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The Use of Cytochalasin B to Distinguish Myoblasts from Fibroblasts in Cultures of Developing Chick Striated Muscle

(electron microscopy/polarized light microscopy/birefringence/arborization)

J. W. SANGER

Pennsylvania Muscle Institute and the Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia, Pa. 19174

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ABSTRACT **Cytochalasin B (5 μg/ml) elicits a differential effect on myoblasts and fibroblasts in culture. After 1 day in culture in the presence of the drug, two types of cells were observed, round cells and cells with elongated arms, designated "arborized" cells. Both cell types were examined in the electron microscope. The round cells contained aggregates of thin and thick filaments as well as a few intact sarcomeres. Within the arms of the arborized cells were bundles of intermediate sized filaments (100 Å in diameter). The round cells could be shaken off the culture dish, washed free of cytochalasin B, and recultured to form myotubes. The remaining arborized cells lost their stellate shape when the drug was removed. The progeny of these cells gave rise to normal fibroblasts. Cytochalasin B, thus could be used to identify and isolate myoblasts prior to their fusion into developing muscle. It is suggested that this differential effect of the drug can be used to prepare pure cultures of fusible muscle cells uncontaminated by fibroblasts.**

During the differentiation of striated muscle, two separate lineages of cells must arise; the myoblasts which will eventually fuse to form the multinucleated myotubes, and the fibroblasts which will not fuse and will eventually become the cells of the endomysium, perimysium, and epimysium. Normally, in cultures derived from developing chick breast muscle, it is not possible to distinguish these two cell types by their respective morphologies. In primary muscle cultures, mononucleated dividing cells vary in morphology from bipolar, spindle-shaped cells to a much flatter, more adhesive cell with notched edges. In early cloning experiments (1, 2) it was demonstrated that most of the colonies of bipolar cells would eventually fuse to form myotubes, but a small percentage of bipolar cells always gave rise to fibroblastic clones. More recently, it has been shown that if clones are grown in conditioned medium, many cells which previously would not have been regarded as potentially myogenic from their morphology do in fact fuse and form striated myofilaments (3–5). Myoblasts can, therefore, only be recognized by their *subsequent* ability to withdraw from the mitotic cycle, fuse, and assemble myofilaments.

In this paper, evidence is presented which demonstrates that these precursor cells can be distinguished by their different reactions to the drug, cytochalasin B, and that this differential response may be used to isolate the two populations from one another. The myoblasts round up in the presence of drug, losing their adhesiveness to the dish, whereas the fibroblasts undergo a stellate transformation in morphology and retain their adhesiveness to the culture dish. This change in morphology of the fibroblasts has been termed arborization (6) since the elongated processes project from the cells like branches from a tree.

MATERIALS AND METHODS

Suspensions of single cells, isolated from the breast of 10- and 11-day-old chick embryos, were cultured as described previously (6). Stock solutions of cytochalasin B (ICI, England) (1 mg/ml) were made up in dimethylsulfoxide and stored at 4°.

For electron microscopy, cells were initially fixed for 2 hr with 2% glutaraldehyde solution (4°) in 0.1 M cacodylate buffer, pH 7.4 on the plastic culture dish. After ethanol dehydration, propylene oxide was added to dissolve the plastic and loosen the cell layer. This layer was then removed to fresh propylene oxide in a glass vial and cut into small pieces several millimeters in length. Pieces of the cell mat were placed in increasing concentrations of Epon plastic dissolved in propylene oxide, ending with three changes of pure Epon with added catalyst (7). The embedded cells were then placed in Beem capsules with fresh Epon mix and polymerized in an oven (1 day at 45° and 3 days at 60°).

Intermediate size filaments were isolated by homogenizing the cells in a 2% solution of Triton X-100. The homogenate was diluted with 0.1 M KCl and centrifuged in an S-30 rotor in the Spinco model L ultracentrifuge (10,000 rpm, 15 min) to remove unbroken cells, nuclei, and mitochondria. The supernatant was then recentrifuged (28,000 rpm, 3 hr) and the pellet resuspended in 0.1 M KCl and negatively stained for examination by electron microscopy.

RESULTS

Formation of arborized cells and round cells

Cells isolated from embryonic chick breast muscle were plated in normal medium and allowed to settle and attach to the culture dishes overnight. Cytochalasin B (5 μg/ml) was then added for 24 hr and two very distinct types of cells were observed. These have been designated round and arborized cells (Fig. 1). About 60–70% of the cells in the culture were round (seven experiments). In addition, some debris was present in the medium, indicating that some cell lysis and enucleation had occurred.

After 48 hr in the presence of the drug, the flattened arborized cells became increasingly branched and adhered tightly to the dish. These cells had from one to four nuclei. The round cells also increased in diameter and could occasionally be observed to contract weakly. They were, however, only tenuously attached, and could be easily shaken from the dish. If these decanted round cells were recovered and placed in medium containing cytochalasin B (5 μg/ml) they remained rounded and were never observed to arborize. Examination of cultures, fixed after 2 days in cytochalasin, with an in-

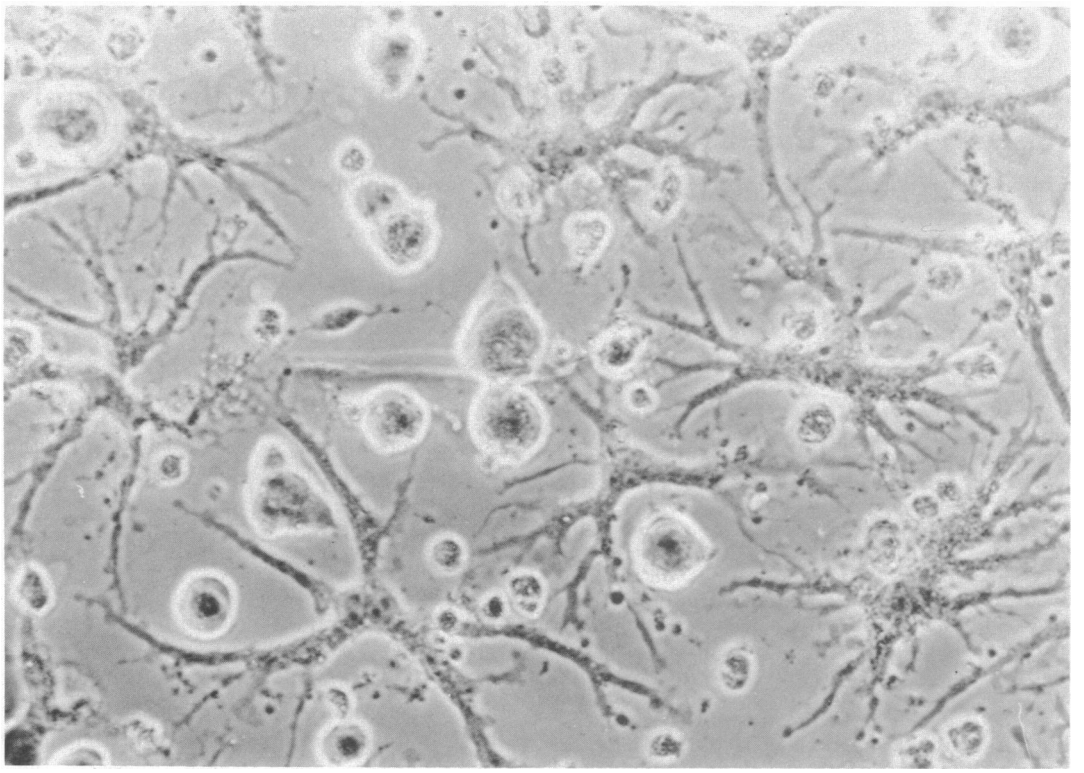


FIG. 1. Field of arborized and round cells after 2 days in cytochalasin B (5 μg/ml). Frequently round cells can be observed to clump together (×390).

verted phase microscope revealed that the round cells were either mononucleated or binucleated. When cultures of fibroblastic cells, prepared by sequential subculturing of primary muscle cultures, were exposed to cytochalasin B, all the cells arborized.

Examination of arborized cells and round cells with the electron and polarizing light microscope

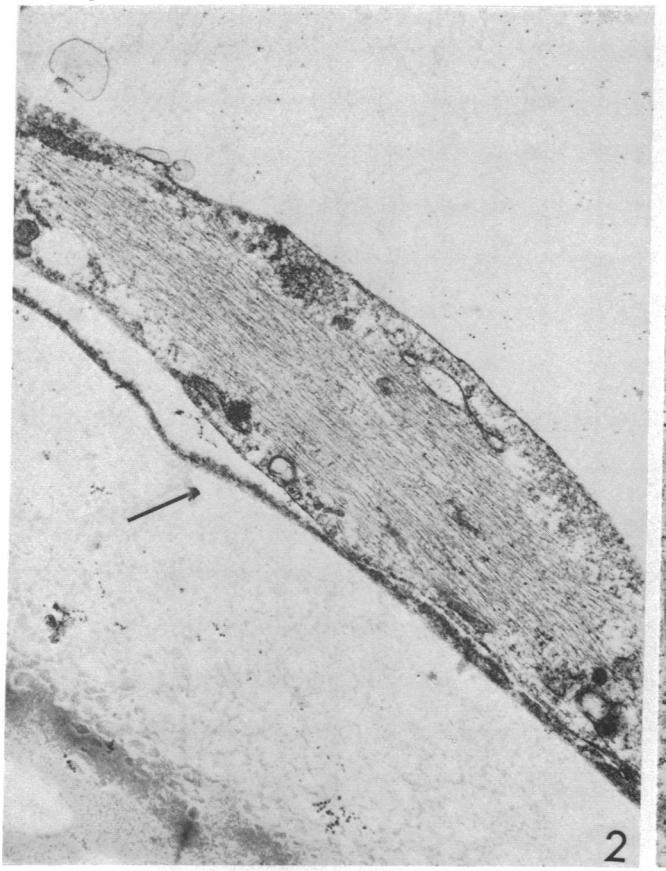
Arborized Cells. Nuclei, other cell organelles, and most of the endoplasmic reticulum were localized in the main cell body of the arborized cells. Large bundles of filaments, about 100 Å in diameter, were in the radiating arms of the cells (Fig. 2). When the filaments were isolated and negatively stained for examination in the electron microscope, they were found to be similar to intermediate-size filaments described in many other cells (8); there was no indication that filaments in one arm were continuous with those of the other arms but rather that they terminated near the cell body. Living cells were then examined with a polarizing microscope. The arms had a large amount of positively birefringent material which usually terminated as it approached the cell body (Fig. 4a and b). In a few cases, however, the birefringent material could be traced from one arm across the body to another.

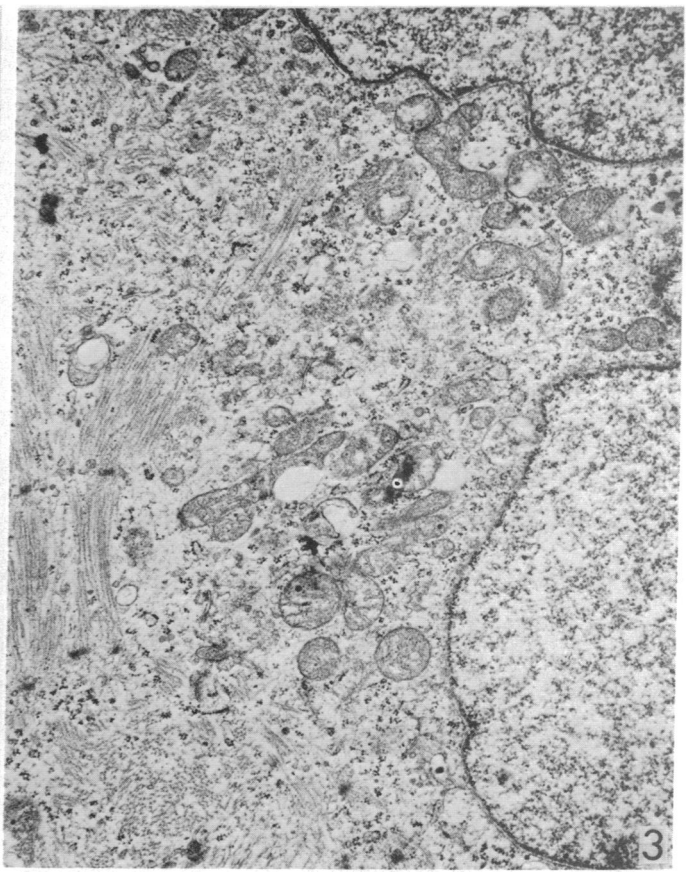
Round Cells. When the round cells were examined in the electron microscope, they were found to contain a disorganized array of thin and thick filaments with Z bands frequently attached to the thin filaments (Fig. 3). In a few cases, reasonably intact sacromeres could be observed. In the polarizing microscope these round cells had a moderate amount of birefringent material but band patterns could not be discerned (Fig. 5a and b).

Behavior of the arborized and round cells on removal of the drug

Arborized cells, when transferred from cytochalasin B medium to normal medium, lost their branched form within 30 min, as has been noted before (6, 9). In the polarizing microscope, the birefringent material in the arms was seen to spread laterally when the drug was removed and the overall birefringence decreased as the arms disappeared. If the drug was reintroduced after 30 min, each cell regained approximately the same arborized shape it had previously and birefringence increased in the arms. No myotubes were ever observed to form from arborized cells once the cytochalasin was removed.

If cultures treated with cytochalasin B for 2 or 3 days were shaken firmly, the rounded cells could be decanted and collected. When these cells were replated at low densities on a collagen substratum in normal medium, almost all of the nuclei were found to be in myotubes by 24 hr and many of the resulting myotubes were up to 300 μm in length. The majority of the myotubes were either mononucleated or binucleated. About 30% of the myotubes contained from three to twenty or more nuclei. Since the round cells were not filtered to remove large clumps before replating, myotubes with more than two nuclei may have resulted from fusion of aggregated round cells. Fusion of the myogenic cells removed from cytochalasin B was very rapid; myotubes with twenty or more nuclei formed within 24 hr from round cells containing one or two nuclei. There was no correlation between the length of the myotube and the number of nuclei within. After 24 hr, the mononucleated myotube in Fig. 6 was about 210 μm long, whereas the length of a myotube with about 18 nuclei was 260 μm (Fig. 6b). The diameter of the multi-nucleated myotube was about five times as great as





FIGS. 2 and 3. (2) An electron micrograph demonstrating the bundle of intermediate sized filaments in the branches of the arborized cell. The tissue mat which coats the bottom of the culture dish is indicated by the arrow (×16,600). (3) An electron microscopic view of a binucleated round cell indicating the scattered alignment of the component myofilaments (×16,600).

the mononucleated one. Frequently, myotubes fused with one another, resulting in branched myotubes. Dispersed among the many myotubes were a few large binucleated or tetranucleated cells. We assumed these to be fibroblasts arising from arborized cells which had been shaken off the dish along with the round cells. The round cells could also be removed individually with a mouth pipette. When such cells were plated, mononucleated or binucleated myotubes were formed exclusively.

DISCUSSION

Cytochalasin B has been shown previously to have an effect on the motility and shape of a variety of cells (10, 11). Initial observations of the effects of cytochalasin B stressed its ability to inhibit forms of motility (11) and its action was attributed to interference with actin-like filaments (11). This view was challenged by Sanger and Holtzer, who demonstrated that cytochalasin B interfered with the incorporation of glucose and glucosamine into glycosaminoglycans and glycogen (6, 12, 13). This effect appeared relatively specific since amino-acid incorporation into protein was not affected. Subsequently, other workers showed that cytochalasin B rapidly interferes with the transport of glucose and glucosamine through the cell membrane (14–16), and as a consequence, the incorporation of exogenous labeled glucose and glucosamine into glycosaminoglycans was inhibited.

Sanger and Holtzer (6) demonstrated that cells isolated from embryonic chick breast muscle when plated immediately in cytochalasin B would become arborized within 2 days. It was concluded then that arborized cells represented both fibroblasts and myoblasts (6). A reexamination of similar noncollagen-coated cultures has now revealed that all of the round cells become detached from the dish. These round cells were then lost with each medium change. In the results reported here, the preplating of cells for 1 day in normal medium, before the addition of cytochalasin B, enabled the resultant round cells to adhere to the collagen-coated dish.

High doses of cytochalasin B (5 μg/ml) clearly induces two distinct shape changes in cultures of cells from embryonic chick breast muscle. Such cultures contain predominately myoblasts and fibroblasts. In this paper, it is shown that the cells which arborize are not myogenic because, when released from cytochalasin B, they failed to form myotubes. Designation of the cells which do round up as myoblasts, is based on their fine structure and their ability to form myotubes. Even low doses of cytochalasin B (0.5 μg), if exposure was for 4 or 5 days, caused a gradual rounding up of elongated binucleated myotubes that had formed initially in the presence of the drug (17).

The induction of round cells by the drug, cytochalasin B, provides a means of early detection of chick myogenic cells in the culture. This is not possible in normal chick cultures, since recognizable myotubes do not form until 3–5 days. Even after 3–5 days, there may be mononucleated myogenic cells which are indistinguishable from fibroblasts. After 1 day in cytochalasin B, we have found that 60–70% of the cells from chick breast muscle are round and, therefore, myogenic. This technique could, therefore, be used to deter-

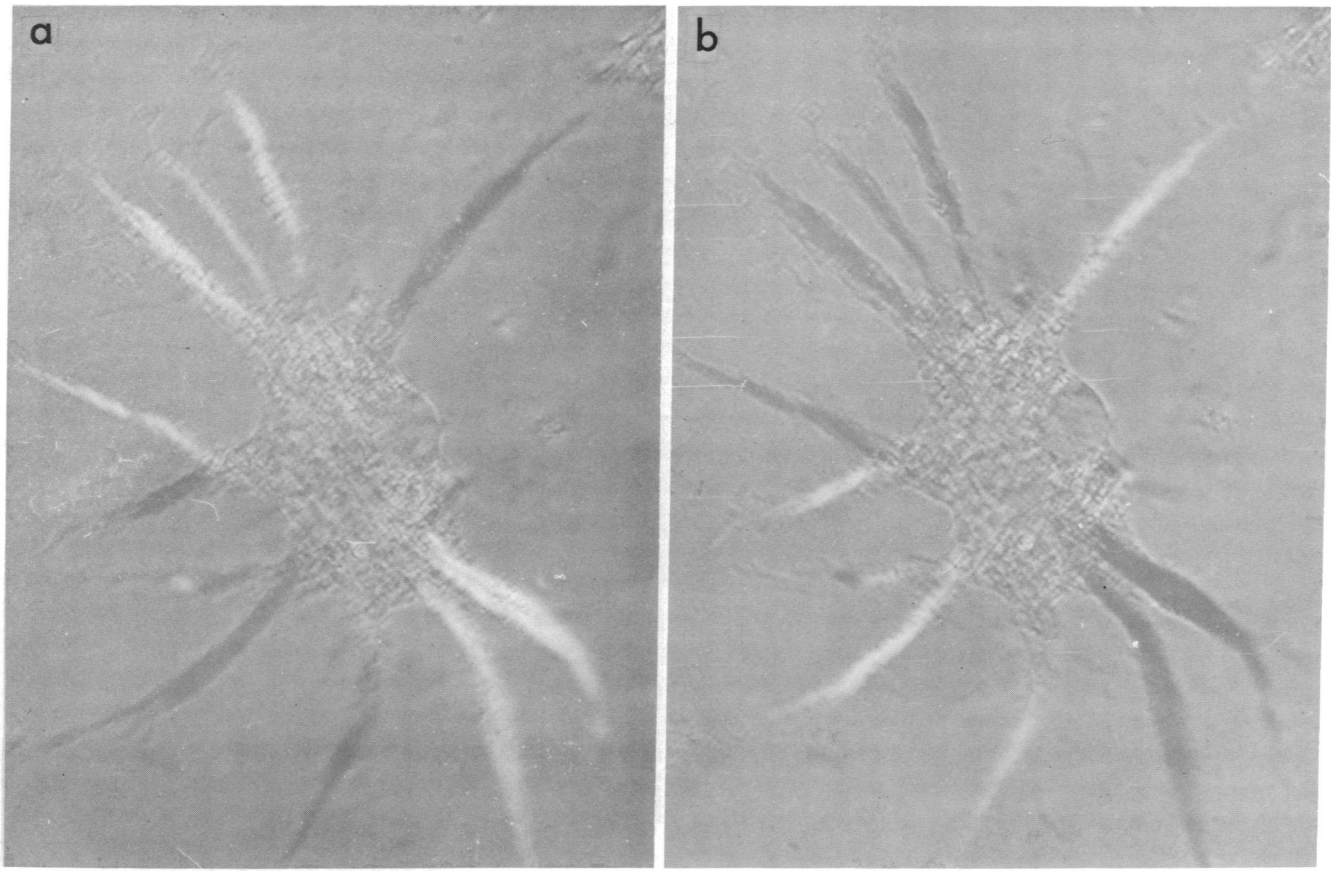


FIG. 4. Two alternate views of a living arborized cell in the polarizing microscope. Note the birefringent material in the branches of the cell (×875).

mine the proportion of myogenic cells in culture after various times of incubation.

The round myogenic chick cells formed in cytochalasin bear a marked resemblance to the spherical post-mitotic, mononucleated myoblasts of lizards formed after 8 days of cell culture in "Growth Medium" (18, 19). Generally there was no sign of thick and thin filaments in lizard cells but, when present (in about 10% of the cells), the filaments were scattered (19). The shape and adhesiveness of the fibroblasts was not affected by this medium. When the spherical lizard myoblasts were placed in "Fusion Medium," the cells fused and formed elongated myotubes within 12–72 hr (18, 19). The fibroblasts were not affected by this medium. "Growth Medium" appears to accentuate the differential adhesiveness of lizard myoblasts and fibroblasts in the same manner that cytochalasin B does with chick myoblasts and fibroblasts.

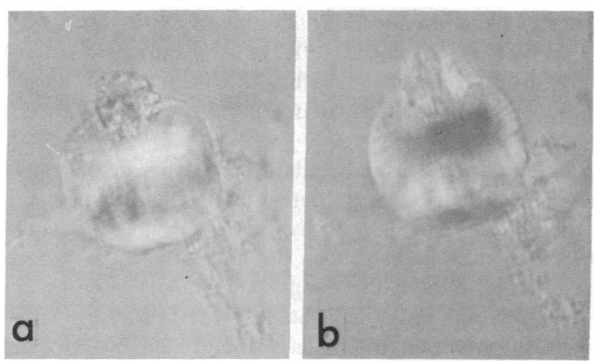


FIG. 5. Alternate views of living round cells in the polarizing microscope. Note the absence of sarcomeric patterns (×875).

Cytochalasin B appears to accentuate the differential adhesiveness of muscle cells and fibroblasts. The reason for the marked differences in adhesiveness of the two cell types is not known. The muscle cells, when deprived of glucose by cytochalasin B (14–16), may be unable to synthesize a membrane component necessary for substrate attachment. A different membrane composition may make fibroblasts less sensitive to glucose deprivation and enable them to remain firmly attached to the substrate when cytochalasin B is present. Careful decanting and replating of rounded muscle cells should result in quantities of unfused muscle cells which are free of fibroblasts. In normal medium, these cells can readily fuse to form large, multinucleated myotubes.

Why the muscle filaments in the chick contractile rounded cells are almost all misaligned from their normal sarcomeric pattern is not known. It may be a result of distortion of the normal elongated shape of the cell induced by the drug. Disruption of the elongated geometry of myotubes by the drug colchicine also leads to a misalignment of the component myofilaments (9). Nevertheless, it is quite clear that cytochalasin B does not halt the synthesis of actin and myosin filaments nor their interaction and resultant contraction of the cell (6, 9, 17). The results reported here and by others (14–16, 20–23) indicate that the membrane may be the major site of action of the drug. The drug might also interfere with the attachment of the Z bands to the cell membrane and

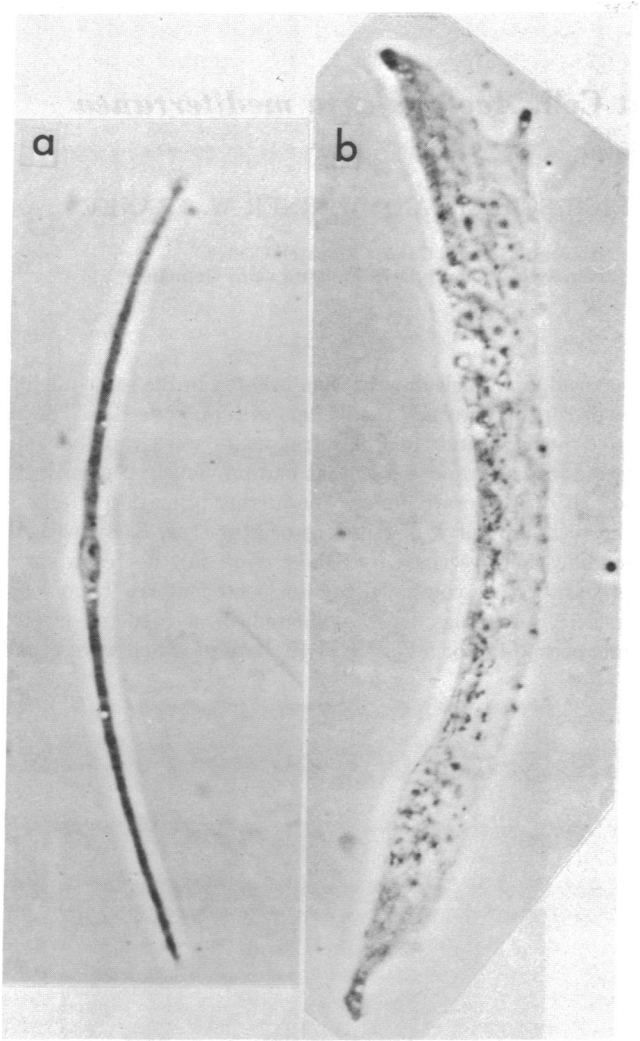


FIG. 6. Round cells were removed from cultures, exposed to cytochalasin B (5 μg) after 2 days, washed in normal medium, and plated on fresh collagen-coated dishes. After 24 hr these two myotubes had formed. (a) mononucleated; (b) about 18 nuclei (×500).

thus cause a misalignment of myofilaments and sarcomeres (24–26).

Accompanying the arborization of the fibroblasts was the appearance in the branches of bundles of 100-Å filaments. The filaments run parallel to the long axis of the branches and thus could serve a cytoskeletal function. Similar filaments are found in the cytoplasm of untreated fibroblasts, but in these untreated cells, the filaments are neither so numerous nor are they aligned in a particular axis. Whether the lack of intermediate filaments prevents branching or vice versa cannot be determined from our results. The use of large doses of cytochalasin B to form arborized cells might also be useful in concentrating large numbers of filaments for biochemical analysis.

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