

CYTOCHALASIN-B: TIME-LAPSE CINEMATOGRAPHIC STUDIES ON ITS EFFECTS ON CYTOKINESIS

AWTAR KRISHAN

With the technical assistance of SALLY WHITLOCK

From the Children's Cancer Research Foundation, Inc., and the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

L cells exposed to cytochalasin-B (cyto-B) show the normal development of deep cleavage furrows in both bipolar and multipolar cell divisions. Due to the drug-induced inhibition of cellular motility, the resulting daughter cells do not move away from each other but reunite to form multinucleate cells. In mitotic cells from cultures exposed to cyto-B for long periods of time, vigorous blebbing and contraction of the cell surface is seen. The evidence from time-lapse studies presented suggests that cyto-B-induced multinucleate cells are formed, not by the failure of the cleavage furrow, but by the drug-induced changes in surface activity and motility of cells after division.

INTRODUCTION

Cytochalasin-B (cyto-B), a mold metabolite (1), was reported by Carter (2) to have a unique inhibitory effect on cell membrane ruffling and the motility of cells in culture. However, cyto-B had no effect on the motility of some protozoa and mouse spermatozoa.

In his original studies, Carter (2) reported that mitotic cells in cultures of Earle's L-strain mouse fibroblasts exposed to cyto-B had normal nuclear divisions which were followed by the development of deep cleavage furrows. However, in many cases, the cleavage furrow failed to separate the cells completely, and the cells reunited to form binucleate cells. Repetition of this process of daughter cell fusion in subsequent divisions resulted in the formation of large multinucleate cells.

In a subsequent report (3) on the asynchrony of nuclear development in cyto-B-induced multinucleate cells (earlier suggested by Carter's observation that many cyto-B-treated cells have odd numbers of nuclei), we observed that in a majority of cases cyto-B, as such, did not prevent the for-

mation of the cleavage furrow but did inhibit the subsequent separation and moving away of the daughter cells.

The presence of 40-70 Å filaments in the cleavage furrow of HeLa cells was reported by Schroeder (4). In cells from cyto-B-treated cultures, he observed the replacement of these filaments by "vague patches of amorphous cortical material." He further reported that HeLa cells "exposed to the drug throughout anaphase never enter a cleavage phase. . . . Furrowing in cell division never occurs and this of course leads to binucleation." In his earlier observations on sea urchin eggs, he had reported an instantaneous regression of the cleavage furrow and the disappearance of furrow filaments upon application of cyto-B (5).

Due to its unique effect on cellular motility and cell surface activity, cyto-B is of particular interest for studies on cell locomotion (6) and embryonic morphogenetic processes (7, 8). Thus, Wessells et al. (9) have claimed that microfilaments, which are

presumably responsible for morphogenetic processes, cell locomotion, and cytokinesis, are either absent or altered in cells treated with cyto-B. In support of their observations, they have quoted those of Schroeder (4) who reported the breakdown of the contractile ring in the cleavage furrow of HeLa cells exposed to cyto-B. However, some other workers have contested the hypothesis that cyto-B acts on the microfilaments (see reference 10 for arguments for and against this hypothesis). For example, Bluemink (11) reported that although exposure of *Xenopus* eggs to cyto-B (5–10 $\mu\text{g}/\text{ml}$) prevented the completion of cytoplasmic cleavage, surface contractions, which are probably caused by microfilaments, were unhampered. In a subsequent report (12), he observed that some membrane-stabilizing agents, e.g. phospholipase-C, had a cyto-B-like effect on the cleavage furrow. From these studies he concluded that cyto-B primarily affects the cell surface and that the microfilaments are affected only indirectly. In a similar report Hammer et al. (13) have shown that in eggs exposed to 5 $\mu\text{g}/\text{ml}$ of cyto-B, the first two cleavage furrows begin at the normal time and with the normal appearance, but third cleavage furrow, which begins at the normal time, is disoriented. From these observations, they have suggested that a cyto-B-sensitive component(s) is present in a pool which is not replenished as this component is utilized during the first two cleavages. In a recent report on the effect of cyto-B on microfilaments of BHK-21 cells, Goldman (14) has concluded that cyto-B does not have a uniform and specific effect on microfilaments of these cells and thus cannot be reliably used to determine the possible role of microfilaments in BHK-21 cell motility.

Schroeder's observations on the effect of cyto-B on the cleavage furrow have been extensively quoted by Wessells et al. (9) and have led to the impression that cyto-B prevents cleavage furrow development by its effect on the microfilaments in the contractile ring which consequently leads to the failure of cytokinesis. Although both Carter's (2) and our own earlier studies (3, 15) have mentioned the formation of a cleavage furrow in cells exposed to cyto-B, neither of these reports included pictures showing the development of the cleavage furrow in cells exposed to cyto-B.

In view of the above-mentioned conflicting reports, we have reexamined some of our earlier time-lapse cinematographic movies, and as well

have prepared some new ones, to analyze the effect of cyto-B on the formation and progression of the cleavage furrow in cultured mammalian cells.

MATERIALS AND METHODS

Earle's L-929 mouse fibroblasts were grown in plastic tissue culture flasks or Sykes-Moore chambers and nourished with Medium-199 containing 10% calf serum and antibiotics, penicillin (100 IU/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$).

For our earlier studies (3), cyto-B was provided gratis by Dr. S. B. Carter of Imperial Chemical Industries, United Kingdom. In our more recent cinematographic studies, cyto-B, purchased directly from Imperial Chemical Industries, was used.

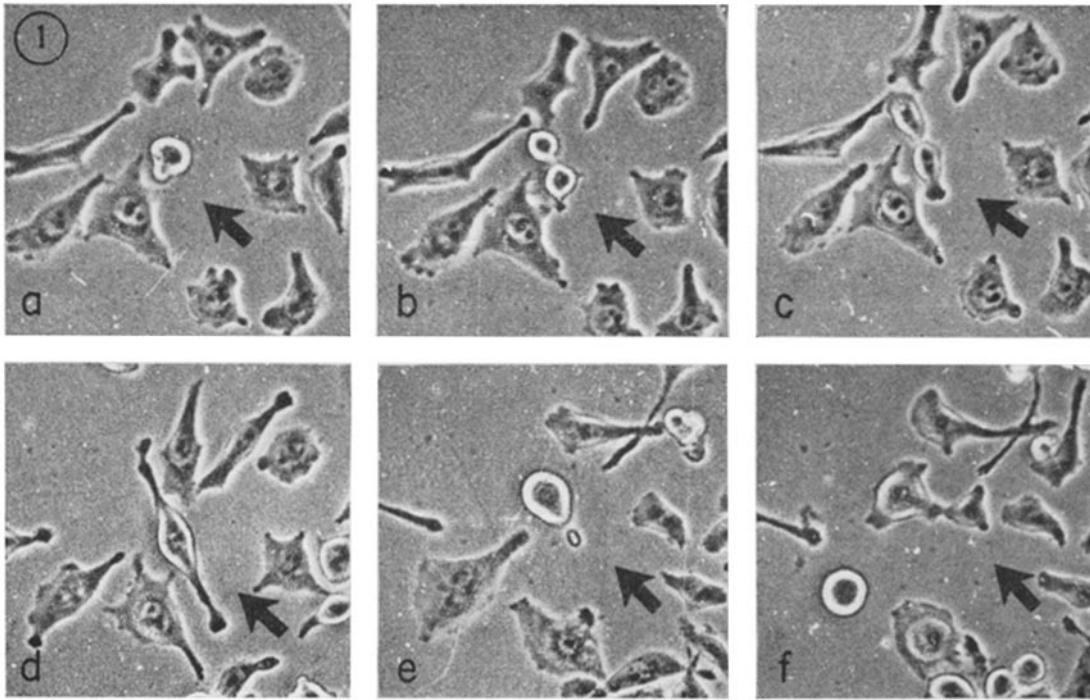
Stock solutions (0.1%) of cyto-B were made in dimethyl sulfoxide (DMSO) and stored at 4°C. Dilutions of the stock solution (1–10 $\mu\text{g}/\text{ml}$) were made in fresh medium immediately before use.

Monolayer cultures were allowed to grow on the substrate for at least one generation cycle (18 hr) before their exposure to cyto-B. Medium was changed approximately every 24 hr. For comparison, control cultures were exposed to medium containing 1% DMSO.

Time-lapse cinematographic movies were made on a Nikon-inverted microscope fitted with a Bolex 16 mm movie camera. Picture taking (one frame per minute) was normally started immediately after exposure of cells to the drug.

OBSERVATIONS

Our earlier reported observations on the formation of the cleavage furrow in cyto-B-treated L cells (3) were based on a movie made in 1967. Fig. 1 shows enlarged prints from this time-lapse movie of Earle's L-929 cells exposed to cyto-B, 1 $\mu\text{g}/\text{ml}$ for 16 hr. In frame *a*, the arrow points to a cell which has partially rounded up and is in mitosis. In frame *b*, a deep cleavage furrow is seen between the two daughter cells. In frame *c*, daughter cell separation has been nearly completed; and although the daughter cells have resumed their spindle shape, they are still connected by a cytoplasmic bridge. Subsequently, these two daughter cells approached each other and reunited to form a large cell seen in frame *d*. After 18 hr, the same cell is seen undergoing a second mitosis in frame *e*, while in frame *f*, a narrow bridge with a dark, dense midbody is seen to connect the two daughter cells. In the subsequent frames (not printed here), the two daughter cells reunited and, after absorbing the bridge and the midbody, formed a larger multinucleate cell.



All figures are approximately $\times 250$.

FIGURE 1 Time-lapse movie prints of an L cell culture exposed to cyto-B ($1 \mu\text{g/ml}$) for 16 hr. Frames *a-c* show a mitosis with deep cleavage furrow dividing the two daughter cells (arrows) which are seen reunited in frame *d*. In frames *e-f*, this cell is seen entering a cell division after 18 hr. A deep cleavage furrow and a midbody are seen in frame *f*. This cell subsequently reunited to form a large multinucleate cell.

Our recent cinematographic movies were made of Earle's L-929 cells incubated in medium containing cyto-B purchased directly from the Imperial Chemical Industries. We have not observed any difference in the *in vitro* effects between this recent batch of cyto-B and that used in our previous studies.

Fig. 2 shows prints from one of the recent movies of cells exposed to $2 \mu\text{g/ml}$ of cyto-B for 30 hr. Arrows point to a mitotic cell which is rounded in frame *a* and is in anaphase in frame *b*. A deep cleavage furrow is seen to develop in frames *c* and *d*. In frames *e-g*, the two daughter cells are seen in the process of reuniting which is completed in frame *h*, giving rise to a single multinucleate cell.

After more than 24 hr of incubation in the cyto-B-containing medium, large numbers of cells are seen to undergo multipolar mitosis; but even in these cells, a deep cleavage furrow develops between the daughter cells in a majority of the cases. Fig. 3 shows prints from a time-lapse movie of

Earle's L cells exposed to $2 \mu\text{g/ml}$ of cyto-B for 4 days. In frame *a*, the arrow points to a rounded mitotic cell which is seen in frame *b* to undergo a multipolar division, resulting in three daughter cells (frame *c*). A deep cleavage furrow is seen between the daughter cells in frames *c* and *d*, and a gradual reunion of the daughter cells takes place in frames *e-h*. The whole process of cleavage furrow development and reunion took an unusually long period of 4.7 hr in this time-lapse sequence.

The majority of cells seen in our time-lapse films show the development of a cleavage furrow which is followed by a partial separation of the daughter cells (which remain connected by the midbody-containing cytoplasmic bridge) and their ultimate reunion. Nevertheless, in some occasional cases, cells are seen to enter anaphase, which is followed by the formation of a shallow cleavage furrow that retracts, and subsequently the incipient daughter cells fuse.

With the continued incubation of the cells in

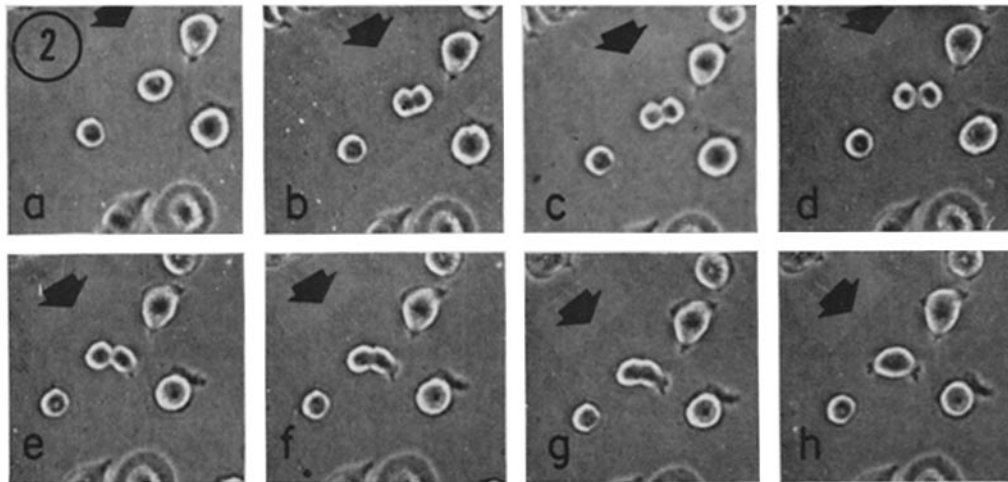


FIGURE 2 Time-lapse sequence from a recent movie of L cells exposed to $2 \mu\text{g}/\text{ml}$ of cyto-B. Cell division accompanied by a deep cleavage furrow is seen to create two daughter cells (arrows) (frame *d*) which subsequently reunite (frames *e-g*) to form a single cell (frame *h*).

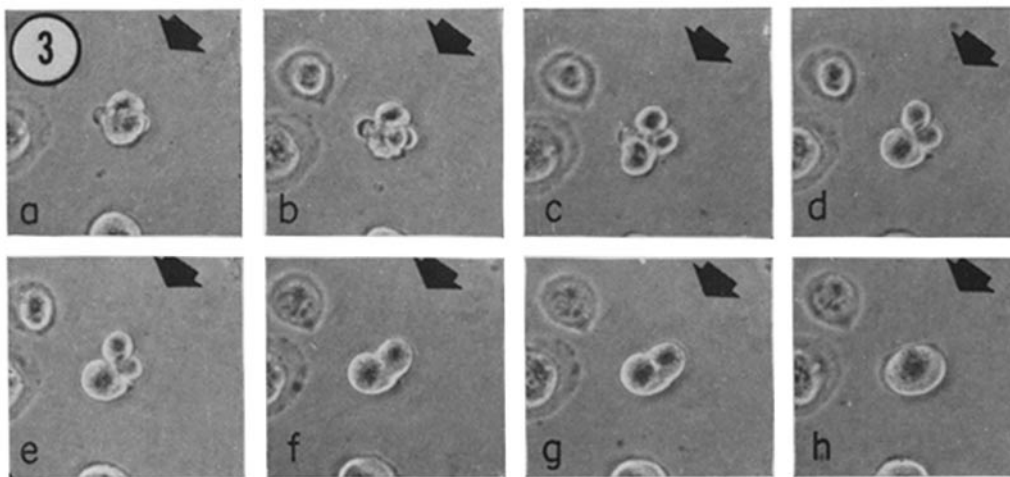


FIGURE 3 This time-lapse sequence shows a tripolar division (frames *b-d*) in a culture exposed to cyto-B ($2 \mu\text{g}/\text{ml}$) for 4 days. Deep cleavage furrows are seen to develop between the three daughter cells (arrows) which subsequently reunite (frames *e-g*) to form a single large cell (frame *h*). Total time from frame *a-h*, 4.7 hr.

the cyto-B-containing medium, a reduction in the number of cells entering mitosis is seen. In cultures incubated for 4-5 days with cyto-B, a number of cells are seen to round up and show, in time-lapse movies, vigorous blebbing of the cell surface for the next 4-6 hr. In many cases this phenomenon is either accompanied or followed by a cell division and cleavage furrow, after which the daughter cells reunite and again resume their

spread-out appearance on the glass or plastic surface. This kind of surface blebbing activity is also occasionally seen in cells incubated with cyto-B for short periods of time.

In cultures incubated with cyto-B for long periods of time (5-11 days), unusual and interesting cells surface activity is seen in cells before and after they complete a cell division. The prints in Fig. 4 illustrate this aspect of the cell surface activity

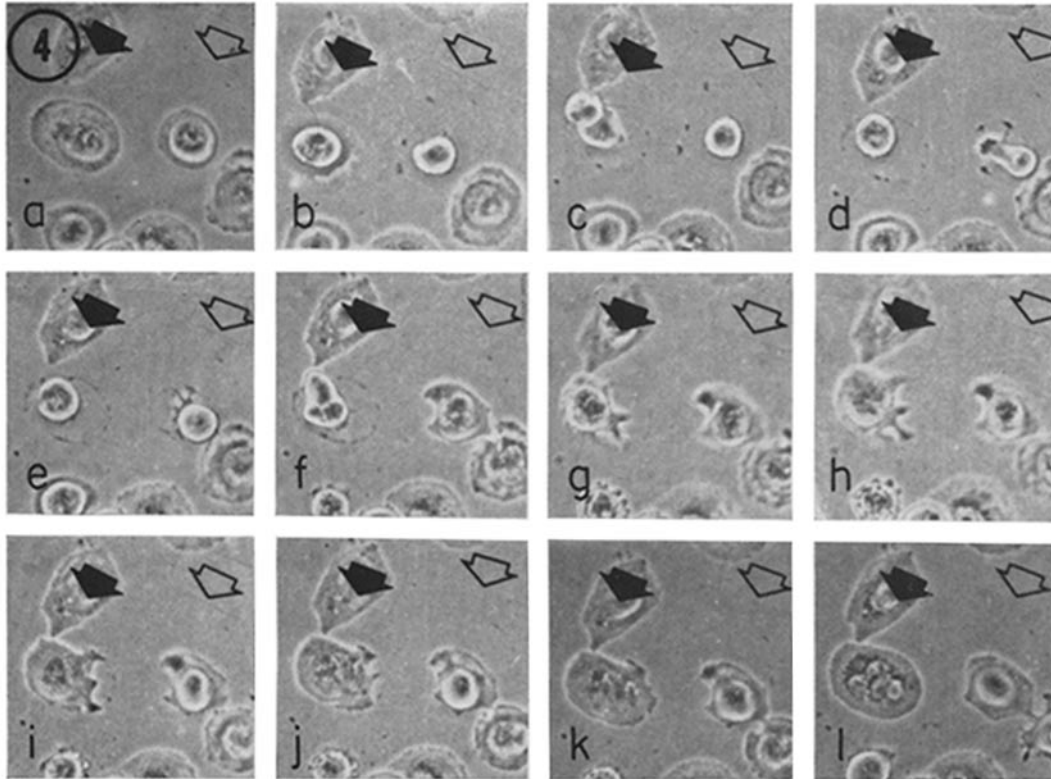


FIGURE 4 Time-lapse sequence showing the unusual cell surface behavior of cells from cultures exposed to cyto-B ($2 \mu\text{g/ml}$) for 7 days. Solid black arrows point to a cell which undergoes mitosis twice (frames *c* and *f*) within 10 hr. A thin line and some densities demarcate the area previously occupied by the cell (frames *e-f*). After reunion of the daughter cells, active pseudopods appear within this demarcated area (frames *g-k*) and the cell spreads to occupy its previous area (frame *l*). The second arrow in these frames points to another cell which shows a similar cell surface behavior.

of cells exposed to cyto-B ($2 \mu\text{g/ml}$) for approximately 7 days. The solid arrow in frame *a* points to a flattened cell which is seen to round up in frame *b* and then to undergo a cell division in frame *c* where two daughter cells with a well-developed cleavage furrow are seen. In the subsequent frames (not shown here), the daughter cells reunited but remained rounded up for the next 8 hr. During this period, vigorous pulling and pushing of the cell surface was seen in the time-lapse movies. In frames *e-f*, a clear zone, demarcated by a thin line and a number of small densities, is seen to mark the area previously occupied by the now rounded-up cell. In frame *f*, which was taken approximately 9 hr after frame *c*, this cell is seen to undergo a second mitosis (tripolar division) which was again followed by the reunion of the daughter cells. In the following frames, this multi-

nucleate cell developed active pseudopodal activity in the area previously occupied by the parent cell and demarcated by the peripheral densities. This sequence of active pseudopod formation is illustrated in frames *g-k*. This activity continued for approximately the next 2 hr, by which time the reunited cell occupied its previously demarcated territory and the surface activity ceased (frame *l*). The second arrow (empty) in Fig. 4 points to a second cell which showed a similar active cell surface activity in conjunction with a mitotic division which, as usual, was followed by the reunion of the daughter cells.

In some of our time-lapse studies, we removed the cyto-B-containing medium from the cell chambers and replaced it with fresh drug-free medium. In time-lapse records of these cells, almost an immediate resumption of ruffled membrane

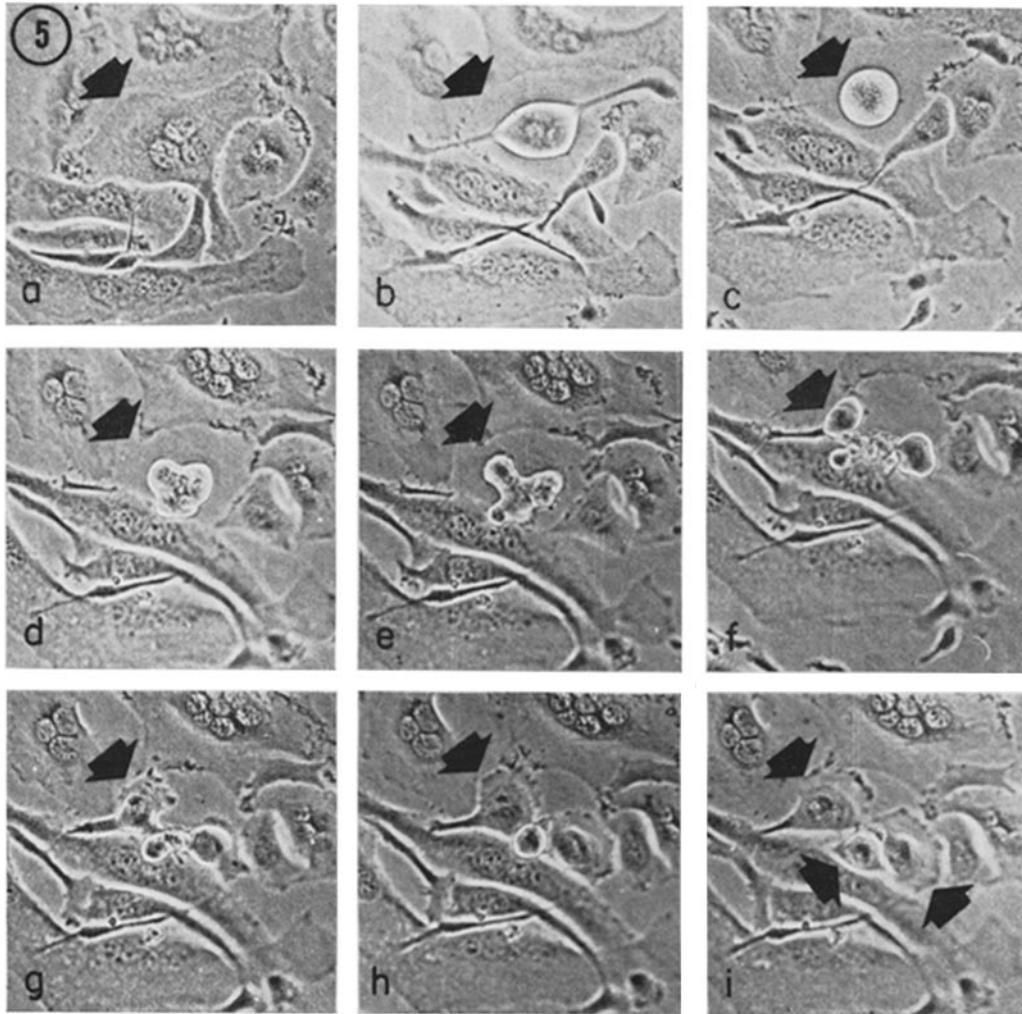


FIGURE 5 Prints from a time-lapse sequence showing mitosis in a cyto B-induced multinucleate cell (arrows) after 24 hr of incubation in drug-free medium. Frames *d-f* show the tripolar mitosis, and in frames *g-i* the three daughter cells, with well-developed, ruffled membranes, are seen pulling away from each other.

and pseudopod formation and cellular motility was seen

In Fig. 5 a series of prints from a time-lapse sequence of cyto-B-induced multinucleate cells ($2 \mu\text{g/ml}$ for 5 days) incubated in fresh, drug-free medium for 24 hr are shown. The arrow in frame *a* points to a cell with three nuclei which is shown to be in mitosis in frames *b* and *c* and to be undergoing a tripolar division in frames *d* and *e*. In frames *f-h*, the three resulting daughter cells are seen to be separated by deep cleavage furrows and to have well-developed, ruffled membranes. In

frame *i*, the three daughter cells, instead of reuniting to form a multinucleate cell, are seen in the process of moving away from each other.

DISCUSSION

A number of theories have been proposed to explain the mechanism of cleavage furrow formation and the resulting cytoplasmic division (cytokinesis) in animal cells. In recent years evidence from ultrastructural studies has been presented in support of both the theory of vesicle fusion (16) and

that of the contractile ring in the cleavage furrow (4). Schroeder's observations on the fine structure of the contractile ring in the cleavage furrow of cultured HeLa cells (4) and the disorganization of filaments in the ring effected by cyto-B have been cited as support for the hypothesis that cyto-B acts on the microfilaments of the cell (9).

Irrespective of what the actual mechanism of cleavage furrow formation is in mammalian cells, evidence from the cinematographic records presented in Figs 1-3 of the present report shows that cyto-B does not prevent the formation or progression of the cleavage furrow in cultured cells. Deep cleavage furrows are seen to develop in a majority of the cells exposed to cyto-B, irrespective of their length of exposure to the drug or its concentration. Cells, both with normal bipolar divisions and with multipolar divisions, form deep cleavage furrows, and in nearly all of these cases the daughter cells, whether two or more than two, reunite to form large multinucleate cells. In occasional cases, a shallow furrow is seen to develop which subsequently regresses, but regardless of whether the furrow is deep or shallow, the daughter cells, in a majority of cases, reunite to form multinucleate cells. These observations rule out the possibility that cyto-B acts on the mechanism responsible for the development or progression of the cleavage furrow. It is more probable that cyto-B acts on the mechanism that is responsible for the moving away of the daughter cells once the bulk of their cytoplasm has been divided by the advancing cleavage furrow. Possibly, the final separation of the postmitotic daughter cells involves the formation of active pseudopods on the surface opposite to that where the cytoplasmic bridge connects the two cells. As cyto-B inhibits cellular motility and the formation of ruffled membranes and pseudopods, it is conceivable that in the absence of these forces for moving the daughter cells apart and causing the final break of the connecting cytoplasmic bridge, the daughter cells reunite. This suggestion is further supported by time-lapse observations which show that after removal from the cyto-B-containing medium, posttelophase cells with well-developed cleavage furrows are seen to regain their pseudopods and move away from each other instead of reuniting (Fig 5).

In an earlier report (15) on the fine structure of cyto-B-induced multinucleate cells, we had observed the presence of large vacuoles (presumably

filled with tissue culture medium) in the peripheral cytoplasm of some of the cyto-B-treated cells. In time-lapse movies, these cells show long fingers of cytoplasm extending between the spaces (vacuoles) and connecting the central nucleus-containing part of the cell with the thin band of cytoplasm on the cell periphery. Although active cytoplasmic streaming and contraction of the cell surface are seen both within the central nucleus-containing region and in the cytoplasm between the vacuoles, the peripheral cytoplasm of the cell remains relatively immobile with apparently firm adhesion to the substrate.

In the present report, Fig. 4 shows the unusual surface activity and the probable adhesive nature of the cell membranes in cells exposed to cyto-B for long periods of time. The cell identified by the solid black arrow assumed a spherical shape in mitosis, but the area previously occupied by the cell is demarcated by a thin line and a number of small densities. Although we have no electron micrographs to show the fine structure of this area surrounding the rounded-up cell, in view of our earlier observations (15) it is probable that this line is a thin layer of cytoplasm which, due to its adhesiveness, did not detach from the substrate.

Wessells and his coworkers (6, 9) have suggested that cyto-B acts by causing disorganization and disruption of the cellular contractile filaments. In time-lapse movies of cyto-B-treated cells, active contractions of the cell surface, which in some cases are vigorous, are regularly seen. Experimental evidence against this hypothesis has also been presented by other workers (10-14). Recent studies with radioactive glucosamine uptake suggest that cyto-B acts by inhibiting the uptake and incorporation of this precursor into mucopolysaccharide and glycoproteins of the cell, thus affecting cell membrane biosynthesis (17). Our time-lapse observations, which essentially show that in tissue culture cells cyto-B does not inhibit cleavage furrow formation and progression but rather the moving away of the daughter cells, are more compatible with studies showing that cyto-B affects the cell membrane than with studies suggesting a cyto-B-induced disruption and disorganization of the contractile microfilaments of the cell (9).

Time-lapse observations reported in the present communication form part of a 400 foot silent movie on the in vitro effects of cyto-B. Duplicate copies are available at cost to bonafide teachers and investigators.

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