Enucleation of Cultured Human Cells[1] (37640)

WOODRING E. WRIGHT AND LEONARD HAYFLICK

Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305

Cytochalasin B (CB), a metabolite produced by the mold *Helminthosporium dematiodium*, has been shown to produce diverse biological effects, inhibiting such processes as morphogenesis (1), cytokinesis (2, 3), phagocytosis (4, 5), glucose uptake (6, 7), and cell locomotion (2, 8). One of its most valuable properties is the ability to cause cells to extrude their nuclei (2). Different sensitivities of several cell types to this action of CB have been reported (9–11). We describe here a quantitative study of the effect of CB concentration, *g* force and temperature on the efficiency of enucleation of mass cultures of human cells. The results suggest the reason for different cell sensitivities, as well as a possible mechanism by which CB effects enucleation.

Methods. The normal human female diploid cell strain WI-38 (20) was enucleated by a method previously described (11, 12). Briefly, cells were plated onto glass coverslips cemented to ¼ in. thick Lucite plates for support. The following day, the Lucite-coverslip plates were placed in an upright position in tubes filled with Eagle's Basal Medium, 10% Calf Serum, and CB. The tubes were then centrifuged for 30 min in an SW27 rotor in a Beckman L2-65B ultracentrifuge. Cells were stained with Giemsa, and the percent enucleation determined at different levels on the Lucite-coverslip plate (Fig. 1). A single set of cytochalasin dilutions was prepared and used throughout these experiments. Thus, although the absolute concentrations of CB reported to produce a given enucleation may contain a small systemic error, the results are internally consistent.

Results. Dose and temperature dependence. Data in Fig. 2 show the efficiency of enucleation of WI-38 cells at 35° and at various concentrations of CB. The centrifugal force necessary for enucleation is highly CB dose-dependent. Furthermore, although most of the cells were stripped off the plates at forces above 80,000*g*, it was possible to enucleate cells even in the absence of CB. The relationship between the concentration of CB and the *g* force necessary to achieve 50% enucleation was obtained from the data in Fig. 2 and from similar data for enucleation at 25°, 15°, 5°, and 0° (Fig. 3).

The curves in Fig. 3 are clearly composed of two different segments: one CB dependent (the sloping lines) and one CB independent (the vertical lines). WI-26 (20) (normal male human diploid fetal lung fibroblasts), Down's syndrome thymus and skin fibroblasts, and VA-13 ($S.V._{40}$ transformed WI-38) were all found to exhibit biphasic curves essentially identical to that obtained for WI-38.

Evidence for a membrane limitation. The limiting *g* force (the *g* force at which enucleation becomes CB independent) for enucleation at different temperatures is plotted on a linear scale in Fig. 4. One explanation for this temperature dependence is that at lower *g* forces, or lower temperatures, the rigidity of the membrane becomes limiting for enucleation. To test this hypothesis, WI-38 cells were treated with agents that either stabilize or destabilize the cell membrane. Treatment with 0.001% glutaraldehyde (10^{-4} *M*)

[1] This study was supported, in part, by research grant HD04004 from the National Institute of Child Health and Human Development, by contract NIH 69-2053 within the Special Virus Cancer Program of the National Cancer Institute, and by Medical Scientist Training Grant No. GM 1922 from the National Institute of General Medical Sciences.

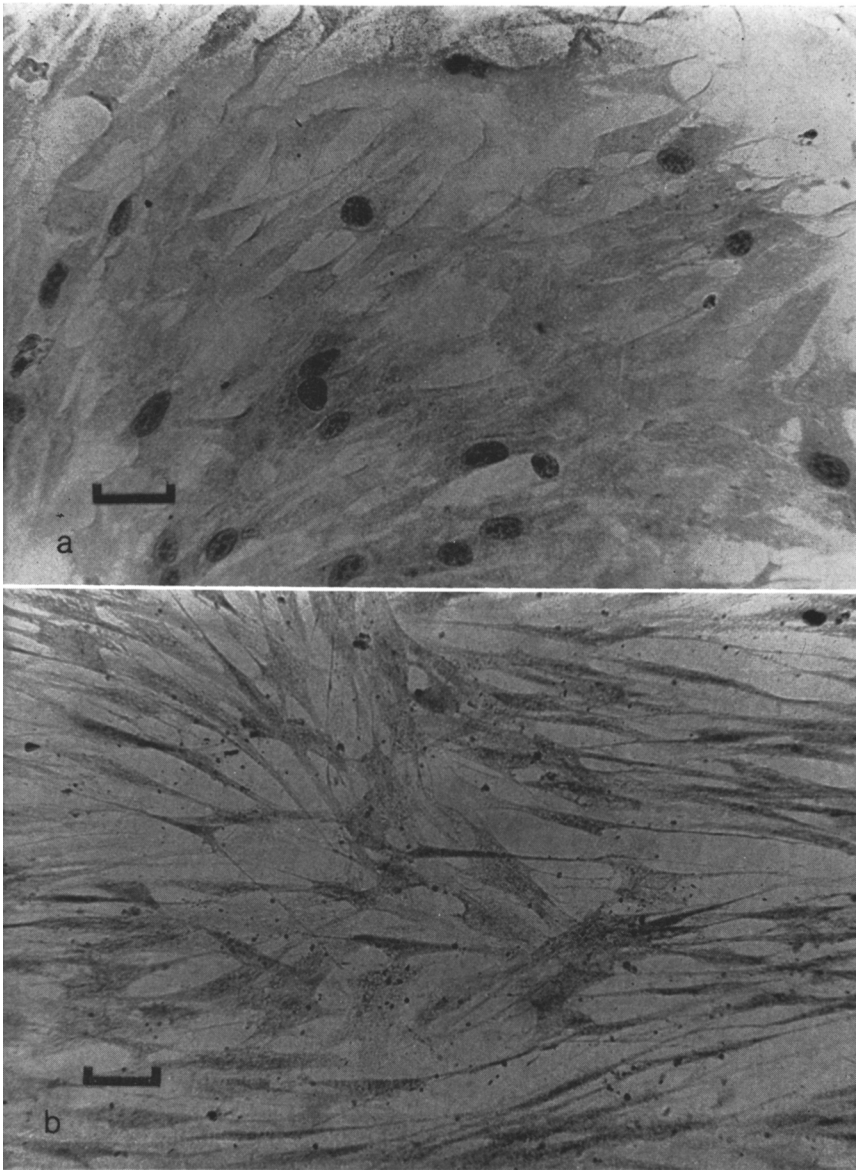

FIG. 1. Enucleated cells. WI-38 cells on Lucite-coverslip plates were centrifuged at 26,000*g* for 30 min at 25°. a) 80% enucleation obtained with 2 μg of CB/ml; b) 100% enucleation obtained with 4 μg of CB/ml. Bar indicates 20 μm.

for 10 min at room temperature increased the limiting *g* force for enucleation at 25° from 10,000*g* to approximately 50,000*g*. A 30-fold higher concentration of formaldehyde was required to produce the same effect, indicating the effect of glutaraldehyde is not due solely to the blocking of amino groups but is also probably due to the stabilizing effect of cross-linking proteins.

Since polyene antibiotics bind to sterols (13) and cause the formation of "pits" in the cell membrane (14), treatment with one such antibiotic was undertaken as a means of destabilizing the cell membrane. Treatment with the polyene antibiotic amphoterocin B (0.05 mg/ml in 0.04 mg/ml sodium deoxycholate) for 20 min at room temperature lowered the limiting *g* force at room temperature from

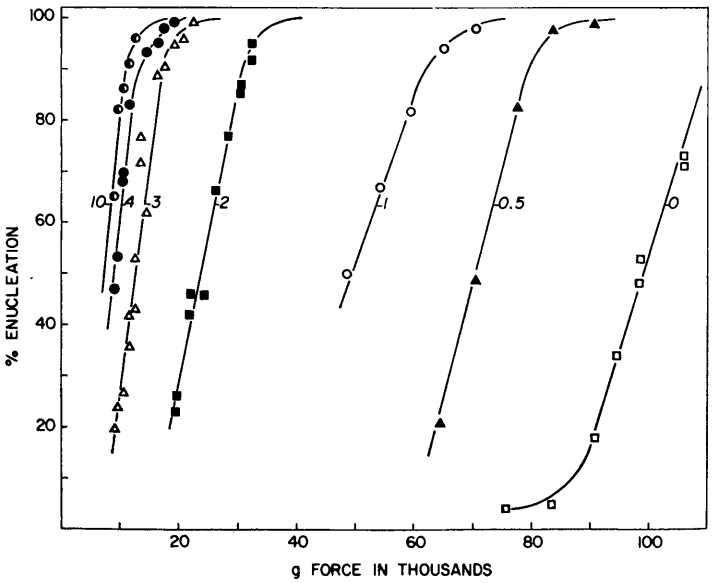

FIG. 2. Enucleation at different g forces and concentrations of cytochalasin B. WI-38 cells on Lucite-coverslip plates were centrifuged for 30 min at 35°. Numbers indicate the concentration of CB in μg/ml. Each point represents a count of approximately 200 cells. Cells were counted at a radius of spin of 9–15 cm at 1-cm intervals, and the g force calculated for each radius of spin. Thus, the effect of a wide range of g forces could be determined in a single plate. Different rpm's were selected to cover different ranges of g forces. Most curves are composed of determinations made from at least 2 Lucite-coverslip plates.

11,700g (range of 3 Lucite-coverslip slides, 11,000–12,500g) to 7500g (range of 4 Lucite-coverslip slides, 7100–7900g). Treatment with lysolecithin (0.125 mg/ml), sodium deoxycholate (0.2 mg/ml), digitonin (0.004%), or ether (0.5 emulsion) failed to decrease the limiting g force. In all cases, the concentration used was the maximum concentraction that did not produce a marked cytopathic effect during a 20 min treatment. If membrane rigidity is the basis for the temperature dependent segment of the enucleation curves in Fig. 3, the failure of agents such as lysolecithin to change the limiting g force suggests that the packing of fatty acid chains (membrane "fluidity") may not be the prime determinant of mammalian membrane "rigidity."

One alternative to the membrane as the basis for the temperature dependent part of the enucleation curves is a temperature dependent change in cytoplasmic rigidity. After centrifugation for 30 min at 25° and at 30,000g in the absence of CB, the nuclei migrate through the cytoplasm and rest against the membrane at the higher g force end of the cell. If cytoplasmic rigidity is limiting, precentrifuging the cells immediately before centrifuging in CB-containing medium should decrease the limiting g force. However, precentrifugation did not alter the limiting g force.

Discussion. Although it is possible to enucleate L-929 at 1g (2, 9), WI-38 is resistant to enucleation under these conditions (11). Extrapolating the CB dependent segment of the curves of Fig. 3 to lower g forces yields a CB concentration of approximately 20 μg/ml for enucleation at 1g. This is close to the 30 μg/ml reported for 40–80% enucleation of L-929 cells (9). This suggests that the different sensitivity to enucleation of WI-38 and L-929 is due to the presence in WI-38 of the CB-*independent* phenomenon, rather than simply representing different degrees of receptor binding of the drug itself. The CB-independent phenomenon prevents WI-38 cells at 35° from enucleating at any force below 10,000g regardless of CB concentration. This limiting g force is temperature sensitive, is in-

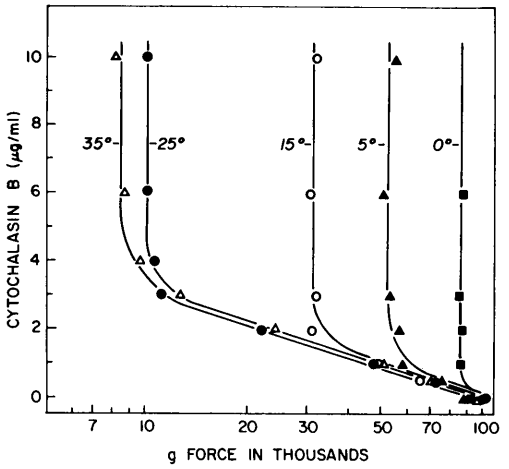

FIG. 3. *g* force necessary to produce 50% enucleation at different temperatures and concentrations of CB. Points determined from the data of Fig. 2 and similar data at 25°, 15°, 5°, and 0°. In all cases the cells were pre-incubated with CB at room temperature for 30 min to allow all CB-cell reactions to reach equilibrium (12).

creased by glutaraldehyde and decreased by amphoterocin B treatment, and is not changed by pre-centrifugation. These observations support the notion that the membrane is the basis for the CB independent segment of the enucleation curves, and thus that it is the basis for the different sensitivities to enucleation by CB.

Some actions of CB have been attributed to its effects on microfilaments (3, 15). While recognizing that these explanations are not yet proven (16–18), we offer the following microfilament model for the enucleation of cells by CB. A subplasmalemmal network of microfilaments has been shown to be disrupted by CB (8). It is possible that this network functions as a barrier preventing cell organelles from coming in contact with the cell

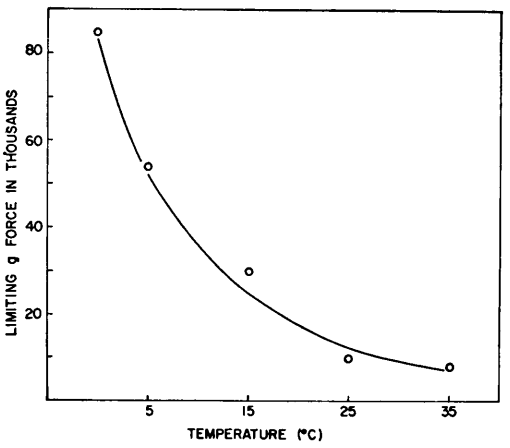

FIG. 4. Limiting *g* force for enucleation at different temperatures. Each point represents the *g* force at which the curves of Fig. 3 become vertical.

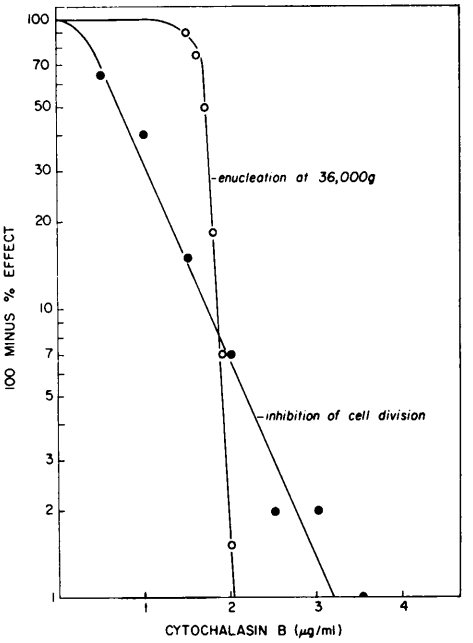

FIG. 5. Target theory plot of the dose dependence for enucleation and inhibition of cell division by CB. 36,000*g* was chosen as the centrifugal force at which the concentration of CB was approximately the same for a 90% enucleation as for a 90% inhibition of cell division. The enucleation curve was calculated by interpolation of the data in Figs. 2 and 3. The *g* force for 50% enucleation at various concentrations of CB (1.5–2.5 μg CB/ml in 0.1 μg increments) was determined from Fig. 3. These points were entered onto a graph similar to Fig. 2, and lines parellel to the curves of Fig. 2 were constructed through each point. The intersection of each of these curves with 36,000*g* yielded the percent enucleation that would have been obtained at that concentration of CB. Inhibition of cell division was calculated from Coulter Counter determinations of cell number after four days continuous cultivation in the presence of CB. Each point represents data from three replicate cultures. The figure shows approximately single-hit kinetics for inhibition of cell division and multi-hit kinetics for enucleation by CB.

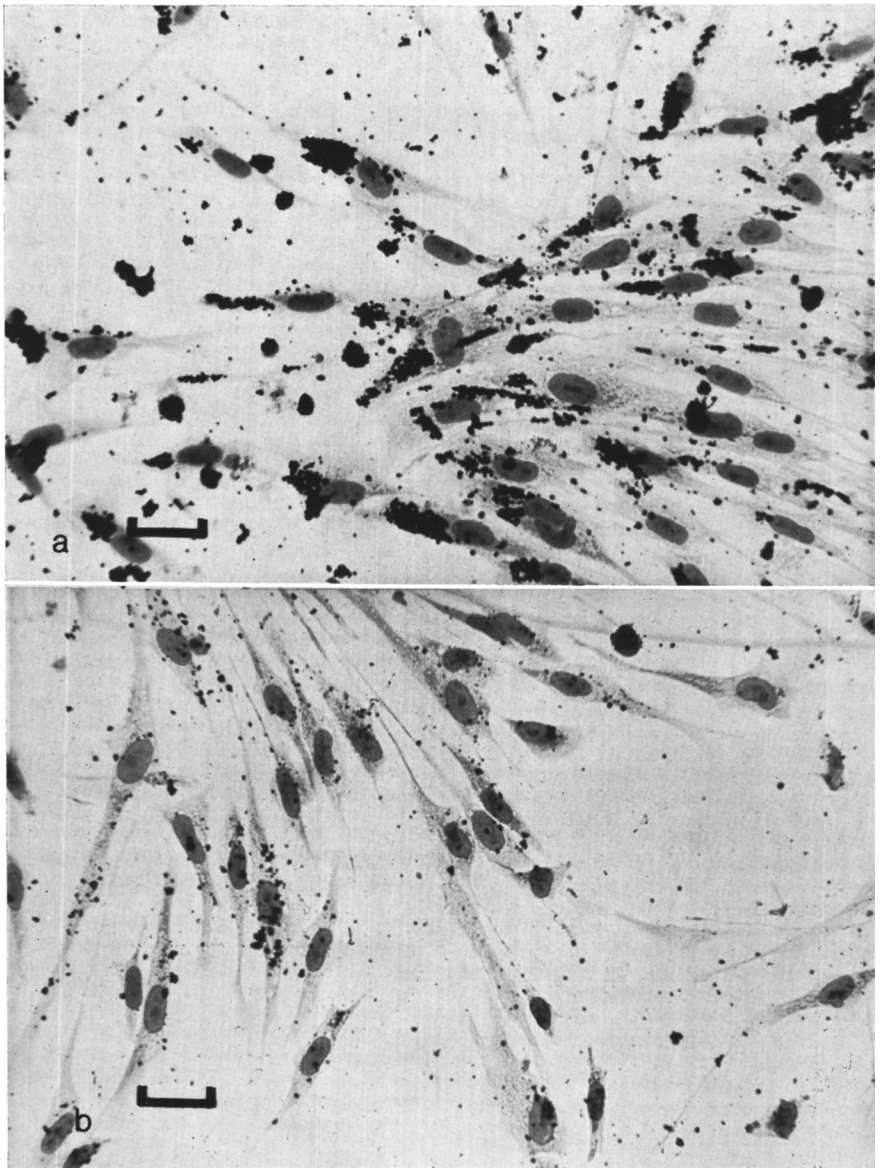

FIG. 6. "Enucleation" of iron particles by CB. 10 ml of a 0.25% (w/v) suspension of carbonyl iron powder (General Aniline and Film Co., Linden, New Jersey) in medium was allowed to settle for 15 min in a 16 × 150 mm test tube. Those particles remaining in suspension (1–4 μm diameter) were added to WI-38 cells growing on Lucite-coverslip plates. Sixteen hr later, the cells were centrifuged for 30 min at 25° and 3,000*g*. a) Medium alone, b) Medium plus 4 μg CB/ml. Bar indicates 20 μ.

membrane (8) where they might be extruded from the cell. Enucleation would occur when this network of microfilaments was sufficiently disrupted to allow the nucleus to come in contact with the plasma membrane. The presence of the network would require its involvement in the normal phagocytic process, since phagosomes would have to be transported across it in order to enter the cell. Disrupting it might thus be expected to inhibit phagocytosis which CB has, in fact, been found to do (4, 5).

An intertwined network may be able to absorb a large number of "hits" before the structure of the network begins to weaken, whereas a parallel array of contractile filaments should display a decremental effect for each "hit." This could explain the much larger dose of CB necessary for enucleation at 1*g* than for actions of CB attributed to its effects on contractile filaments, such as the inhibition of cytokinesis. Although the mechanism for this inhibition was originally attributed to the effect of CB on a specific "contractile ring" (3), this interpretation is now questionable (16–18). Nevertheless, within the constraints of a microfilament model, the inhibition can still be considered to be the result of the disruption of some contractile filament mechanism necessary for cytokinesis. The hypothesis that enucleation is caused by the disruption of a subplasmalemmal network of microfilaments predicts the multi-hit kinetics of enucleation as compared with the single-hit kinetics of the inhibition of cell division (Fig. 5).

One further prediction of this model is that the process involved in enucleation should not be specific for the nucleus. This is demonstrated by the fact that the extrusion of phagocytized particles is facilitated by CB (Fig. 6). Further confirmation of the lack of nuclear specificity is provided by the observation of lysosomal exocytosis by polymorphonuclear leukocytes in the presence of CB (19).

The evidence supporting this hypothesis is now circumstantial, but it is nonetheless a useful working model to describe the mode of action of CB in the enucleation of mammalian cells.

Summary. Human cells were found to have a biphasic response to high *g* force enucleation in the presence of cytochalasin B (CB). At higher *g* forces, enucleation is exquisitely CB dependent. At lower *g* forces, enucleation becomes independent of the concentration of CB and highly dependent upon temperature. Because of the ability to manipulate this part of the response with temperature, glutaraldehyde and amphoterocin B, it is suggested that at lower *g* forces the rigidity of the cell membrane becomes limiting for enucleation. Cells that are sensitive to enucleation at 1*g* would thus be sensitive because of a difference in the physical characteristics of their membranes, rather than a different sensitivity of their receptors to CB itself.

The processes involved in enucleation were found to exhibit multi-hit kinetics, and were not specific for the nucleus. A model based on the ability of CB to disrupt a subplasmalemmal network of microfilaments is presented to explain these observations.

1. Wrenn, J. T., and Wessells, N. K., Proc. Nat. Acad. Sci. USA **66**, 904 (1970).

2. Carter, S. B., Nature **213**, 261 (1967).

3. Schroeder, T. E., Z. Zellforsch. Mikrosk. Anat. **109**, 431 (1970).

4. Davis, A. T., Estensen, R., and Quie, P. G., Proc. Soc. Exp. Biol. Med. **137**, 161 (1970).

5. Allison, A. C., Davies, P., and De Petris, S., Nature New Biol. **232**, 153 (1971).

6. Kletzien, R. F., Perdue, J. F., and Springer, A., J. Biol. Chem. **247**, 2964 (1972).

7. Estensen, R. D., and Plagemann, P. G., Proc. Nat. Acad. Sci. USA **69**, 1430 (1972).

8. Spooner, B. S., Yamada, K. M., and Wessells, N. K., J. Cell Biol. **49**, 595 (1971).

9. Poste, G., and Reeve, P., Nature **229**, 123 (1971).

10. Prescott, D. M., Myerson, D., and Wallace, J., Exp. Cell Res. **71**, 480 (1972).

11. Wright, W. E., and Hayflick, L., Exp. Cell Res. **74**, 187 (1972).

12. Wright, W. E., *in* "Methods in Cell Physiology" (D. Prescott, ed.) Vol. 7. Academic Press, New York (1973).

13. Feingold, D. S., Biochem. Biophys. Res. Commun. **19**, 261 (1965).

14. Kinsky, S. C., Luse, S. A., Zopf, D., Van Deenen, L. L. M., and Haxby, J., Biochem. Biophys. Acta **135**, 844 (1967).

15. Wessells, N. K., Spooner, B. S., Ash, J. F., Bradley, M. O., Luduena, M. A., Taylor, E. L., Wrenn, J. T., and Yamada, K. M., Science **171**, 135 (1971).

16. Carter, S. B., Endeavor **31**, 72 (1972).

17. Bluemink, J. G., Z. Zellforsch. Microsk. Anat. **121**, 102 (1971).

18. Estensen, R. D., Rosenberg, M., and Sheridan, J. D., Science **173**, 356 (1971).

19. Davies, P., Allison, A. C., Fox, R. I., Polyzonis, M., and Haswell, A. D., Biochem. J. **128**, 78 (1972).

20. Hayflick, L., Exp. Cell Res. **37**, 614 (1965).

Received May 21, 1973. P.S.E.B.M., 1973, Vol. 144.