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Selection of reconstituted cells from karyoplasts fused to chloramphenicol-resistant cytoplasts

(cytochalasin B/enucleation)

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ABSTRACT **Murine Balb/3T3 and murine A-MT-BU-A1 mammary tumor cells were separated in the presence of cytochalasin B into enucleated cytoplasmic components (cytoplasts) and nucleated subcellular components (karyoplasts). Karyoplasts were derived from 3T3 cells, while cytoplasts were derived from A-MT-BU-A1 cells that were both chloramphenicol-resistant (CAP^r) and sensitive to hypoxanthine/aminopterin/thymidine (HAT^s). CAP^r has been shown to be cytoplasmically transmitted (possibly a mitochondrial gene mutation), while sensitivity to medium containing HAT has been shown to be transmitted by the nucleus (i.e., nuclear gene mutation). Such CAP^r cytoplasts derived from A-MT-BU-A1 cells were then fused, using polyethylene glycol, to HAT-resistant 3T3 karyoplasts. The mononucleated reconstituted cells produced by such procedures were cloned in medium containing both HAT and CAP. Some of the reconstituted cells survived, because they were resistant to both drugs, while the nuclear and cytoplasmic whole cell contaminants were killed by one or the other of the two drugs. The results of these experiments indicate that reconstituted cells that are derived from two different cell lines are viable, as indicated by their ability for long-term proliferation in culture. Most of the clones derived resembled morphologically the 3T3 nuclear donor parent cells, but some of the clones did not resemble either parental cell line. It is anticipated that such selection techniques will permit more complete analysis of interrelationships between nucleus and cytoplasm.**

Cytochalasin B can be used to separate animal cells growing in monolayer culture into nuclear [karyoplasts (1, 2) and minicells (3)] and cytoplasmic [cytoplasts (1, 2)] components. As previously reported (2), these cell fragments die within 48–72 hr after separation, but in the past 2 years, workers in several laboratories have reported reconstitution and subsequent viability of mammalian cells by fusing karyoplasts from a specific cell line to cytoplasts from the same cell line (4–6) (homospecific) or to other cell lines (7, 8) (heterospecific). These studies indicate that such reconstitution experiments are feasible, but convincing evidence that such reconstituted cells after several mitotic divisions are not whole cell contaminants that detached during enucleation or that failed to enucleate during the centrifugation is still lacking. The main reason such reconstituted cells have not been satisfactorily identified, as opposed to whole cell contaminants, is due primarily to the lack of both nuclear and cytoplasmic markers that would permit the identification of reconstituted cells, at a time when the properties of such cells may have been modified by the influence of a new combination of nucleus and cytoplasm.

In this report, evidence is presented for the selection of viable reconstituted cells between karyoplasts, derived from nontumorigenic mouse Balb/3T3 cells, fused to chloramphenicol-resistant (CAP^r) cytoplasts, derived from the mouse mammary tumor cell line A-MT-BU-A1. The methods presented may be generally applicable to a wide range of investigations on the functional relationships of the nucleus and cytoplasm.

Abbreviations: HAT, hypoxanthine/aminopterin/thymidine; CAP, chloramphenicol; PEG/Me_2SO, polyethylene glycol/dimethyl sulfoxide.

MATERIALS AND METHODS

Cell Cultures. Murine Balb/3T3 clone A31 cells derived from Balb/c mouse embryos were grown in Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum (Flow Labs, Inc., Rockville, MD). The Balb/3T3 cells are contact-inhibited and nontumorigenic, and the modal chromosome number is 44. Murine A-MT-BU-A1 cells, a mammary tumor cell line obtained from Harry Malech and Nelson Wivel (National Cancer Institute, Laboratory of Cell Biology), were also grown in Dulbecco's modified Eagle's medium supplemented with glucose (3.5 mg/ml), 10% fetal calf serum, and 50 μg of chloramphenicol (CAP) per ml. The A-MT-BU-A1 cells were derived from MT-29240, an uncloned, epithelial, highly contact-inhibited line containing intracisternal A particles and adapted to tissue culture from a transplantable mammary tumor that originally arose spontaneously in a female Balb/c mouse. The modal chromosome number is 59, and these cells contain a metacentric marker chromosome (9) that allows them to be distinguished from the 3T3 cells. Cells to be enucleated were plated in Falcon 3013, 25-cm^2 flasks (Falcon Plastics, Oxnard, CA) as described (10).

Procedures. The 3T3 cells that served as nuclear donors were grown in medium containing approximately 10^6 large latex spheres per ml (1.0 μm diameter) (Duke Standards, Palo Alto, CA). Each cell took in by phagocytosis approximately 10 spheres, as previously described (4). During the enucleation, almost all of the spheres remained with the cytoplasts and were used as morphological markers for whole 3T3 cells that detached during enucleation. In some experiments, 0.8 μm fluorescent (fluorescein isothiocyanate) latex spheres were used so as to permit quick identification of cybrid contaminants (i.e., whole 3T3 cells fused to A-MT-BU-A1 cytoplasts). The A-MT-BU-A1 cells that served as cytoplasmic donors were not prelabeled but contained useful cytoplasmic and nuclear genetic markers. The cytoplasmic gene marker is a resistance (i.e., the cells survive) to medium containing high levels (50 μg/ml) of chloramphenicol (CAP) (9–12). The nuclear gene marker is a sensitivity (i.e., the cells die) to medium containing hypoxanthine/aminopterin/thymidine (HAT). In these experiments, 10^{-4} M hypoxanthine, 10^{-5} M aminopterin, and 4×10^{-5} M thymidine were used (glycine is already supplied in Dulbecco's minimal essential medium). When reconstituted cells were tested for viability in selective medium containing both HAT and CAP, only donor calf serum was used so as to avoid the possible problems associated with fetal calf serum containing nucleotides.

Fusion Procedures. The 3T3 cells were first enucleated by cytochalasin B/centrifugation procedures (2, 10, 13, 14), and the karyoplasts and contaminants were then subjected to a 2-hr

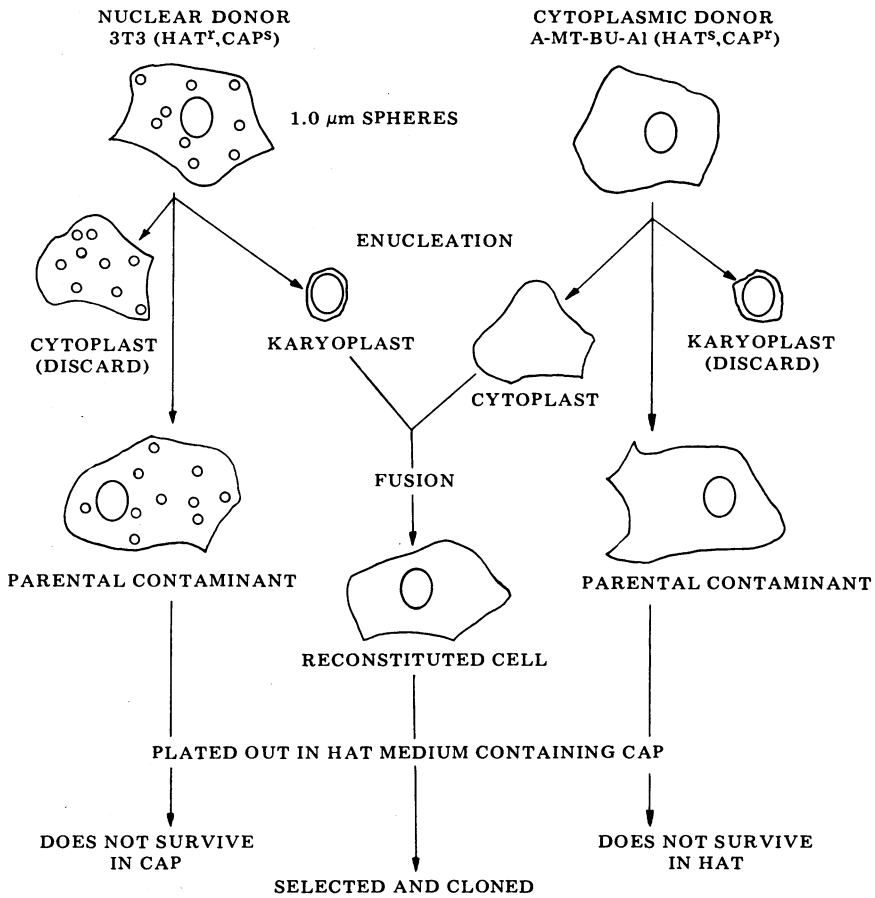

FIG. 1. Schematic representation of the experimental design. HAT-resistant (HAT^r) karyoplasts were derived from 3T3 cells labeled with 1.0 μm diameter latex spheres and fused to CAP-resistant (CAP^r) cytoplasts derived from A-MT-BU-A1 cells. After fusion, mononucleated cells not containing latex spheres were cloned in medium containing both HAT and CAP. The whole cell contaminants from the 3T3 cells die in the presence of CAP, while the whole cell contaminants from the A-MT-BU-A1 cells die in the presence of HAT. Fusions between whole 3T3 cells and A-MT-BU-A1 cytoplasts were not studied because of the presence of large latex spheres. Fusion between A-MT-BU-A1 whole cells and 3T3 karyoplasts were not studied because they were not mononuclear. Some mononucleated cells survived in the selective medium and after karyotyping were considered reconstituted cells.

differential adhesion step. This procedure, originally designed to separate fibroblasts from myoblasts in primary muscle cultures, also purifies the karyoplasts from whole cells and cytoplasmic contaminants, as suggested by Lucas and Kates (7). The karyoplasts do not appear to attach and spread as well as whole cells or cytoplasts. Therefore, by lightly tapping the flask after 2 hr, a highly purified karyoplast population (usually less than 1% whole cell contaminants) was obtained. During the 2-hr differential adhesion procedure, the A-MT-BU-A1 cells were enucleated and the cytoplasts were trypsinized, washed, and then kept on ice until the completion of the differential adhesion step. In some experiments, an additional purification procedure was used for the cytoplasts, which involved layering the cytoplasts and whole cell contaminants on a step Ficoll gradient similar to the procedure described by Wigler and Weinstein (15). The purified cytoplasts and karyoplasts were then mixed and fused by a modification of the polyethylene glycol (PEG)/dimethyl sulfoxide (Me_2SO) technique previously described (16, 17). The basic procedure was to take 8.5 ml of minimal essential medium and 1.5 ml of Me_2SO (Sigma Chemical Co., St. Louis, MO) which were then sterilized by filtration. Nine grams of PEG 6000 (J. T. Baker Chemical Co., Phillipsburg, NJ) were then sterilized by autoclaving and mixed with the minimal essential medium/Me_2SO. Precisely 1 ml of this PEG/Me_2SO solution was then added to the karyoplast/cytoplast pellet for 1 min and then 9 ml of minimal essential medium was added. The suspension was quickly centrifuged, and the pellet was then washed several times to ensure complete removal of the PEG/Me_2SO. Such experiments resulted in approximately a 40% fusion index (the number of nuclei in polykaryons divided by the total number of nuclei with a correction for control cultures).

Selection of Reconstituted Cells. Immediately after PEG/Me_2SO fusion, the cells were placed on small glass fragments in several 60-mm petri dishes. Approximately 30 min later, only the individual fragments containing single cells were placed in multiwell tissue culture plates. Only mononucleated cells not containing latex beads were considered possible reconstituted cells and subsequently studied. These cells were then grown in medium containing both HAT and CAP so that only reconstituted cells would survive.

Plan of Experiment. The plan for the entire experiment is shown in Fig. 1. The 3T3 cytoplasts and 3T3 whole cells contain large latex spheres (1.0 μm diameter), while the 3T3 karyoplasts usually contained no, or occasionally one or two, latex spheres. The 3T3 cells, as described, were HAT^r and CAP^s, while the A-MT-BU-A1 cells were HAT^s and CAP^r. The 3T3 karyoplasts and A-MT-BU-A1 cytoplasts were fused using PEG/Me_2SO and placed in medium containing both HAT and CAP. (See legend for Fig. 1.) The potential whole 3T3 cell revertants were eliminated from the start by the presence of latex spheres, while the potential whole cell revertants from the A-MT-BU-A1 cells

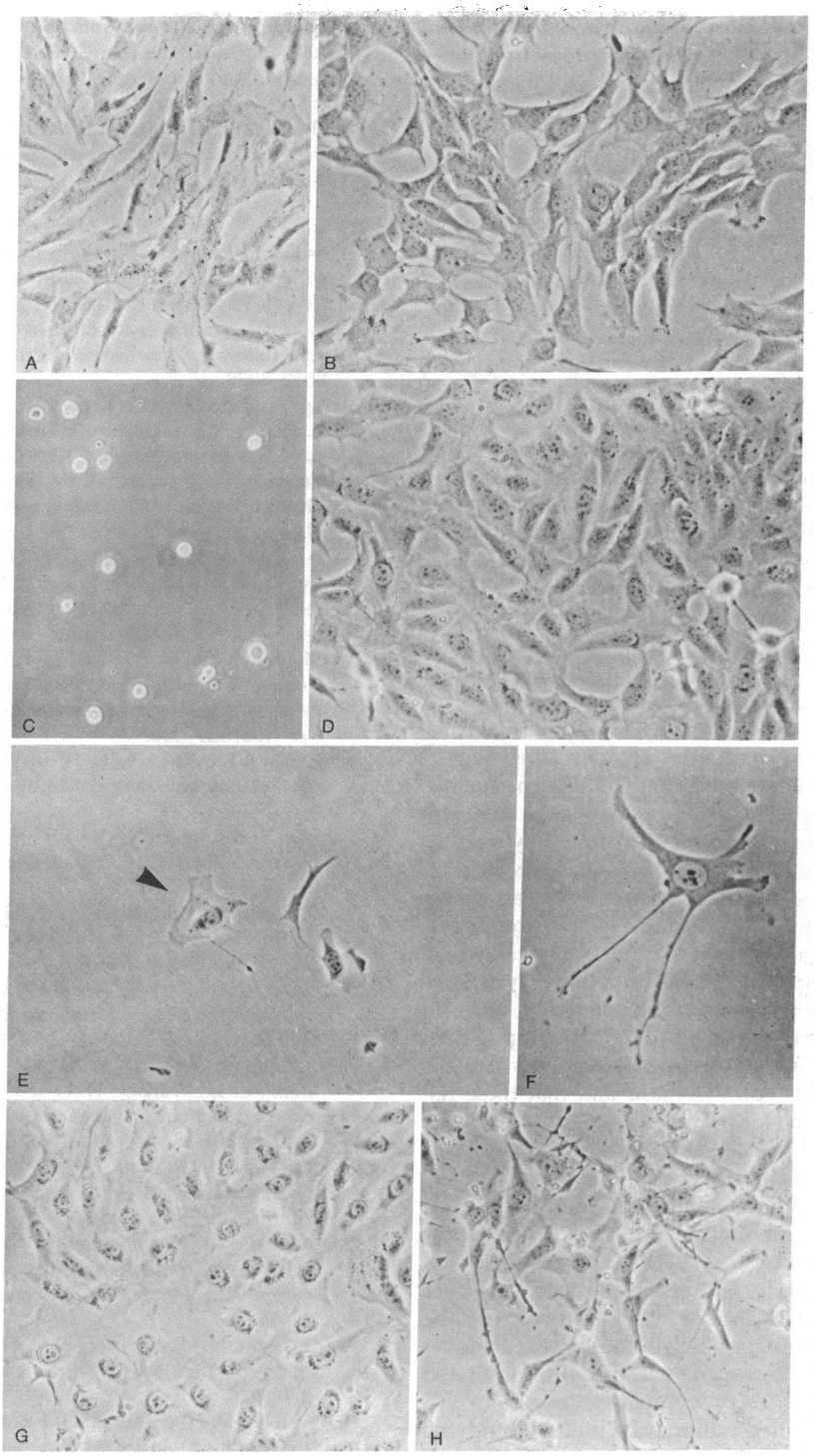

FIG. 2. Phase contrast photomicrographs of parental cells, cell components, and products of the fusion reaction. (*A*) Cytoplast derived from A-MT-BU-A1 cells (*B*); (*C*) karyoplasts derived from 3T3 cells (*D*); (*E*) 3T3 cell containing latex spheres (arrow), a cytoplast, and a cell without latex spheres; (*F*) reconstituted cell; (*G*) population of cells resembling 3T3 parents that were derived from a reconstituted cell; (*H*) population of cells that do not resemble the 3T3 parents but that were derived from a single reconstituted cell.

were identified by a different karyotype from the reconstituted cells containing a 3T3 karyotype. Thus, these drug-resistant markers, in combination with karyotyping and the use of latex spheres as cytoplasmic markers, allow clean isolation of viable reconstituted cells.

RESULTS AND DISCUSSION

The cytoplasts (Fig. 2*A*) derived from A-MT-BU-A1 cells (Fig. 2*B*) and karyoplasts (Fig. 2*C*) derived from 3T3 cells (Fig. 2*D*) were mixed together without PEG/Me_2SO to determine the amount of whole cell parental contaminations and the amount of spontaneous fusion. The A-MT-BU-A1 cells enucleated very easily, and only preparations containing less than 1% whole cell contaminants were used (the flasks could be centrifuged a second or third time to ensure this low percentage of whole cell contaminants, or a Ficoll gradient purification procedure could be used). Approximately 6–8% whole cell contaminants detached during the 3T3 karyoplast preparation. These whole cell

contaminants plus karyoplasts were then subjected to a 2-hr differential adhesion procedure (7). During this procedure most whole 3T3 cells, containing 1.0 μm diameter latex spheres, attached and spread out while the spherical or ellipsoidal karyoplasts, containing no or occasionally one or two spheres, did not spread. The weakly attached karyoplasts were then removed from the flasks and fusion experiments were initiated. In these experiments, the karyoplast preparation was never contaminated by more than 1% whole cells. It is important to note, however, that these whole cell contaminants almost always die in CAP; but to eliminate possible revertants, these cells also contained latex spheres (Fig. 2*E*, arrow) so that they were eliminated from consideration immediately after fusion procedures.

In addition to karyoplasts and cytoplasts that did not fuse during the PEG/Me_2SO procedure, 85% mononucleated cells, 11% binucleated cells, and 4% multinucleated cells were observed in a typical experiment. The overall fusion index was approximately 40%. From the standpoint of the experimental objective, the most important type of cell contained a single nucleus and a cytoplasm without latex spheres (Fig. 2*F*). These cells were numerous, but after 2 weeks of culture in HAT medium containing CAP, only a small percentage subsequently divided to produce viable clones (Fig. 2*H*). That these clones were derived from single cells without latex spheres, contained a 3T3 karyotype, and survived in selective medium was convincing evidence that these were indeed reconstituted cells.

The most important observation in these initial experiments was the survival and long-term proliferation of cells reconstituted between 3T3 karyoplasts and A-MT-BU-A1 cytoplasts. Most of the clones obtained, resembled morphologically contact-inhibited Balb/3T3 cells (Fig. 2*G*). However, a few clones (Fig. 2*H*) morphologically did not resemble either parental cell line. The reasons for this are unclear, but it is conceivable that the cytoplasm carries information for cell form that may express itself independently of nuclear stored information.

Other researchers have presented data that imply that homospecific and heterospecific reconstituted cells can be prepared from karyoplasts and cytoplasts and that these reconstituted cells are viable (4–8). These studies, however, did not use a cytoplasmically inherited gene marker and, thus, one is always aware of the possibilities of studying whole cell parental contaminants instead of reconstituted cells. Bunn *et al.* (11), Wallace *et al.* (12), and Malech and Wivel (9) have presented convincing evidence that CAP^r cytoplasts can be transferred to other whole cells, but these investigators have not reported fusing CAP^r cytoplasts to other karyoplasts. The present work, therefore, combined both of the above techniques into one that permitted the fusion and selection of reconstituted cells from A-MT-BU-A1 CAP^r cytoplasts and Balb/3T3 karyoplasts. Such reconstituted cells may be useful in studying such interesting phenomena as gene regulation, cell differentiation, nucleocytoplasmic interactions, virus replication, and cytoplasmic inheritance.

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