Cytochalasin B
I. Effect on Cytokinesis of Novikoff Hepatoma Cells[1] (35470)

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Aldridge and co-workers (1) first isolated and described the structure (Fig. 1) of a mold metabolite, Cytochalasin B (CB) extracted from cultures of *Helminthosporium dematioideum*. Carter (2) studied the effects of 1 μg/ml of CB (2.1×10^{-6} *M*) on mammalian cells in culture and found: cytokinesis or division of the cytoplasm is inhibited while nuclear division proceeds; a cleavage furrow develops but is not completed; if the compound is removed the cells undergo "multiple cytoplasmic divisions" following the next nuclear division; and finally cell motility and ruffling of cell margins are inhibited. He implicated a change in surface properties, namely an increase in adhesion to glass surfaces, as a possible mode of action for the drug.

Ridler and Smith (3) subsequently studied the effects of CB on phytohemagglutinin-stimulated lymphocytes in suspension culture. They noted that cytokinesis was inhibited consistently only at CB concentrations of 6 μg/ml. They reasoned that the difference in sensitivity may be due to differences in culture method or cell type. They further observed that increase in cell size was not accompanied by a proportional increase in cell cytoplasm. Such a disproportionate increase might indicate a selective toxicity to cytoplasmic constituents.

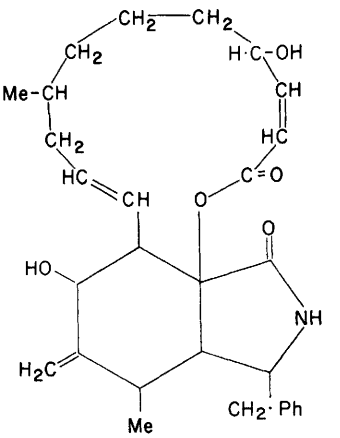

FIG. 1. Structure of Cytochalasin B.

The foregoing experiments led Carter (2) to suggest that the drug was a specific inhibitor of cytokinesis because it seemed to have little effect on cell growth. The evidence for this was, however, based exclusivly on morphology. Studies involving precursors of DNA, RNA, protein, and membranes should indicate if true growth occurred or if other causes were responsible for apparent nuclear division and cytoplasm growth and also if there were proportionate increases in nucleus and cytoplasm. Further, if cells grown in suspension cultures were used, the postulate that increased adhesion to glass surfaces is a mode of action of CB could be tested.

A system of rapidly growing cells in suspension culture, N1S1-67 (Novikoff rat hepa-

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toma) cells, was chosen in which cell number, cell size and uptake of precursors could be easily assayed. We report the results of experiments which demonstrate that the CB is an effective inhibitor of cytokinesis at levels of 2.1×10^{-6} *M* (1 μg/ml) while allowing both nuclear division and cell volume to increase at rates comparable to control. Uptake of radioactive precursors for DNA, RNA, protein, and membrane suggest that there is true growth of both nucleus and cytoplasm. Further our results indicate that the drug will act during mitosis. The inhibition of membrane fusion is suggested as a mode of action of drug inhibition of cytokinesis.

Materials and Methods. A stock solution containing 1 mg/ml of CB in dimethyl sulfoxide (DMSO) was split into 10- to 20-ml aliquots which were placed in brown prescription vials, frozen, and stored at —20° or below prior to use. The culture of N1S1-67 cells has been described previously (4): The cells were grown in Swims 67 medium (Grand Island Biol. Co.) with 5% calf serum and 2–4 m*M* glutamine and kept continuously in log growth. N1S1-67 cells were seeded at a concentration of 2×10^5 cells/ml and 24 hr later were used in the experiments described below. Since the doubling time in log phase is 12 to 14 hr the concentration for the experiments described below was close to 8×10^5 cells/ml. The growth rate of N1S1-67 cells was not affected by DMSO at concentrations of 0.1%, while 1% DMSO caused a longer lag before growth resumed at the same rate as that of controls. For the remainder of the experiments 0.1% DMSO was used as a control.

Incorporation of ^{3}H-thymidine (1 μCi/ml) and ^{3}H-uridine (5 μCi/ml) was measured by first precipitating aliquots of cells with cold (0°) 0.5 *N* perchloric acid and subsequently treating and counting the samples as described previously (5). Samples assayed for incorporation of ^{3}H-L-leucine (2 μCi/ml) were heated with 0.5 *N* trichloroacetic acid at 70° for 30 min and subsequently treated and counted as described previously (5). Samples assayed for ^{3}H-choline (2 μCi/ml) were treated and counted as described previously (6) except that sodium pyrophosphate was omitted in the initial precipitation with cold (0°) 0.5 *N* perchloric acid. In the experiments involving use of radioactive precursors, both cell numbers and number of nuclei/cell were checked at 4-hr intervals. In all CB-treated cultures, the number of cells remained the same as at time zero while the number of nuclei/cell increased. These measuremnts were similar from experiment to experiment and indicated similar effects of the CB.

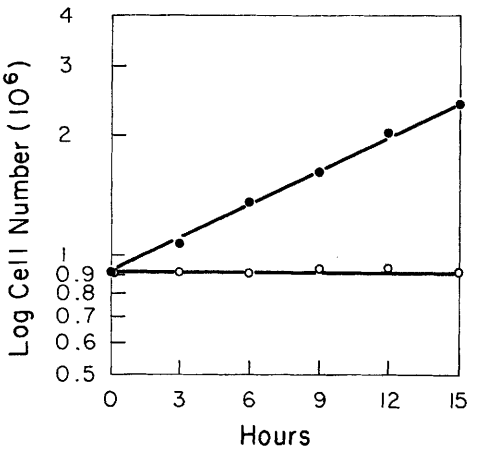

FIG. 2. Growth of N1S1-67 cells in the presence of: 0.1% DMSO (●); and 1 μg/ml of CB (○).

Results. We measured first the effects of 1 μg/ml of CB on cell number. Figure 2 demonstrates that CB completely inhibited the increase in cell number. Since our counting procedure involved dissociation of the cells with trypsin which may destroy some nonviable cells, we compared the response of CB-treated cultures and controls to 0.12% aqueous trypan blue. Both CB-treated and control cultures were similar in that less than 1% of cells took up the dye. Further, an aliquot of N1S1-67 cells was treated with CB for 24 hr, spun down, and resuspended in fresh media not containing CB. Twenty-four hr later the cell concentration was the same as in an aliquot of control N1S1-67 cells similarly manipulated. These results indicated that cell death was not the reason that the CB-treated cells failed to divide.

Following a short lag, total cell volume per fixed aliquot of culture (10 ml) increased at a rate equal to the control (Fig. 3a), suggesting that the cells grow in size at a normal rate. Relative rates of increase in cell number

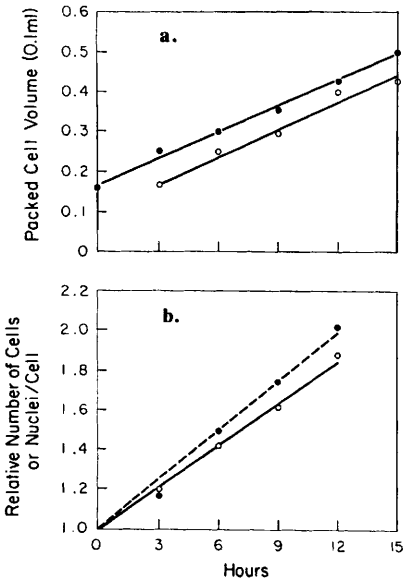

FIG. 3a. Packed cell volume of N1S1-67 cells determined simultaneously with growth curve in Fig. 2: Cell volume was determined on 10 ml of culture in Constable centrifuge tubes under identical conditions of centrifugation (1000*g* for 5 min); (●) 0.1% DMSO control; and (○) 1 μg/ml of CB. (b) Ordinate represents either number of cells divided by number of cells at time zero [0.1% DMSO control (●)] or number of nuclei/cell divided by the number of nuclei/cell at time zero [1 μg/ml of CB (○)]. Mitotic cells were omitted from counts of number nuclei per cell.

in controls and the increase in number of nuclei per cell in CB-treated cultures (Fig. 3b) were also nearly equal. These results indicate that for at least the first 12 hr the CB-treated cells increase in volume and in the number of nuclei at a rate comparable to normally dividing cells.

Division of nuclei and increase in cell size do not by themselves indicate normal growth since division of the nucleus might occur without increase in DNA and cytoplasmic expansion could occur without RNA, protein, or membrane synthesis. Figures 4a–d indicate that uptake of tritiated thymidine, uridine, leucine, and chloline take place at rates comparable to increase in number of nuclei and cell volume. These uptake rates are consistent with true nuclear, cytoplasmic, and membrane growth in these cultures. The observations on precursor uptake have been limited to the first 12 hr after CB addition to the culture since most if not all of the cells should have divided during this period. If a profound effect on macromolecular synthesis exists, then it should coincide with the apparently absolute inhibition of cytokinesis. Further Krishan and Ray-Chaudhuri (7) and Carter (personal communication) have demonstrated asynchronous ^{3}H-thymidine uptake and nuclear division in multinucleate cells.

Since CB apparently prevents any increase in cell number, *i.e.*, prevents any cell division, one could expect the drug to act during the mitosis. To test this assumption N1S1-67 cells were treated for 7 hr with 2×10^{-7} *M* *N*-desacetyl-*N*-methylcolchicine, (Colcemid) resulting in arrest of about 70% of the cells in mitosis. Two aliquots of the cells were removed, centrifuged, washed, and resuspended in fresh media, one aliquot in 1 μg/ml of CB and the other in 0.1% DMSO. The results are shown in Fig. 5a and b. Figure 5a demonstrates that the Colcemid block was reversed in both CB and control culures. Figure 5b demonstrates that the CB-treated cultures demonstrated an increase in number of nuclei/cell which is consistent with failure of

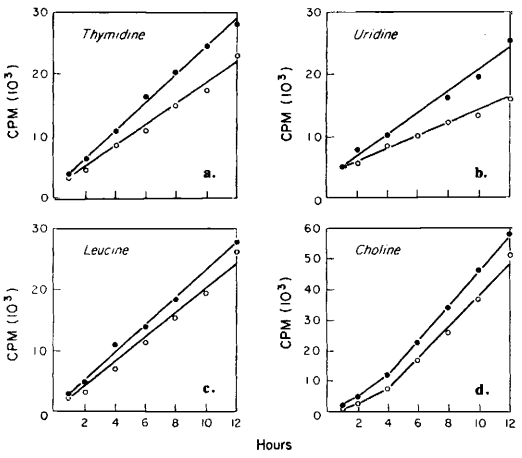

FIG. 4. Results of a typical experiment on cells from a single culture. The cells were split into equal aliquots and exposed to tritiated precursors: Uptake of tritiated precursors into acid precipitable fractions by N1S1-67 cells as indicated: (●) 0.1% DMSO control; (○) 1 μg/ml of CB. Inhibition of cytokinesis was complete in this experiment and the increase in number of nuclei/cell was comparable to that in Fig. 3b.

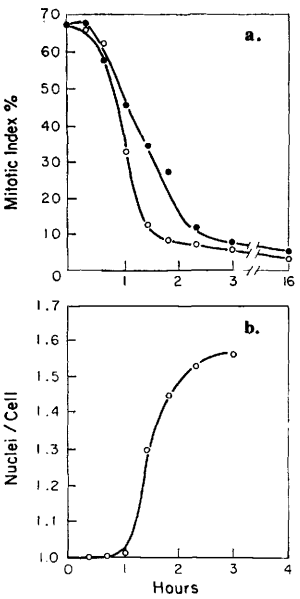

FIG. 5a. Mitotic index of N1S1-67 cells in: 0.1% DMSO (●) control; or 1 μg/ml of CB (○) on recovery from Colcemid treatment. (b) Number of nuclei/cell in CB-treated culture in same experiment described in (a). The number of nuclei per cell in the 0.1% DMSO control remained 1.

the CB-treated cells to divide their cytoplasm. This indicates that CB acts during mitosis.

Discussion. Our data supports Carter's (2) observation that CB has little toxic effect on mammalian cells at concentrations of 2.1×10^{-6} *M*. Indeed nuclear division and cytoplasmic growth are relatively undisturbed during the first 12 hr of exposure to CB. It is not surprising that uptake of precursors of DNA, RNA, protein, and membrane are also only slightly affected. Since these functions are not disturbed one could reasonably infer that major metabolic pathways for energy production are not markedly inhibited. The differences between controls and CB-treated cells for uptake of nucleic acid precursors, thymidine and uridine, seem to be more marked than those for leucine and choline. While the incorporation of radioactivity into an acid precipitable fraction can be considered to represent an indication of synthesis, one can expect some variations in such radioactivity based upon variations in transport and endogenous pool size (8). Further CB acts as late as the M (mitosis) phase of the cell cycle, whereas neither RNA nor protein synthesis are necessary for the completion of mitosis once a cell has entered prophase (9). In amphibian embryos, DNA (10) or RNA synthesis (11) is not necessary for cleavage, yet CB is effective in preventing cytokinesis in *Xenopus laevis* eggs. The inhibition of cytokinesis can take place as late as metaphase in the cell cycle as indicated by results illustrated in Fig. 5, but our results do not allow us to establish a forward boundary of sensitivity to CB. Data from *Xenopus laevis* eggs (12) suggest that drug sensitivity may occur entirely within the time period of mitosis, although sensitivity may extend to the premitotic gap (G_2).

Increased adhesiveness to glass can also not account for CB action in suspension culture although it can be argued that an alteration in surface properties might occur which could produce the same result.

Schroeder has noted an immediate reversal of cleavage in sea urchin eggs [Schroeder, T. E., *Biol. Bull.* **137**, 413 (1969)] and in HeLa cells (Schroeder, T. E., personal communication). We have found that cleavage occurs and proceeds normally with subsequent reversal in *Xenopus laevis* embryos (12). Carter and others have made similar observations on mammalian cells (2, 7). It is not immediately apparent why such a discrepancy exists; however, our observations lead us to propose that CB does not interfere with either nuclear division or furrowing but with the final division of the cytoplasm.

This cytoplasmic division must involve processes of membrane fusion. Phagocytosis and pinocytosis are example of such fusion. Other examples might be found in budding of virus from plasma membrane, transmission at the chemical synapse, secretion of intracellular material, and cell fusion with Sendai virus. Our experiments indicate that CB, indeed, inhibits phagocytosis (Davis, A. T., Estensen, R. D., and Quie, P. G., manuscript in preparation) and pinocytosis (Wagner, R., Rosenberg, M., and Estensen, R., manuscript in preparation). All of the aforementioned processes are thought to involve the plasma membrane. Other membrane assembly or fu-

sion processes such as assembly of the nuclear membrane or division of mitochondria (Johnson, R., personal communication) are apparently not affected at these low concentrations. The drug, therefore, may also be useful in classifying fusion processes. The extent to which CB inhibits membrane fusion processes remains to be determined. Further investigation of the mode of action of CB will hopefully shed more light on these functions of membranes.

Summary. The mold metabolite Cytochalasin B (CB inhibits cytoplasmic division (cytokinesis) while allowing nuclear division to proceed. N1S1-67 (Novikoff rat hepatoma) cells exposed to drug concentrations of 2.1×10^{-6} *M* do not undergo cytoplasmic division, but cell volume and number of nuclei per cell increase at rates which are comparable to the rates of similar processes in controls. Incorporation of ^{3}HTdr, ^{3}HUdr, ^{3}H-L-leucine and ^{3}H choline suggest that such increases represent true nuclear and cytoplasmic growth.

These results indicate that inhibition of macromolecular synthesis does not account for the action of CB. The inhibition of a process of membrane fusion is suggested as a possible mode of action of the drug.

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