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LIPOSOME-CELL INTERACTIONS

A RAPID ASSAY FOR CELLS IN SUSPENSION CULTURE

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Summary

A method has been developed for the rapid separation of cells in suspension from non-cell associated lipid vesicles in various assays for vesicle-cell interaction. Separation is achieved on a discontinuous Ficoll-Paque gradient. Cells and free vesicles are totally separated, as evidenced by both radiolabelled vesicles, and vesicles containing the fluorescent dye 6-carboxyfluorescein. The main advantages of this method are the rapidity, efficacy, and gentleness of the separation. Viability of the cells remains consistently high (greater than 96%) throughout the separation. Since this method involves a one-step centrifugation, it precludes the necessity for repeated washings of cells which have been incubated with lipid vesicles.

Introduction

Recent studies [1—7] have shown that cultured mammalian cells are able to incorporate artificial phospholipid vesicles (liposomes) without significant cytotoxicity. There is significant interest in the mechanisms of liposome-cell inter action [10,11,13,17—19] and since a variety of water-soluble compounds can be entrapped within liposomes [3—8], there is also widespread interest in lipo-

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somes as 'carriers' to enhance the incorporation of enzymes, drugs, steroids, vitamins, etc. into cells [9,12,14—16].

Assays for the interaction of liposomes with fibroblastic cells present no technical difficulty, since the incubation media and washing solutions are decanted directly from the monolayers. The most commonly used method for the separation of liposomes from cells in suspension, however, involves repeated washings by centrifugation and resuspension of the cells. Such techniques impose time constraints on the frequency of sampling and the processing of multiple samples. In addition, glassware must be siliconized to minimize the damage to, and loss of, the cells during the repeated centrifugations.

Our method for the separation of liposomes from cells in suspension culture provides a rapid and gentle technique which overcomes many of the problems in the repeated centrifugation of cells to remove non-cell associated vesicles.

Materials and Methods

Murine leukemia (L-1210) cells were maintained in spinner culture with RPMI 1640 medium containing 10% fetal calf serum and extra glutamine (2 mM). The cells were harvested from log-phase growth and were washed three times by gentle ($500 \times g$, 10 min) centrifugation and resuspension in Ca^{2+}-Mg^{2+} free Hanks buffer, pH 7.4. After the final washing, the cells were adjusted to $5 \cdot 10^6$/ml and were held at 37°C for a maximum of 15 min. Viability was monitored by trypan blue exclusion.

Unlabelled egg phosphatidylcholine in ethanol was obtained from Avanti Biochemicals, Birmingham, AL; choline-methyl-[^{14}C]phosphatidylcholine in ethanol/toluene (1 : 1) (spec. act., 50 Ci/mol) was obtained from New England Nuclear, Boston, MA; 6-carboxyfluorescein was purchased from Eastman Chemicals, Rochester, NY.

10 mg phosphatidylcholine in ethanol were rotoevaporated to dryness and lyophilized overnight. The dried lipid was rehydrated in 1 ml of the above mentioned buffer, allowed to swell at room temperature for 20 min and sonicated in a bath-type sonicator (Branson Model B-12) for 35 min. The sonicated suspension was spun at 10 000 rev./min ($8500 \times g$) for 60 min in a Beckman J-21 B centrifuge, and the supernatant was retained.

Radiolabelled liposomes were prepared by adding choline-methyl-[^{14}C]-phosphatidylcholine ($5.0 \cdot 10^5$—$1 \cdot 10^6$ cpm) to the unlabelled phosphatidylcholine before rotoevaporation.

The fluorescent dye 6-carboxyfluorescein was entrapped in liposomes by sonicating the lipid in the buffer containing 200 mM 6-carboxyfluorescein,

In all instances, the supernatant from the centrifugation was passed over a 1×40 cm Sephadex G-50 column equilibrated with the buffer. The void volume (containing small unilamellar liposomes) was retained.

Ficoll-Paque was purchased from Pharmacia, Inc., Piscataway, NJ, as a sterile 5.7% (w/v) solution. For clarity, the dilutions mentioned hereafter refer to v/v dilutions of the original 5.7% Ficoll-Paque solution with the buffer.

All gradients were prepared in 5 ml cellulose nitrate centrifuge tubes (No. 305050, Beckman Instruments, Palo Alto, CA). Gradients of various dilutions of Ficoll-Paque (70, 60, 50, 35 and 30%) were prepared, and each tube received

2.5 ml of one of the gradients. A 1 ml cushion of undiluted Ficoll-Paque was underlaid by placing a capillary pipette through the gradient to the bottom of the tube. The pipette was connected to a peristaltic pump, and the cushion was added at a flow rate of 0.3 ml/min.

Details on the quantitation of liposome-cell interactions, as well as time-course studies, will be presented elsewhere. For the purposes of this study, we wish to demonstrate the rapidity and the efficacy of the technique. Cells were therefore incubated with liposomes containing either ^{14}C-labelled phospholipid or quenched (200 mM) 6-carboxylfluorescein and were immediately centrifuged and fractionated.

Equal volumes of cells and liposomes (1 ml of each, to yield $2.5 \cdot 10^6$ cells and approx. 3.0 mg phospholipid) were mixed, and 0.75 ml of the mixture was gently layered on top of the Ficoll gradient. The gradients were spun at 1200 rev./min ($300 \times g$) for 1 min in a table-top centrifuge (IEC Model HNS) equipped with a swinging-bucket rotor. As controls, cells at the same density but without liposomes were also spun through gradients.

Immediately after centrifugation, the tubes were punctured and fractionated in a downard-flow direction, with 0.25 ml fractions being collected. Each fraction was examined for cells by counting in triplicate in a Neubauer hemacytometer. Radioactivity was determined by liquid-scintillation counting; for studies utilizing 6-carboxyfluorescein, each fraction was lysed by the addition of 1 ml of 10% Triton X-100 (Rohm and Haas, Philadelphia, PA) in the buffer. The relative fluorescence of each fraction was obtained on a Perkin-Elmer Model MPF-3 spectrofluorometer with λ_{ex} = 480 nm, λ_{em} = 520 nm, excitation and emission bandpass of 4 nm and a vertically polarized excitation beam.

Trypan blue exclusion was performed before and after the washing and resuspension of cells in the buffer; before and after incubation with liposomes and after centrifugation and fractionation. At no time did the viability fall below 96%. We have incidentally noted an artefactual increase in the viability of the cells after centrifugation (e.g., from 96% to 98—99%). This is apparently related to the properties of the gradient and the migration of dead cells through the gradient; cells which are dead, swollen and lysed remain at the top of the gradient, and those cells which take up trypan blue but appear intact and of the same size as viable cells migrate with the viable cells through the gradient. The centrifugation therefore removes the grossly aberrant dead cells from the general population.

Results

The distribution of cells shows a marked dependence on the concentration of the Ficoll-Paque which overlays the cushion (Fig. 1). In a 70% gradient, the cells do not band at the gradient-cushion interface (Fig. 1A), but as the concentration of the gradient is lowered a larger proportion of the cells accumulate at the interface (Figs. 1A, B). Maximal banding is observed on 30—35% gradients, in which 80—90% of the cells are recovered in fractions 4—6 (Figs. 1B, 2, 3). This area corresponds to the cushion/gradient interface.

Although cells will band on a 30% gradient, in the presence of liposomes there is an incomplete separation (Fig. 2A). We believe this phenomenon is due

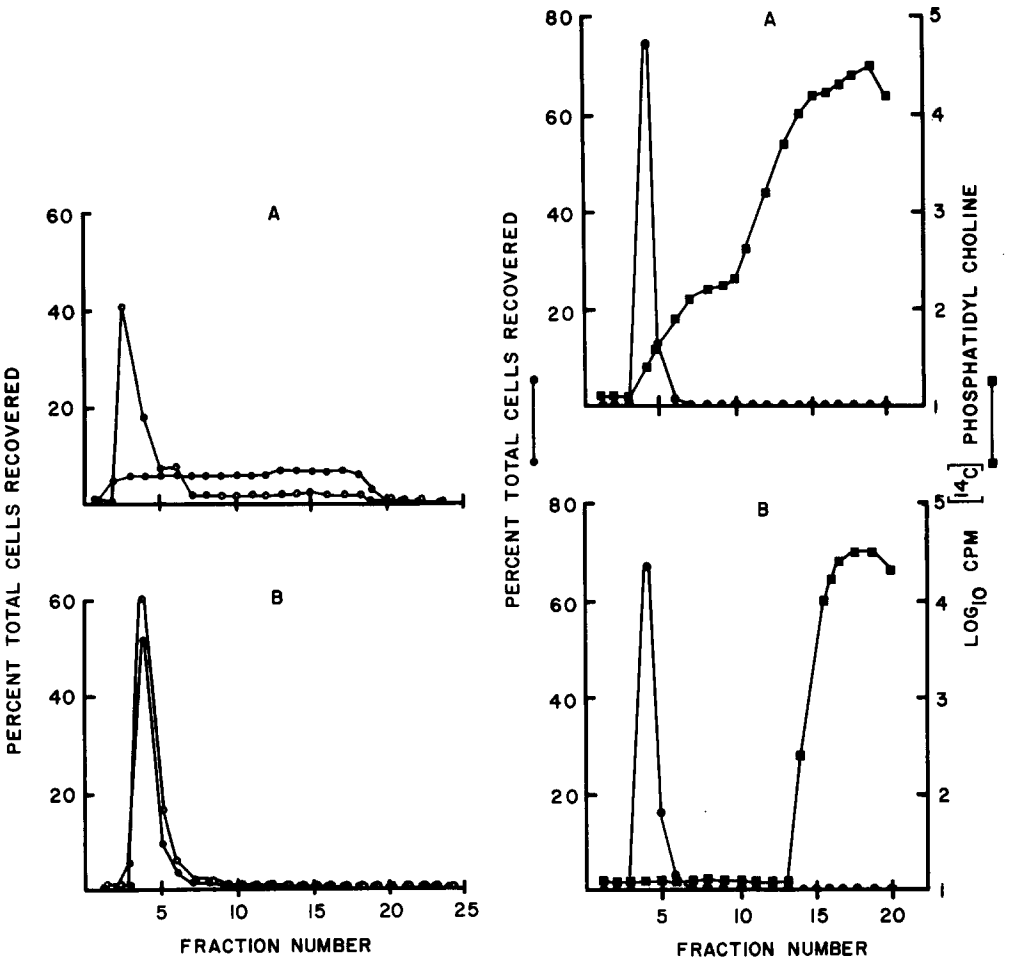

Fig. 1. Distribution of L-1210 cells on gradients of varying concentrations of Ficoll-Paque. All gradients contained a 1-ml cushion of undiluted Ficoll-Paque and a 3.5-ml overlay of the indicated concentrations. Washed cells ($5 \cdot 10^6$/ml) were diluted 1 : 1 with the buffer, and 0.75 ml of cells was layered over each gradient. Centrifugation and fractionation were as listed in Materials and Methods. (A): ●———●, 70%; ○———○, 60% Ficoll-Paque. (B): ●———●, 50%; ○———○, 35% Ficoll-Paque.

Fig. 2. Distribution of L-1210 cells (●———●) and choline-methyl [^{14}C]phosphatidylcholine labelled liposomes (■———■) on Ficoll-Paque gradients. Preparation of gradients, centrifugation and fractionation were as listed in Materials and Methods. Separation on (A) 30% and (B) 35% Ficoll-Paque.

to turbulence as a result of the cells migrating through the gradient. The liposomes are apparently carried through the gradient behind the cells. The problem is easily rectified by increasing the gradient to 35% (Fig. 2B).

There are minor variations among gradients, but parallel experiments demonstrate equivalent separations of cells from both [^{14}C]phosphatidylcholine liposomes and fluorescent liposomes containing 6-carboxyfluorescein (Compare Figs. 2B and 3); the cells are separated from free liposomes by at least seven fractions.

Our single-step centrifugation of liposome/cell suspensions has several

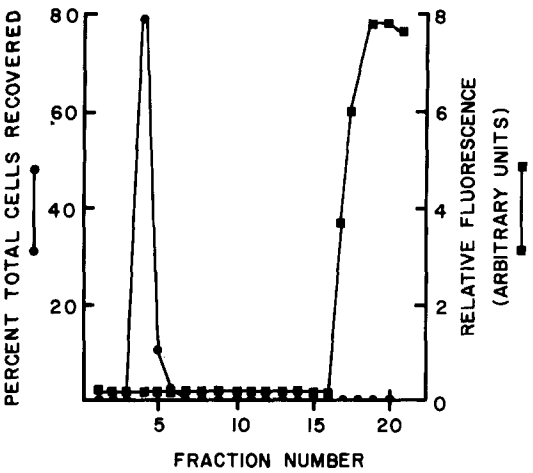

Fig. 3. Separation of L-1210 cells (●———●) and liposomes containing 200 nM 6-carboxyfluorescein (■———■) on a 35% Ficoll-Paque gradient. Preparation of gradients, centrifugation and fractionation were as listed in Materials and Methods. After they were examined for cells, all fractions were lysed with 1 ml of 10% Triton X-100 in the buffer and were assayed for relative fluorescence, as listed in Materials and Methods.

advantages over the more commonly used methods of repeated washings. It is much more rapid, requiring only the 60-s centrifugation. Since the walls of the cellulose nitrate tubes are non-wettable, there is no need to siliconize the tubes, which is normally required to minimize the damage to cells by shear forces in glass centrifuge tubes. If cells are incubated with liposomes containing 6-carboxyfluorescein, they may be taken directly from the gradient and examined via epifluorescence microscopy. There is no detectable background fluorescence to interfere with photomicrography. Also, the technique is quite gentle. We can detect no decrease in the viability of the cells at any point in the procedure.

Acknowledgements

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