an inhibition of enucleation, while cultures that had cytochalasin D washed out exhibited increases in numbers of both free nuclei (Fig. 12 *A*) and reticulocytes (Fig. 12 *B*).

None of the cytoskeletal inhibitors used in this study seriously compromised the metabolism of FVA cells. This was confirmed by the fact that cells treated with inhibitors were benzidine positive, indicating that hemoglobin was accumulating during the time of exposure to the agents. Further, FVA cells continued to incorporate iron into heme in the presence of each of the cytoskeletal modifiers (Table I).

## Discussion

The results of this study clearly demonstrate that FVA cells undergo a series of enucleation steps in liquid suspension *in vitro* that parallel those of erythroblasts *in vivo*. This is a significant finding in that an interaction with the extracellular

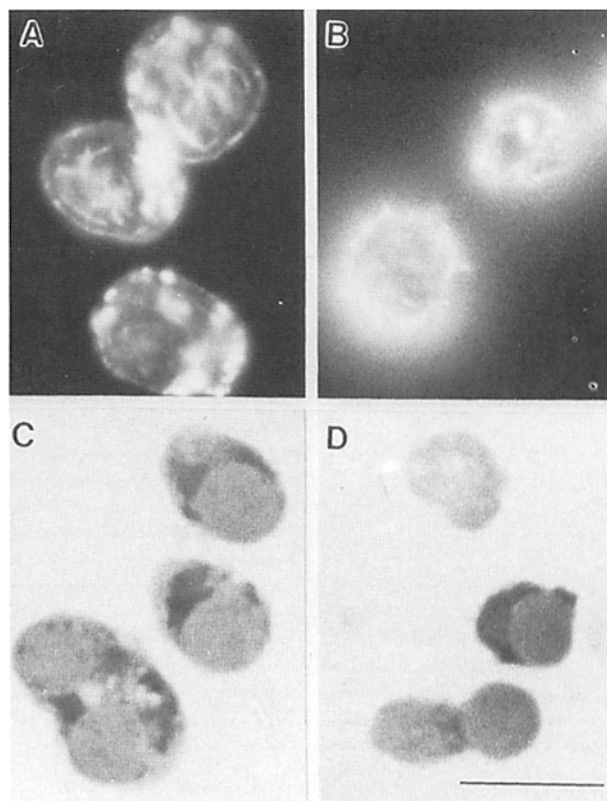


**Figure 10.** Effects of taxol and cytochalasin D on enucleation. After 30 h of culture in the presence of EP, FVA cells were treated with either 5  $\mu$ M taxol ( $\square$ ), 5  $\mu$ g/ml cytochalasin D ( $\Delta$ ) or DMSO vehicle alone ( $\bullet$ ). The accumulation of free nuclei (A) and reticulocytes (B) resulting from treatment with each are shown. Data points represent mean  $\pm$  1 standard deviation of triplicate measurements.

matrix and/or accessory cells is not a requirement for enucleation to occur. Patel and Lodish (16) have found that a fibronectin matrix is required for murine erythroleukemia (MEL) cells to enucleate *in vitro*. Even though FVA cells are grown in the presence of 30% serum, which would expose them to fibronectin, they do not attach to their culture plates, they remain rounded and move about freely when their culture plates are agitated. When immature FVA cells are grown on fibronectin-coated plates, they attach strongly and spread during the first 24 h of culture. They become detached from the plate in increasing numbers after this time and resemble cells grown in suspension throughout the culture period (our unpublished results). FVA cells grown under both conditions enucleate equally well.

The localization of spectrin using immunofluorescence microscopy confirms our earlier biochemical observations concerning spectrin accumulation in FVA cells (10). The complete segregation of spectrin to the membrane of the incipient reticulocyte during enucleation is in agreement with the findings of Geiduschek and Singer (5) for enucleating cells isolated directly from the marrow of mice. The present study indicates that FVA cells will be useful in determining the mechanism whereby spectrin is segregated to the membrane of the incipient reticulocyte before enucleation.

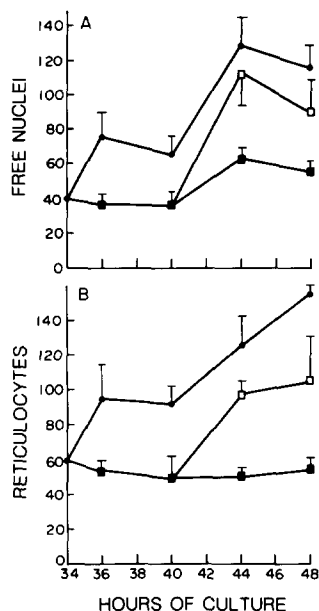
One of the interesting findings of this study was the discovery that vimentin filaments were absent from FVA cells during the period of maturation studied. Dellagi et al. (4) have reported that vimentin expression is lost at variable stages of differentiation between the committed erythroid cell and the mature erythrocyte by observing vimentin fluorescence in murine bone marrow cells, while Ngai et al. (14) have found that vimentin gene expression is rapidly repressed in a MEL cell line when it is chemically induced to differentiate. In contrast to mammalian erythrocytes, the erythrocytes of other vertebrate species maintain their nuclei while in the circulation. Intermediate filaments appear to function in anchoring the nucleus in place in these species (6, 31) and it



**Figure 11.** Effect of cytochalasin D on F-actin and FVA cell morphology. A, FVA cells labeled with rhodamine-conjugated phalloidin after 2 h in the presence of 5  $\mu$ g/ml cytochalasin D. Numerous foci of F-actin and aberrant rodlike F-actin structures are present in the cytoplasm. B, Control cells incubated for 2 h in the presence of 1% DMSO and stained with rhodamine-conjugated phalloidin. No abnormal F-actin inclusions are visible. C, FVA cells stained with 3,3'-dimethoxybenzidine after 40 h in the presence of EP and 10 h in the presence of 5  $\mu$ g/ml cytochalasin D. Free nuclei and reticulocytes are absent and a binucleated cell is present. D, In FVA cells stained as in C but exposed to DMSO instead of cytochalasin D. A normal looking enucleating cell and reticulocyte are illustrated. Bar, 10  $\mu$ M.

has been hypothesized that the loss of vimentin filaments facilitates enucleation (14). However, our results indicate that the enucleation of mammalian erythroblasts cannot be explained simply by the loss of vimentin filaments. During the course of their maturation *in vitro*, without vimentin filaments as a component of their cytoskeleton, FVA cells undergo an average of two rounds of cell division (9) and proceed through a complex series of morphological changes (11) before shedding their nuclei. In addition, vimentin filaments are no longer a component of the MEL cell cytoskeleton after 48 h of induction, but these cells do not normally enucleate (14). Repression of vimentin gene expression may be a prerequisite for enucleation to occur, but there clearly must be other factors involved in initiating the enucleation process.

Microtubules underwent dramatic changes in distribution during the course of maturation of FVA cells. Throughout the first 24 h of culture, microtubules were radially arranged just under the plasma membrane and appeared to form a basket around the cell nucleus. There was no evidence of



**Figure 12.** Demonstration of the reversibility of the cytochalasin D-induced inhibition of enucleation. Control cultures (●) received addition of 1.0% DMSO after 34 h of culture in the presence of EP. After 6 h in the presence of DMSO, control cells were collected by centrifugation, washed twice with fresh medium and then replated with fresh medium containing 1.0% DMSO. Experimental cultures received the addition of 5  $\mu$ g/ml cytochalasin D after 34 h of culture in the presence of EP (■). At 40 h, the cytochalasin D-treated cells were collected by centrifugation, washed twice with fresh medium to remove the cytochalasin D, and then replated at the same cell density as before the wash. Half of the cultures had 5  $\mu$ g/ml of cytochalasin D added back (■) while the remaining cultures received the addition of 1.0% DMSO (□). The accumulation of free nuclei (A) and reticulocytes (B) resulting from treatment with each of these are shown. Data points represent mean  $\pm$  1 standard deviation of triplicate measurements.

had 5  $\mu$ g/ml of cytochalasin D added back (■) while the remaining cultures received the addition of 1.0% DMSO (□). The accumulation of free nuclei (A) and reticulocytes (B) resulting from treatment with each of these are shown. Data points represent mean  $\pm$  1 standard deviation of triplicate measurements.

microtubules forming a marginal band in any FVA cell using fluorescence or electron microscopy. Microtubule distribution changed as the nucleus became eccentric before enucleation. When microtubules were present in enucleating cells, they were found in the incipient reticulocyte. Two experimental approaches were taken to test the roles microtubules might play in the enucleation process. The first of these was to accelerate the loss of microtubules by adding the microtubule inhibitors colchicine and vinblastine to the cultures to determine whether enucleation could be enhanced by removing the basket of microtubules from around the nucleus. Both of these agents caused microtubules to disappear completely, but they did not have any major effects on enucleation. The second approach was to use taxol to prevent microtubule disassembly to see if enucleation could be inhibited. FVA cells were observed to enucleate normally despite the taxol-induced accumulation of large numbers of microtubules. The resulting reticulocytes contained abnor-

mally high numbers of polymerized microtubules. These observations indicate that microtubules are not directly involved in the extrusion of the nucleus.

Our results appear to contradict those of Skutelsky and Danon (26) who used erythroid spleen colonies in lethally irradiated mice as a model to study enucleation. They found no enucleating erythroblasts in the spleen colonies if colcemid was injected into mice 1.5 h before killing them, but they did observe numerous nuclei that were deformed by constrictions similar to those seen in enucleating erythroblasts. They hypothesized that microtubules were required for correct positioning of the constriction so that enucleation could occur. However, Skutelsky and Danon used only electron micrographs in their analysis and did not present quantitative data of percentages of enucleating cells seen in control- versus colcemid-treated mice. It is unlikely that our cytocentrifuge preparations caused artifactual shedding of nuclei from colchicine or vinblastine treated FVA cells because microtubules were completely absent from colchicine and vinblastine treated FVA cells within 2 h of their addition and free nuclei continued to increase in the cultures beyond that point. In addition, colchicine- and vinblastine-treated FVA cells enucleate after being allowed to settle onto coverslips without being centrifuged (not shown). However, since we were observing erythroblasts growing in suspension culture in vitro and Skutelsky and Danon were using cells growing in compacted colonies in vivo, one cannot rule out the possibility that microtubules play some role in vivo in the correct positioning of the nucleus and/or the constriction to better facilitate enucleation.

F-actin also underwent dramatic rearrangements during the course of maturation of FVA cells as indicated by fluorescence microscopy. F-actin had a diffuse cortical location in FVA cells at the initiation of culture, and it became patchy and organized into small bundles in cells by 24 h of culture. An intense area of F-actin fluorescence was found in the region of the constriction between the extruding nucleus and incipient reticulocyte in enucleating cells. These results, along with the observations of others who have proposed that enucleation may be a process similar to cytokinesis (3, 19, 24–26) suggests that F-actin is present in the form of a ring in the constriction, much in the way that it is in the cleavage furrow of mitotic cells (17, 23). Cultures of FVA cells were treated with cytochalasin D to determine whether F-actin was essential for enucleation. Cytochalasin D was an effective inhibitor of enucleation in FVA cells. It also inhibited cytokinesis in FVA cells as indicated by the large number of mul-

**Table I.**  $^{59}\text{Fe}$  Incorporation into Heme in Control and Experimental Cultures

Treatment <sup>‡</sup>	Total culture period (cpm/0.5 ml culture)*		
	36 h	42 h	48 h
Saline	4,395 $\pm$ 202	6,672 $\pm$ 92	5,561 $\pm$ 130
5 $\mu$ g/ml colchicine	3,338 $\pm$ 127	4,963 $\pm$ 171	5,062 $\pm$ 227
5 $\mu$ g/ml vinblastine	3,337 $\pm$ 81	4,358 $\pm$ 390	4,479 $\pm$ 232
1.0% DMSO control	3,049 $\pm$ 136	4,420 $\pm$ 360	4,197 $\pm$ 347
5 $\mu$ g/ml cytochalasin D <sup>§</sup>	2,793 $\pm$ 129	4,205 $\pm$ 663	4,925 $\pm$ 690
5 $\mu$ m taxol <sup>§</sup>	3,289 $\pm$ 373	4,542 $\pm$ 476	5,165 $\pm$ 932

\* Values represent mean  $\pm$  SD of triplicate measures.

<sup>‡</sup>  $^{59}\text{Fe}$  and cytoskeletal modifiers were added to all cultures at 30 h.

<sup>§</sup> Final concentration of DMSO in media = 1.0%.



tinucleated cells seen in cytochalasin D-treated cultures. This observation lends further support to the hypothesis that cytokinesis and enucleation are similar processes and that actin filaments are essential for enucleation. The cytochalasin D-induced inhibition of enucleation was largely reversible since extruded nuclei and reticulocytes were seen in the cultures upon removal of cytochalasin D. Repasky and Eckert (20) found that cytochalasin B caused decreases in free nuclei and enucleating cells when erythroblasts isolated from newborn mouse spleen were incubated for short periods in vitro. However, they could not follow enucleation for periods greater than ~1 h and thus were unable to determine if the effects of cytochalasin B were reversible. Furthermore, cytochalasin B has documented metabolic effects (7) as well as its effect on F-actin, so that the cause of the inhibition of enucleation was not clear. Our results using cytochalasin D, which has no (18) or minimal (15) metabolic effects at the concentration used in this study, suggest that the cytochalasin-induced inhibition of enucleation is because of its direct effect on F-actin. Evidence that cytochalasin D did not seriously compromise the metabolism of FVA cells includes the unaffected incorporation of iron into heme during the period of treatment of FVA cells with cytochalasin D and the morphological observation of normal hemoglobin accumulation during the same period by benzidine staining.

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