A PREPARATIVE METHOD FOR OBTAINING ENUCLEATED MAMMALIAN CELLS

Michael H. Wigler and I. Bernard Weinstein

Institute of Cancer Research and Departments of

Medicine and Microbiology, College of Physicians and Surgeons

Columbia University, New York, New York 10032

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SUMMARY

Mouse L cells can be enucleated in suspension by centrifugation in discontinuous Ficoll density gradients while in the presence of Cytochalasin B. Greater than 50% of the cytoplasts thus obtained attach to glass or plastic and undergo morphologic recovery within 2-4 hours of replating. Protein synthesis in cytoplasts undergoes a biphasic decay from an initial rate of approximately 50% of control nucleated cells. This method can yield up to 5 x 10^8 cytoplasts with consistently low levels of contamination by nucleated cells (less than 0.2%), and is well suited for obtaining quantitative amounts of cytoplasts or karyoplasts for physiologic or biochemical studies.

INTRODUCTION

In 1967 Carter reported that treatment of mouse L cells with the mold metabolite Cytochalasin B (CB) caused profound morphologic effects, including nuclear extrusion and, in a small number of cells, enucleation (1). Since that time, methods utilizing CB have been developed for obtaining large numbers of enucleated cells, or cytoplasts. According to those methods, when cells adhering to glass or plastic coverslips are inverted in media containing CB and centrifuged, the cells which remain attached are predominantly cytoplasts (2,3). The yields obtainable from such methods are limited by the total surface area which can be conveniently centrifuged, and are insufficient for many purposes. We have explored, therefore, the possibility that cells can be enucleated by centrifugation in isoosmotic density gradients where the theoretical limit of yield is determined by the band capacity of the gradient.

EXPERIMENTAL PROCEDURES

Materials Cytochalasin B (Aldrich Biochemicals) solutions were made from stock solutions of 2.0 mg/ml in dimethyl sulfoxide (Me_2SO), stored at 4°C. Ficoll (Pharmacia Fine Chemicals), a synthetic polymer of high molecular

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weight, was dissolved by stirring overnight in twice distilled water to make a final concentration of 50% w/w, sterilized by autoclaving, and stored at -20°C. Radiochemicals, 4,5 [^{3}H]-L-leucine (30-50 Ci/mmole), 5,6 [^{3}H]-uridine (40-50 Ci/mmole) and thymidine-methyl-[^{3}H] (40-50 Ci/mmole) were obtained from New England Nuclear.

Cells Mouse L cells were grown as monolayer cultures at 37°C in an humidified atmosphere of 5% CO_2. Cultures were fed Dulbecco's Modified Eagle's Medium (GIBCO) supplemented with 5% fetal calf serum (GIBCO) and antibiotics, hereafter referred to as "growth medium". Cells were negative for mycoplasma when tested by the method of Todaro and Aaronson (4).

Enucleation Procedure Cells were seeded at 2 x 10^6 cells per 9 cm plastic petri dish. Three days later, confluent cell sheets were washed once with phosphate buffered saline (PBS) and detached by incubation with 0.5 mM ethylenediamine tetraacetic acid (EDTA) in PBS for 15 minutes at room temperature followed by vigorous pipetting. Cell suspensions were pooled, centrifuged at 1000 rpm for 5 minutes and resuspended in 12.5% Ficoll in Eagle's Minimal Essential Medium for spinner culture (GIBCO) containing 10 µg/ml CB and 0.5% Me_2SO to a final concentration of 0.5-2.0 x 10^7 cells/ml. Three ml of this cell suspension, essentially free of cell clumps, were then layered onto Ficoll density gradients that had been previously prepared as follows. Cellulose nitrate tubes were sterilized by UV light, and then carefully filled with the following layers of Ficoll: 2 ml of 25%; 2 ml of 17%; 0.5 ml of 16%; 0.5 ml of 15%; and 2 ml of 12.5% Ficoll, all in spinner culture medium containing 10 µg/ml CB and 0.5% Me_2SO and preequilibrated with CO_2 to pH 7.0-7.4. The refractive indices of these successive layers were: 1.380, 1.365, 1.363, 1.361 and 1.355. The discontinuous gradients were then incubated at 37°C for at least six hours. Three ml of the freshly prepared cell suspension (see above) was then applied and overlayed with 3 ml of spinner culture medium containing CB. Gradients were centrifuged in a Beckman SW 41 swinging bucket rotor in an L 3-50 ultracentrifuge for 60 minutes at 25,000 rpm and 31°C. The centrifuge and rotor were prewarmed to 31°C by running at 25,000 rpm for four hours prior to use.

After centrifugation, visible materials at appropriate banding interfaces were collected separately from the top of the tube with a syringe and cannula, diluted into 20 ml of growth medium and harvested by centrifugation at 1000 rpm for 5 minutes. The 1000 rpm pellets were resuspended in growth medium, replated into plastic petri dishes or glass coverslips at the desired density and incubated at 37°C in a CO_2 incubator. A similar scaled up procedure could be used with the larger volume SW 27 rotor.

RESULTS

After centrifugation of L cells in Ficoll gradients under conditions described above, the major bands of cellular material were the 0-12.5% Ficoll interface which contained primarily cellular debris; the 15-17% Ficoll region which contained the cytoplasts; and the 17-25% Ficoll interface which contained nuclei, or karyoplasts. In a series of six experiments we found that 50-70% of the input cells were ultimately recovered as cytoplasts from the 15-17% Ficoll region.

Within 15 minutes of replating into plastic petri dishes, 90% of the cytoplasts were attached to the dish. Within two to four hours, most cyto-

plasts had flattened, and, but for the absence of nuclei, they had the morphology of intact L cells. The remainder of attached cells were round and did not have the thinly spread membrane edges of fully recovered cytoplasts. The quality of morphologic recovery was enhanced by replating cytoplasts at high density (2×10^6 cells per 5 cm dish). The morphologic half-life of cytoplasts prepared in this way was greater than 24 hours. Degeneration was marked by cytoplasmic vacuolization, cytoplasmic retraction and eventually detachment from the dish.

Feulgen staining done on six separate preparations demonstrated that nucleated cells were present less than once for every 500 cytoplasts. Feulgen staining of the nuclear fraction collected from the 17-25% Ficoll interface (as described above) indicated that the nuclei were virtually free of intact cells and cytoplasts.

A number of experimental parameters were explored in order to arrive at our present enucleation protocol. Some of these will be discussed briefly.

Detaching L cells with either 0.1% trypsin or 0.5 mM EDTA and 0.1% trypsin resulted in decreased recovery of cytoplasts. On the other hand, three other cell lines, NRK (Normal Rat Kidney), Vero (African Green Monkey fibroblasts), and a rat liver epithelial cell line, tolerated enucleation well when detached with 0.25% trypsin.

It was found necessary for cell suspensions to be free of cell clumps prior to enucleation, as cell clumping reduced enucleation efficiency. The cellular load on the Ficoll gradient was found to be an important variable. If more than 6×10^7 cells were applied to a preincubated gradient, cell clumping occurred. If less than 1.5×10^7 cells were applied, morphologic recovery of the cytoplasts was poor. Preincubation of the discontinuous Ficoll gradient at 37°C increased the permissable cell load.

CB was an absolute requirement for enucleation. Cells suspended in media and centrifuged in Ficoll gradients containing 0.5% Me_2SO without CB did not enucleate. Similarly, centrifugation was an absolute requirement for

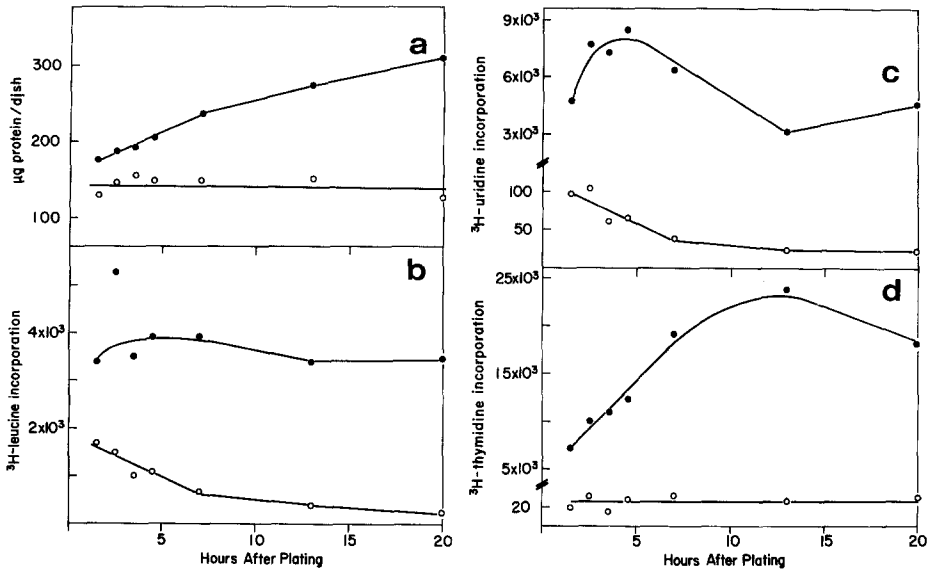

Figure 1. Incorporation by cytoplasts and control nucleated cells of [^{3}H]-leucine, [^{3}H]-uridine and [^{3}H]-thymidine into acid precipitable counts. L cells (3 x 10^7) were layered on each of six Ficoll density gradients containing CB and centrifuged as described in Experimental Procedures. The total yield of recovered cytoplasts was 1.2 x 10^8 cells. These were replated at 2 x 10^6 cytoplasts per 5 cm petri dish in growth medium. Control nucleated cells were L cells that were suspended in Ficoll and CB but were not centrifuged. They were replated at 1 x 10^6 cells per 5 cm dish. Duplicate cultures of either cytoplasts or control nucleated cells were incubated with either 30 µCi/ml [^{3}H]-leucine, [^{3}H]-uridine or [^{3}H]-thymidine for 60 minutes at various times after replating and the incorporation of radioactivity into acid precipitable counts determined by previously published methods (5). Total protein per dish was determined by the Lowry method (6). A) Total protein per dish. B) Incorporation of [^{3}H]-leucine. C) Incorporation of [^{3}H]-uridine. D) Incorporation of [^{3}H]-thymidine. Incorporation data is expressed as cpm per µg of cell protein. (●-●) control nucleated cells; (o-o) cytoplasts.

enucleation. Virtually 100% pure L cell cytoplasts could be obtained by centrifuging at 30,000 rpm rather than 25,000 rpm, but such cytoplasts did not recover when replated.

The time courses of macromolecular synthesis in cytoplasts isolated by our standard Ficoll gradient procedure are indicated in Figure 1. As controls we used L cells that were exposed to CB and Ficoll under conditions used for enucleation with the omission of centrifugation. They therefore remained nucleated and were replated in parallel with the cytoplast preparation obtained after centrifugation. When compared to nucleated control cells, the cytoplasts

had negligible [^{3}H]-thymidine incorporation (less than 0.2% per mg protein of control) and [^{3}H]-uridine incorporation (less than 2% per mg protein of control) into acid precipitable material. These low values provide evidence for the high efficiency of enucleation. In contrast to these results, [^{3}H]-leucine incorporation by cytoplasts was considerable and underwent a biphasic decay with an initial rapid decline (4 hour half-life) followed by a decline with a much longer half-life. The [^{3}H]-leucine incorporation appears to be a valid measure of protein synthesis by cytoplasts since both at early (4 hours) and late (24 hours) time points it was inhibited more than 90% by 10 µg/ml cycloheximide.

DISCUSSION

The present study demonstrates that mammalian cells can be enucleated in suspension with high efficiency by centrifugation in Ficoll density gradients while in the presence of cytochalasin B. Cytoplasts obtained by this method will attach to glass or plastic, undergo morphologic recovery, remain morphologically intact for at least 24 hours, and continue to incorporate [^{3}H]-leucine into protein for several hours after enucleation.

Greater centrifugal forces are required for enucleation in suspension than are required for enucleation of cells adherent to coverslips (2). This is in part attributable to the fact that, in suspension, the shearing force between cytoplasm and nucleoplasm is proportional to the difference in buoyant density of cytoplasm and nucleoplasm, whereas the shearing force is proportional to the difference in density of nucleoplasm and aqueous medium in the case of cells adhering to a coverslip.

The method described in the present study was developed for the L cell line. Similar procedures, with modifications, have worked well for other cell lines and will be the subject of a more comprehensive report.

Enucleation of cells in suspension has several advantages over previously published methods. These include: 1) A significant scale-up of the number of cells that can be enucleated. With the use of all six buckets of the Beckman

SW 27 rotor, 5 x 10^8 cytoplasts can be obtained with a single preparation. Similarly, large numbers of pure karyoplasts are obtainable. 2) Single cell suspensions may be used directly without the prerequisite attachment to a solid surface required by other methods. Possible disadvantages of this method are: 1) It is more complicated. 2) It may be a more traumatic procedure. The latter aspect requires further investigation.

We are hopeful that this preparative method will facilitate studies on various aspects of nucleo-cytoplasmic functions.

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