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Inhibition of cytokinesis in Friend leukemia cells by membrane mobility agents

(multiple nuclei/giant cells/A_2C/Flomol fluorescent probe)

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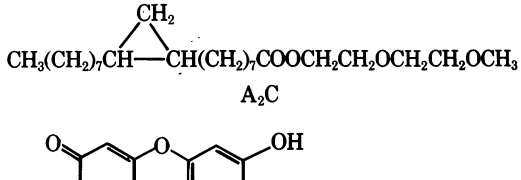
ABSTRACT **Treatment of a line of Friend leukemia cells with a dispersion of the membrane mobility agent, A_2C, yields cells that undergo successive nuclear divisions without cytokinesis, resulting eventually in cells with as many as 30 nuclei. Neither the DNA replication rate of the cells nor the generation time is different after treatment; in addition, the multiple nuclei divide synchronously, and the chromosome number corresponds to the number of nuclei in the cell. Inhibition of cytokinesis is not observed if the cells are washed with reagent-free medium within 1 hr of treatment, but is observed if washing is delayed for 24 hr. Membrane mobility agent loaded with the fluorescent probe, Flomol F20C, leads to fluorescent membrane; fluorescence disappears from the membrane after a change of medium within 1 hr, but not after a change of medium within 24 hr.**

Some stages in the overall development resemble those seen for cytochalasin B inhibition of cytokinesis, although the mechanisms may well be different for the inhibition promoted by membrane mobility agent. The inhibition of cytokinesis by A_2C provides a potentially interesting means for studying cytokinesis and the regulation of differentiation.

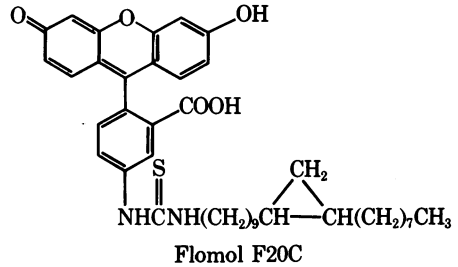
As the result of an attempt to cause the differentiation of a line of Friend leukemia cells towards hemoglobin production (1–4) with membrane mobility agents (5) we have discovered that the membrane mobility agent A_2C (5–7) inhibits cytokinesis without interfering with DNA replication or nuclear division. Viable giant, multinucleate cells can be produced. In the present article, we describe our results, note potential applications of the findings, and point out possible mechanisms.

MATERIALS AND METHODS

Cells. Friend leukemia cells (FL) (clone 39, derived from a clone identified as 745) were grown in suspension culture in Dulbecco's modified Eagle's medium (8) supplemented with glucose (22 mM) and 15% fetal calf serum. The cells (initial inocula 1×10^5 cells per ml) were cultured in plastic petri dishes (50 mm diameter, Nunc, Denmark) containing 4 ml of medium at 37° under humidified 5% CO_2/air. The generation time under these conditions was about 24 hr.



A_2C



Flomol F20C

Mobility Agent. A_2C (chromatographically pure synthetic material, available from Makor Ltd., P.O.B. 6570, Jerusalem, Israel) (2.31 μmol/μl) was placed in a tube with a Hamilton 10-μl syringe. Enough modified Eagle's medium was added to give concentrations of 0.05–0.5 μl of A_2C per ml of modified Eagle's medium. The mixture was sonicated (MSE sonicator) for 15–30 sec, yielding a suspension of particles (0.4–1.6 μm diameter).

Flomol-Loaded A_2C. Flomol F20C, a fluorescent probe for following the interaction of A_2C with membranes (E. M.

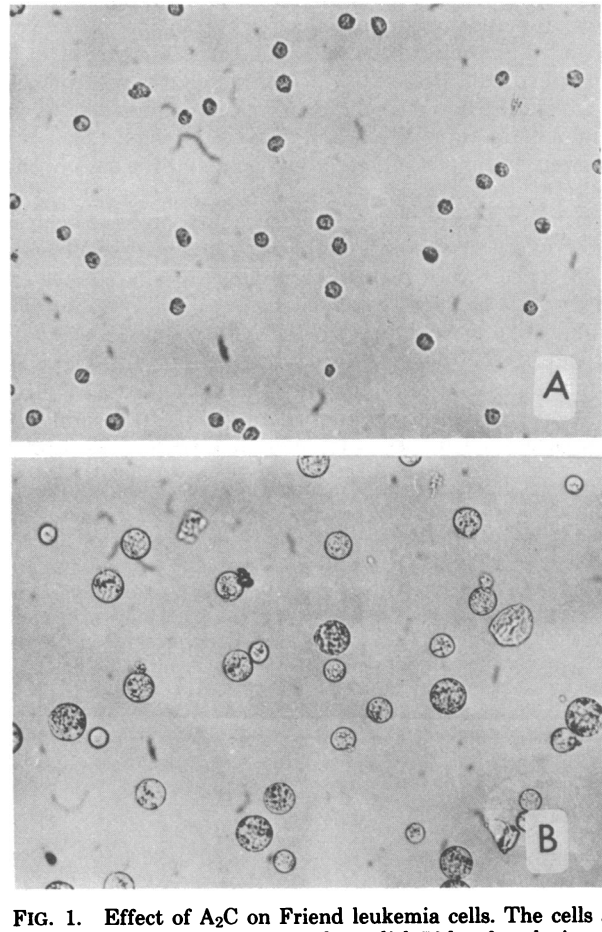


FIG. 1. Effect of A_2C on Friend leukemia cells. The cells are viewed in suspension on a tissue culture dish 72 hr after the inocula have been added to the medium in the dish. (×200.) (*A*) Control sample. (*B*) A_2C-treated cells. Medium contained 0.125 μl of A_2C per ml. Many giant cells are seen.

Abbreviation: FL cells, Friend leukemia cells.

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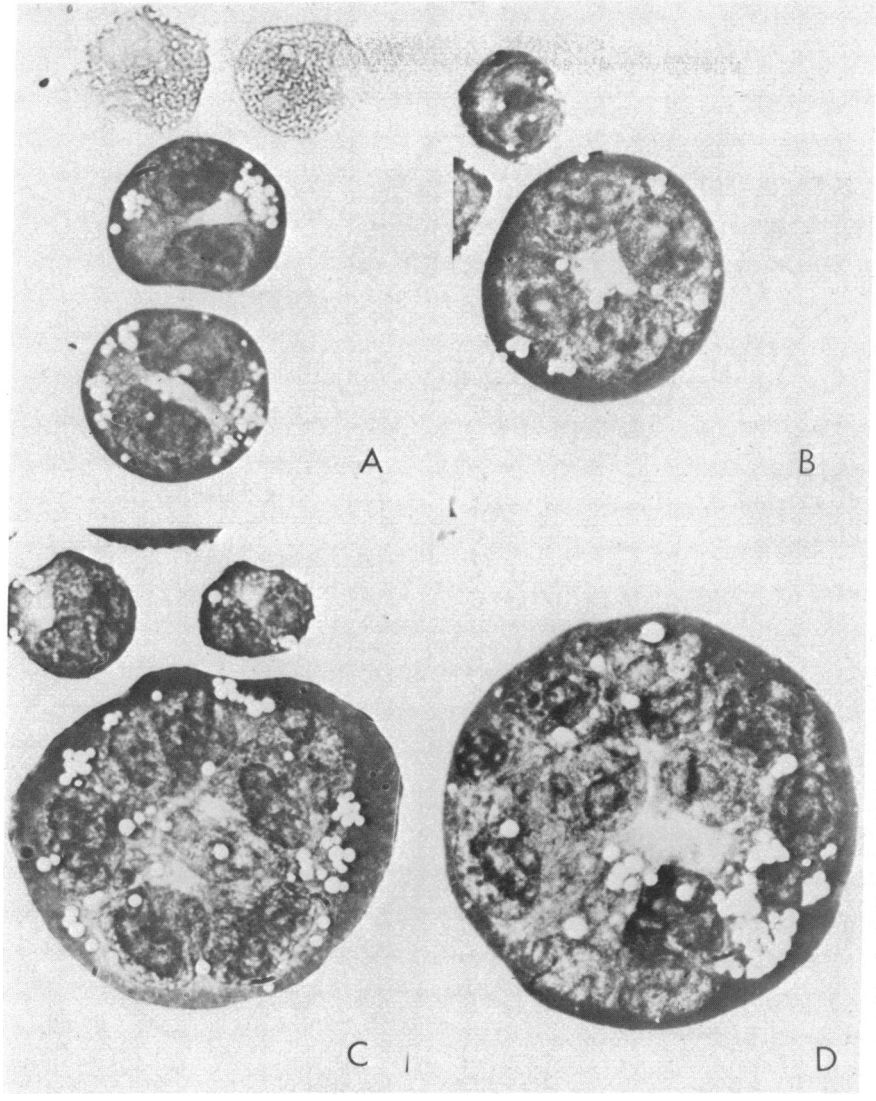

FIG. 2. Nuclei in A_2C-treated cells. Cells were grown in medium to which 0.125 μl of A_2C per ml had been added. Cells were fixed and stained after centrifugation in a cytocentrifuge. Photographs were taken at intervals. (×1080.) (*A*) 24 hr; (*B*) 48 hr; (*C*) 72 hr; (*D*) 96 hr.

Kosower, N. S. Kosower, and P. Wegman, unpublished data; N. S. Kosower, E. M. Kosower, S. Lustig, and D. H. Pluznik, unpublished data), was mixed as a methanol solution (2×10^{-3} μmol/μmol of A_2C) with A_2C. The methanol was removed by a stream of N_2, modified Eagle's medium was added, and the mixture was sonicated as above. The dispersed A_2C is thus "loaded" with Flomol F20C, in the form of fluorescent particles.

Treatment of Cells with Mobility Agent. The A_2C suspension (or the Flomol-loaded A_2C suspension) was mixed with an equal volume of cell suspension in modified Eagle's medium containing 30% fetal calf serum to give a final cell concentration of 1×10^5 cells per ml in medium containing 15% fetal calf serum and A_2C volumes from 0.025 μl to 0.25 μl/ml. (Zero A_2C = control.)

Inspection of Cells. We examined (*a*) cells in tissue culture dishes; (*b*) cells in suspension stained with 0.1% trypan blue (live cells are not stained); (*c*) cells on slides after centrifugation in

Table 1. Extent of DNA synthesis* in control and in A_2C-treated† cultures of Friend leukemia cells

Culture, hr	Control, $\times 10^{-5}$/ml	A_2C†-treated, $\times 10^{-5}$/ml		[^{3}H]Thymidine incorporation, cpm $\times 10^{-3}$	
	Cell (nuclei) no.	Cell no.	Nuclei no.	Control	A_2C-treated
0	1	1			
24	2.7	1.2	2.3	230	237
48	6.8	1.5	5.7	587	550
72	11	1.4	10.8	900	770

* Determined by incorporation of tritiated thymidine into the cold trichloroacetic acid-precipitable material of the whole cell population of a culture dish.

† A_2C was 0.125 μl/ml of medium.

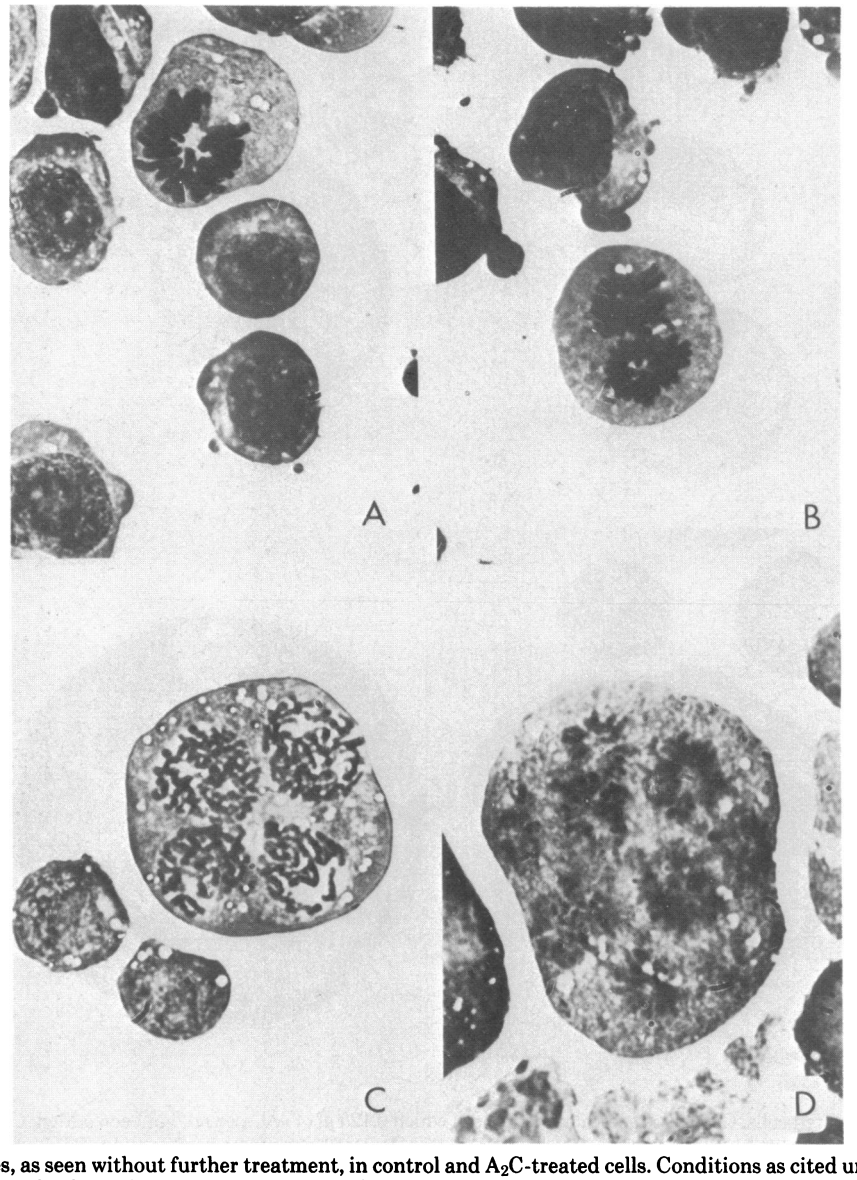

FIG. 3. Mitotic figures, as seen without further treatment, in control and A_2C-treated cells. Conditions as cited under Fig. 2. *No colchicine was added.* (*A*) Cell in control culture. Metaphase figures in A_2C-treated cells at various times: (*B*) 48 hr; (*C*) 72 hr; (*D*) 96 hr.

a cytocentrifuge, fixation in methanol, and staining with Giemsa's solution; and (*d*) cells fixed after addition of colchicine (to observe metaphase chromosomes for counting with an expanded image projected from a photograph) [2.5 μM colchicine (4–5 hr), 0.55% KCl (10 min), CH_3OH–CH_3COOH fixation, Giemsa stain].

Thymidine Incorporation. [*methyl*-^{3}H]Thymidine (Amersham, specific activity 19 Ci/mmol) was added to the tissue culture dishes at the beginning of the culturing procedure. At intervals, the total cell population of a dish was filtered onto Millipore filters (0.45 μm) and the cells were washed with cold saline and 5% trichloroacetic acid. The acid-denatured material was dried and radioactivity was measured in the usual way with a Tricarb scintillation counter. Parallel inspection of cells was made at the same time on aliquots of cells from another dish.

RESULTS

Inhibition of Cytokinesis. The addition of A_2C to FL cells results in the formation of giant cells (Fig. 1). The percentage of affected cells varies with the concentration of A_2C used: 90% with 0.25 μl/ml, 70–80% with 0.125 μl/ml, and 25–50% with 0.03–0.06 μl/ml. The highest concentration of A_2C normally used did not affect the viability of cells, as judged by the trypan blue exclusion test and thymidine incorporation (see below).

To determine the nature of the giant cells produced by the A_2C treatment, we fixed, stained, and examined the cells. Binucleated cells were found after 24 hr, tetranucleated cells after 48 hr, and cells containing 8 and 16 nuclei on the third and fourth days, respectively (Fig. 2). Viable cells containing over 30 nuclei can be seen in 5- to 6-day-old cultures. Synchrony of nuclear division is suggested by the fact that most of the multinucleated cells contain 2*n* nuclei, and is further supported by the observation that nuclei within a cell are synchronized with regard to chromosome condensation (Fig. 3). The number of chromosomes in multinucleated cells corresponds to the number of nuclei observed in cultured cells (Fig. 4): 40 to 48 chromosomes in mononuclear cells, 80 to 96 in cells containing 2 nuclei, and 160 to 188 in cells containing 4 nuclei. A metaphase chromosome spread taken at the time when 8 nuclei were seen in cultured cells revealed 350 to 400 chromosomes (not illustrated).

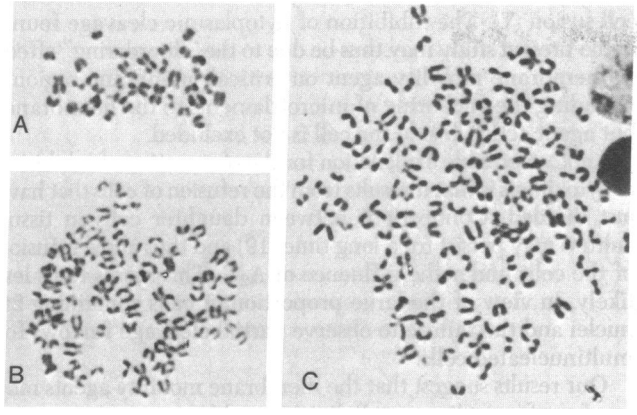

FIG. 4. Mitotic metaphase chromosomes of control and A_2C-treated Friend leukemia cells. Chromosome spreads were prepared by routine colchicine treatment. Photographs were projected onto a large gridded screen for counting. (*A*) Control. A_2C-treated: (*B*) 24 hr; (*C*) 48 hr.

Careful examination of the cultures especially between the first and second divisions, revealed that a partial cleavage furrow could be seen in some cells. An unusual feature in the methanol-fixed, A_2C-treated cells is that many vacuoles are present, in comparison with few in the control cells (Figs. 2 and 5).

DNA Synthesis in A_2C-Treated Cells. Cell cultures treated with A_2C incorporated thymidine into cold trichloroacetic acid-precipitable material to about the same extent as control cultures. Parallel experiments for control and treated cells showed that the extent of incorporation was similar in the two cases even though the number of cells was markedly lower in the A_2C-treated cells (Table 1).

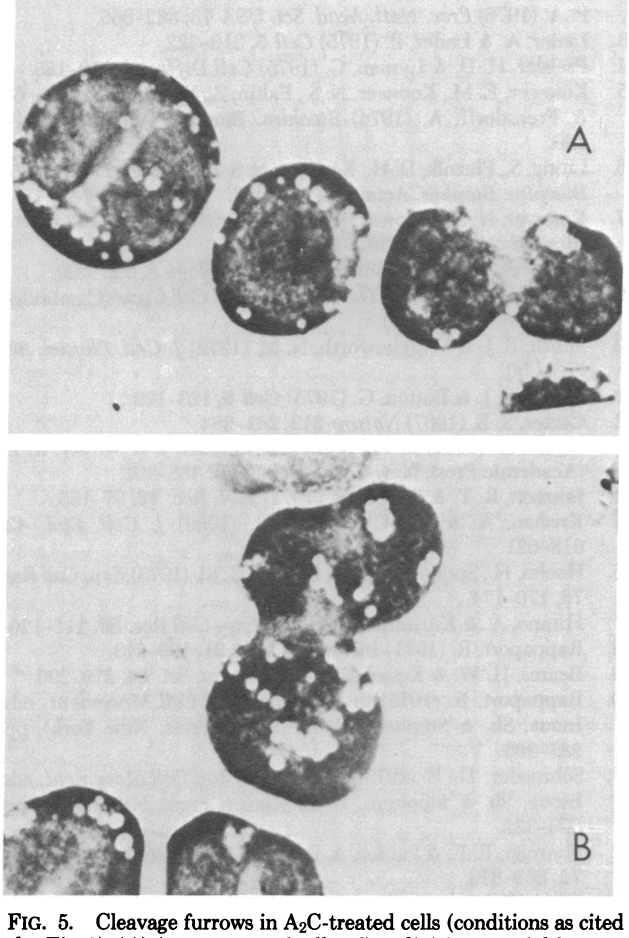

FIG. 5. Cleavage furrows in A_2C-treated cells (conditions as cited under Fig. 2). (*A*) Appearance of cell at first division: partial furrow. (*B*) Partial furrow at second division.

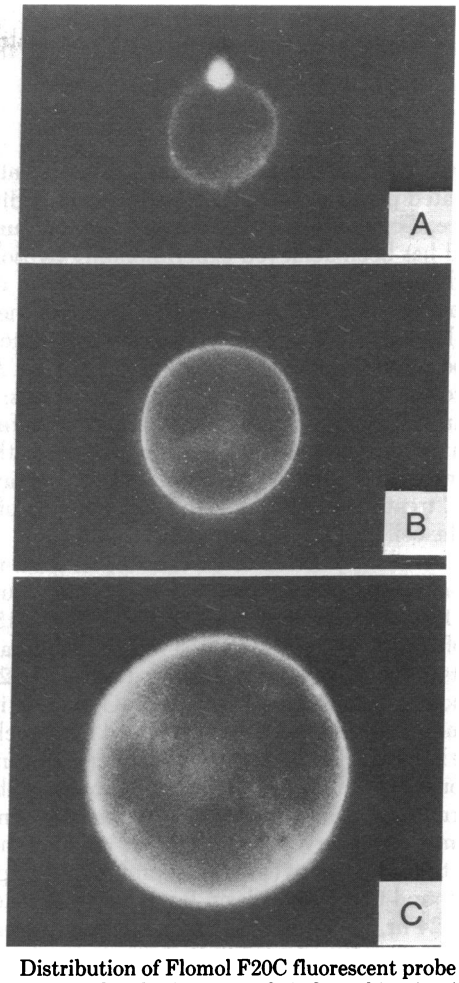

FIG. 6. Distribution of Flomol F20C fluorescent probe in Friend leukemia cells treated with Flomol F20C-A_2C combination (for details see *Materials and Methods*). (*A*) 1 hr after addition. A particle of Flomol-loaded A_2C is seen attached to the cell. (*B*) 48 hr after treatment with F20C-A_2C combination. (*C*) 96 hr after treatment.

Reversibility of A_2C Effect and Distribution of A_2C in Cells. If the A_2C-treated FL cells are washed free of the original medium within 1–2 hr after treatment and resuspended in A_2C-free medium, normal cell division is observed after 24 hr. However, if the washing is delayed for 24 hr, nuclear division without cytokinesis is still observed and the cells continue as they do in the absence of a washing step.

Flomol F20C-loaded A_2C provides visualization through the fluorescence of the F20C probe of the initial distribution of the A_2C in the FL cells. (The chemical nature of the fluorescent probe F20C is such that its distribution in the cell will initially resemble that of A_2C.) Within a few minutes of mixing F20C-A_2C suspension with cells at 37°, the membrane of the FL cells is fluorescent. The membrane remains fluorescent through subsequent nuclear divisions (Fig. 6). If the cells are washed and resuspended in fresh medium within 1–2 hr after treatment with the F20C-A_2C combination, no fluorescence is seen in the membrane. If washing is delayed for 24 hr, the fluorescence remains in the membrane, not only for the first nuclear division without cytokinesis but for all subsequent nuclear divisions until multinucleated cells with fluorescent membranes are produced. Our observations suggest that the

fluorescence is always associated with the membrane, although some fluorescence may be distributed through the cytoplasm.

DISCUSSION

Karyokinesis and cytokinesis are usually highly integrated, interregulated processes in the cell cycle. Nuclear division in the absence of cytokinesis may, however, occur naturally (see ref. 9) and has been achieved experimentally by isolation of mutants defective in cytokinesis (10, 11) and by chemical means, notably by use of cytochalasin B (12). In the present work, we have shown that the formation of multinucleate FL cells can be induced by the membrane mobility agent A_2C. The overall process has the following specific properties: (*i*) cytokinesis is inhibited without nuclear division being affected; (*ii*) the nuclei divide synchronously; and (*iii*) DNA synthesis and generation time are unchanged from that of untreated cells. Thus, A_2C treatment does not affect the progress of the cell through the cell cycle.

Mitotic synchrony seems to be the rule in most naturally occurring multinucleate cells (13, 14) but may not occur in some mutants (11) or in cytochalasin B-treated cells (12, 15). In addition, polyploidy and a diminished rate of mitosis are found in mutants and in cytochalasin B-treated cells (10–12, 15, 16). Another notable difference between the A_2C-induced inhibition of cytokinesis and the inhibition produced by cytochalasin B is that the latter is reversible by washing after a multinucleated cell has formed, whereas the A_2C effect is not reversible at this stage. Firm conclusions on the differences between the two reagents must be reserved until the same cell lines have been subjected to both reagents (14, 17). In preliminary experiments we have found that cytochalasin B produces binucleated FL cells after 24 hr, but we have noted abnormal nuclear masses of various sizes in the giant cells that appear on further incubation.

The processes underlying cytoplasmic division, including the induction and the progressive development of the cleavage furrow, are not well understood, and the factors that are responsible for defective division are obscure. Much attention has been directed towards a topographical analysis of the progressive changes in membrane and submembrane structures. Microdissection studies of dividing cells have indicated that the cleavage furrow region is stronger and more rigid than the rest of the cell (18). Scanning electron microscopic studies of the fine structure of the cell surface in the cleavage furrow region show a very active region, with many microvilli and folds. The folds become larger with the development of the furrow, a change assumed to be associated with increased tension within the cleavage furrow cortex (19). A dense layer containing microfilaments closely associated with the membrane encircles the dividing cell in the region of the cleavage furrow; the layer is thought to act as a contractile ring, drawing the membrane inwards and continuously deepening the furrow (13, 18–21).

The foregoing observations point to the importance of ordered, "rigid," organized structures for the normal progression of cytoplasmic cleavage. Membrane mobility agents were designed to be easily incorporated into cell membranes, to increase the "disorder" within the hydrophobic portion of the bilayer and thereby facilitate motion of membrane components. The effects thus far found for membrane mobility agents are in accord with these expectations: increased lateral mobility of antibody–membrane receptor complexes in lymphocytes (5), promotion of cap formation at the expense of agglutination in lectin-treated malignant cell membrane (6), and promotion of cell fusion (7). The inhibition of cytoplasmic cleavage found in the present study may thus be due to the "disordering" effect of membrane mobility agent on critical membrane regions, including the anchoring of microfilaments to the membrane, but agent action inside the cell is not excluded.

Another possible explanation for the observation of inhibition of cytokinesis is that it results from the refusion of cells that have just divided. Connections between daughter cells in tissue culture may persist for a long time (19) and thus allow refusion of the cells under the influence of A_2C. This seems to us less likely, in view of the large proportion of cells containing $2n$ nuclei and the failure to observe partial cleavage furrows for multinucleated cells.

Our results suggest that the membrane mobility agents may be of use for studies on cell division and for investigations into the regulation of cell cycle phases (22) and on the interaction of nuclei with cytoplasm. Problems related to cell differentiation (23), particularly in FL cells (24), may be probed in a new way with membrane mobility agents.

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