Comparison of the Coverslip and the Discontinuous Percoll Density Gradient Methods of Enucleation of Mouse Cells

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Abstract. Two methods of enucleation of LB 10 cells, a subline of mouse L cells, were used: the method of enucleation of cells growing in monolayers, and the newly improved method of enucleation in discontinuous Percoll gradients. The second method was more effective and, as shown by incorporation of ^{3}H-lysine, protein synthesis in cytoplasts was prolonged twice when compared with that in cytoplasts obtained by the coverslip method.

Key words: Enucleation — Cytoplasts — Density gradient centrifugation — Protein synthesis

Introduction

In recent years enucleated cells (cytoplasts) have become widely used for virological and physiological studies in experimental cell biology. After fusion of such cell fragments with complete cells or other cell fragments (e. g. karyoplasts), enucleated cells have been used to study cytoplasmic inheritance, affection of nuclear gene expression by cytoplasmic genes, etc. (for a review, see Ringertz and Savage 1976). A method of enucleation was described by Prescot et al. (1972). This method is based on the centrifugation of cells which adhere to glass, plastic or other surfaces in the presence of cytochalasin B. The relatively heavy nuclei are removed from the cytochalasin-treated cells by centrifugal force with subsequent separation of cytoplasmic and nuclear components. The advantage of this method is its simplicity and reliability. However, by this method it is extremely difficult to enucleate poorly adhering cells, or those of suspension cultures. In addition, the yield of cytoplasts obtained by the method of enucleation in monolayers is limited by the relatively small total surface area of coverslips in one centrifugation run. Wigler and Weinstein (1975) introduced therefore a method of cell enucleation based on Ficoll density gradient centrifugation in the presence of cytochalasin B.

For enucleation of cells in suspensions continuous Percoll gradients were also

used (Bossart et al. 1975; Schaap et al. 1981), but further purification of resulting cytoplasts was necessary. On the other hand, discontinuous Percoll gradients used in the experimente presented here, give relatively pure fractions of cell fragments and enable good spatial separation of cytoplasts and karyoplasts on the basis of their different densities. This improved method of cell enucleation was compared with that of classical coverslip method and the cytoplasts were characterized.

Materials and Methods

LB 10 mouse cells, a subline derived from mouse L fibroblasts were used. The cells were cultured in minimal Eagle's medium supplemented with 10 % calf serum (MEM) and harvested for experimental use using trypsin (DIFCO) (0.25 % in phosphate buffered saline solution). Two methods of enucleation were employed (methods A and B).

Method A of cell enucleation was the slightly modified monolayer method of Prescott et al. (1972). Briefly, round coverslips (21 mm in diameter), containing a monolayer of LB cells confluent to 80 %, were put cell-side-down into plastic centrifugal test tubes containing phosphate buffered saline solution supplemented with 10 % calf serum and 10 μg cytochalasin B (Calbiochem Behring Corp.) per ml. The suspension was centrifuged for 50 minutes at 23.000 $\times$ g in a fixed-angle rotor prewarmed to 37 °C. Coverslips with the attached cytoplasts were then removed, washed twice in MEM without serum, placed in plastic Petri dishes in MEM, and cultured at 37 °C to allow recovery of cytoplasts.

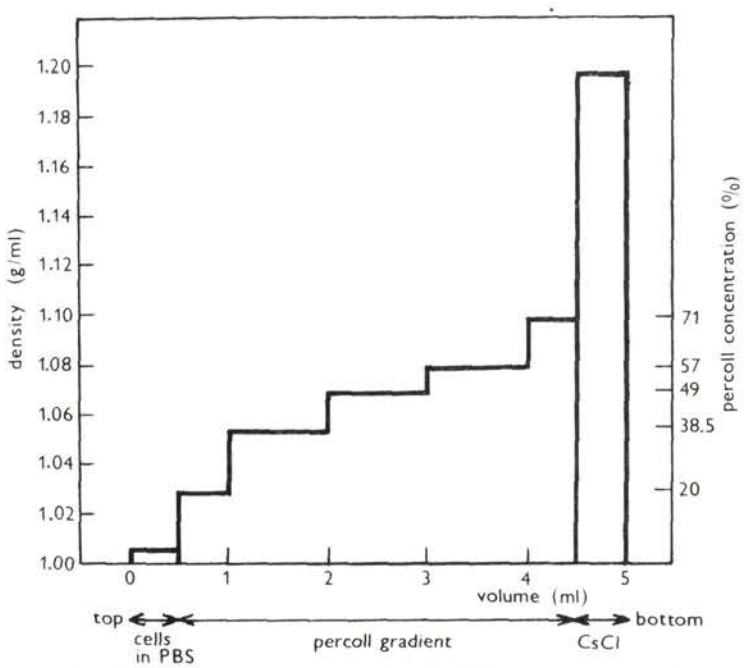

Fig. 1. Density profile of a discontinuous gradient of Percoll. LB 10 cells resuspended in 0.5 ml of PBS were layered on the top of the gradient. To prevent formation of Percoll aggregate sediment, 0.5 ml of CsCl solution was added the bottom of the tube.

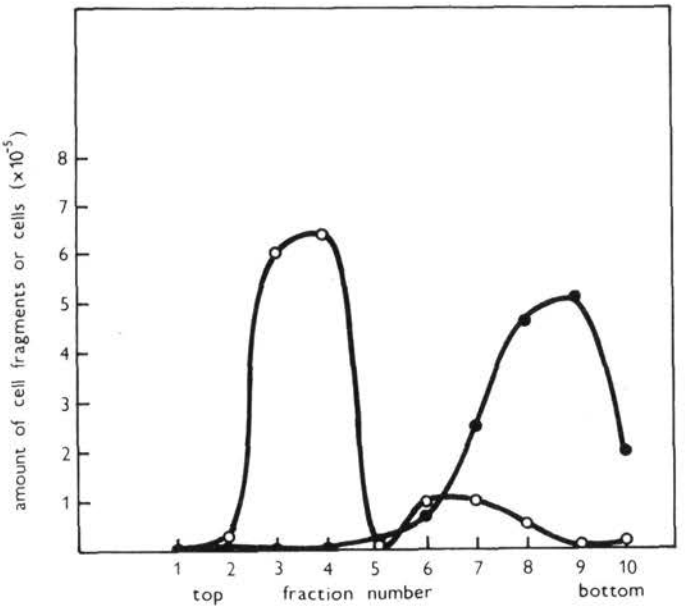

Fig. 2. Enucleation of LB 10 cells by Percoll gradient centrifugation. Abscissa: fractions (0.5 ml); ordinate: amount of cell entities. (○—○) cytoplasts, (●—●) karyoplasts and intact cells.

Method B of enucleation was based on the Wigler and Weinstein (1975) Ficoll density gradient centrifugation method with Percoll used as a separation medium. Percoll (Pharmacia Fine Chemicals) was diluted with a 10 times concentrated physiological saline solution (1.5 mol.l^{-1} NaCl) 9:1 (v:v) to obtain a solution with a normal osmolarity and a density of 1.14 g/ml. This stock solution was diluted again with normal physiological NaCl solution (0.15 mol . l^{-1}) to obtain following densities of the Percoll solution: 1.03; 1.055; 1.07; 1.08; and 1.10 g/ml. To these dilutions cytochalasin B was added to a final concentration of 10 μg/ml. The solutions were layered into cellulose nitrate tubes (5 ml volume) on 0.5 ml of CsCl solution (density 1.2 g/ml) according to Fig. 1. The gradients were incubated for 16 to 18 hours at 37 °C. LB 10 cells, obtained by trypsinization of logarithmically growing cultures and resuspended in 0.5 ml of phosphate buffered saline ($1 - 3 \times 10^6$ cells per test tube) were layered on the top of the gradients with subsequent centrifugation for 40 minutes at $35{,}000 \times g$. Fractions (0.5 ml) were then tested for the amount of cytoplasts and karyoplasts, or whole cells, respectively. To obtain pure cytoplasts, the respective band with cytoplasts was sucked up using an injection syringe; the cytoplasts were diluted in MEM, low-speed centrifuged and seeded onto glass coverslips in Petri dishes.

Protein synthesis in cells enucleated by the method A and B, respectively, was determined after cell recovery in MEM over 2 hours. During this 2-hour period, attachment of cytoplasts obtained by method B in the presence of 10 % of fetal calf serum in the medium occurred. The cytoplasts were labelled with ^{3}H-lysine (Radiochemical Centre Amersham, 3.7×10^5 Bq) for 2 hours. Intact LB 10 cells served as controls. Cells and cytoplasts were labelled at 2-hour intervals, fixed and coated with Ilford K2 nuclear emulsion (exposure 1 month).

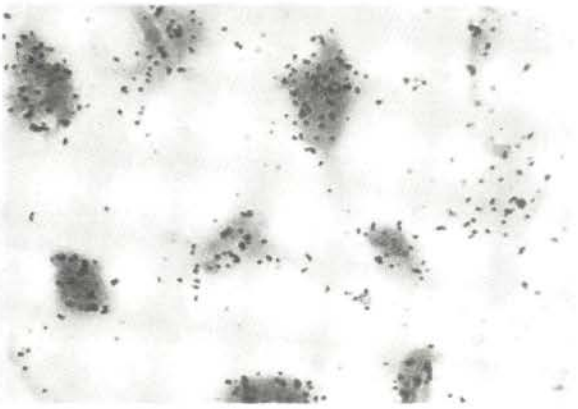

Fig. 3. Autoradiography of cytoplasts obtained by the method B of enucleation after 2 hours of recovery and subsequent labelling with ^{3}H-lysine for 2 hours.

Results

Method A. The yield of cytoplasts obtained by the method A is limited by the size of the area of the glass coverslips used. Slips having a diameter of 21 mm with approximately 2.5 to 6.6×10^4 cells attached give 1 to 3×10^4 cytoplasts. The amount of cytoplasts depends on the monolayer density: a density of about 2.5 to 6.5×10^4 cells per slip yields 60 % of input cells, whereas a total monolayer (8×10^4 cells per slip) yields only 30—40 %. The physiological condition of the cells affects the enucleation efficiency, and the presence of serum in the enucleation medium is crucial. Intact cell contamination of cytoplasts obtained using method A of LB 10 cell enucleation was 1 to 3 %.

Method B. A total of 5×10^6 cells were layered on three gradients of Percoll in one centrifugation procedure. Sixty five per cent of these cells were yielded after centrifugation. Cell densities exceeding 4×10^6/ml of PBS resulted in formation of cell clumps and in a decrease in the enucleation efficiency. For this reason (appearance of cell clups as a result of increasing cell densities at sharp Percoll interfaces) layered gradients had to be incubated at 37 °C prior to enucleation. The cytoplast content in the fractions and their contingent contamination by intact cells, karyoplasts and cell debris are shown in Fig. 2. The cytoplasts-richest fraction was band 1.055 g/ml after centrifugation. In this fraction, intact cells and karyoplasts were only rarely observed (less that 1 %).

Proteosynthesis was monitored in cytoplasts obtained by both methods, and in control LB 10 cells, using autoradiography after ^{3}H-lysine incorporation (Fig. 3). Results are summarized in Figs. 4 and 5. Cytoplasts obtained by the method A were capable of protein synthesis at least 6 hours after the enucleation procedure

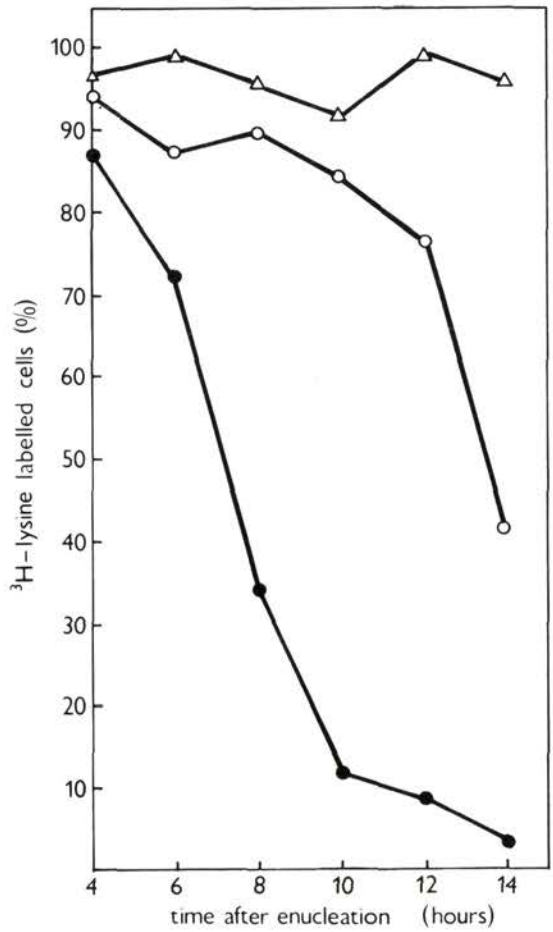

Fig. 4. Incorporation of ^{3}H-lysine into cytoplasts and control cells. (△—△) intact LB 10 cells, (●—●) cytoplasts obtained by the method A, (○—○) those obtained by the method B. Each point represents the result of evaluation of 500 cells or cytoplasts.

(73 % of cytoplasts); at other time intervals, the frequency of labelled cytoplasts decreased (Fig. 4), as did the intensity of the labelling (Fig. 5). Of the cytoplasts of LB 10 cells obtained by the method B, 85 % and 77 % were labelled after 10 and 12 hours of cultivation, respectively. In this case (method B-cytoplasts), no decrease in the intensity of labelling was observed in the course of the 10 hours (Fig. 5).

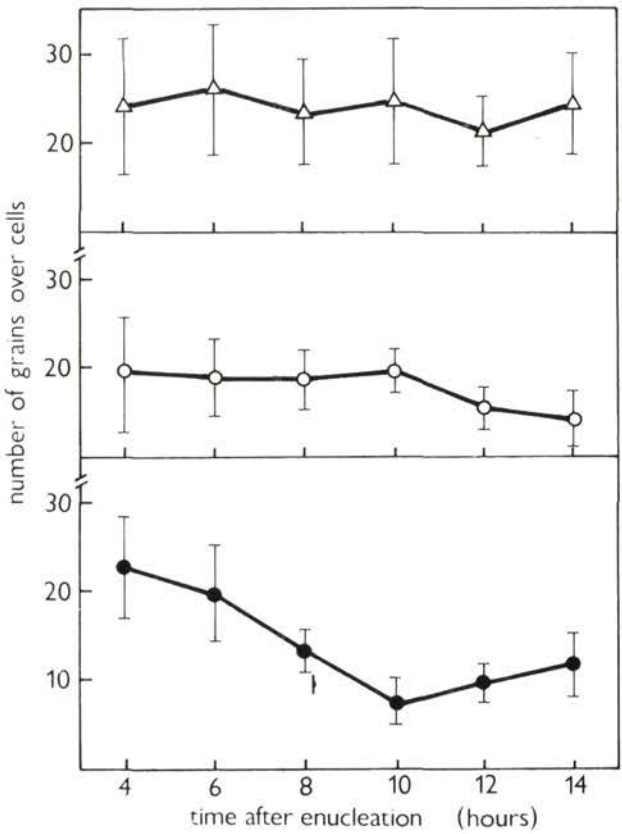

Fig. 5. Intensity of ^{3}H-lysine labelling of cytoplasts and control cells. (△—△) intact control cells, (●—●) cytoplasts obtained by the method A, (○—○) those obtained by the method B. Each point represents the mean grain count over a cell calculated from 40 mesurements. Standard deviations of the mean are indicated.

Discussion

The method (B) of mouse somatic cell enucleation using centrifugation in discontinuous gradients of Percoll in the presence of cytochalasin B is based on the method of Wigler and Weinstein (1975), who used discontinuous gradients of Ficoll, and on that of Bossart et al. (1975), who employed self-generating gradients of Percoll. Mixing HEP-2 human cells with Percoll solutions and subsequent centrifugation yielded cytoplasts in gradients formed in situ, though purification by further centrifugation was necessary (Bosart et al. 1975). Another design of the experiment was proposed by Schaap et al. (1981) who used gradients of Percoll. Resuspended cells are layered in a small volume of Percoll on the top of a 40 %

gradient solution. After centrifugation, a mixture of intact cells, cytoplasts and karyoplasts appears at the bottom of the test tube, their separation being possible using a cell sorter.

In experiments presented here Percoll discontinuous gradients were used. Wigler and Weinstein (1975) found only 0.2 % of intact cells in the cytoplast fraction, 50 to 70 % of the input cells being yielded. The effectiveness of the method B used in our experiments (65 %) is comparable to that of Wigler and Weinstein, the purity of the cytoplast-fraction being 100 %, since intact cells occurred only very rarely in the cytoplast population.

Detachment of cells by means of trypsin resulted in a decreased viability of the cytoplasts obtained by Wigler and Weinstein (1975), while a single cell suspension obtained by trypsinization of monolayer cultures (Bossart et al. 1975) did not tend to form cell clups; moreover, the yield of cytoplasts was higher than that with cells obtained from a suspension culture. No effect of trypsin on cell viability was observed in our experiments, in agreement with the results of Bossart et al. (1975).

Preincubation of gradients at 37 °C prior to centrifugation was essential to prevent formation of cell clumps at the sharp interfaces of the different densities of Percoll. Instantaneous use of Percoll gradients, in which partial diffusion of neighbouring layers does not take place, resulted in a decreased effectiveness of enucleation.

Intensity and duration of protein synthesis in enucleated cells are dependent on the conditions of enucleation, cell lines used, and other factors (Goldmann and Pollack 1974; Poste 1972). The prolongation of the ^{3}H-lysine incorporation interval to 10 hours in cytoplasts obtained by Percoll gradient centrifugation as compared to 4—6 hours in cytoplasts obtained by the coverslip method (Široký and Spurná 1982) is probably due to a better physiological condition of cytoplasts obtained by the former method. The ability of cytoplast attachment after seeding was substantially better in Percoll-enucleated cells.

The advantage of the use of discontinuous gradients of Percoll is the possibility of piling up any densities along a gradient, whose range may extend over that in Percoll gradients formed in situ. In contrast to other authors using Percoll (Bossart et al. 1975; Schaap et al. 1981) no contamination with complete cells or cell debris was found in the cytoplast fraction. In addition, contrary to Wigler and Weinstein (1975), no trypsin-induced decrease in cytoplast viability was observed. Compared to the classical coverslip method (Prescott et al. 1972), it is possible to obtain substantially more cytoplasts (and karyoplasts) in one centrifigation procedure; the fractions obtained contain neither intact cells nor cell debris, and it is possible to enucleate cells of suspension cultures as well as cells poorly adhering to a glass surface.

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