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ISOLATION OF CELL PLASMA MEMBRANES ON MICROCARRIER CULTURE BEADS *

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A simple, efficient method for the purification of plasma membranes from cultured cells is presented. Membrane purification is effected by attachment of viable cells to commercially available microcarrier culture beads, followed by lysis of the cells, agitation on a vortex mixer and sonication. Optimal conditions for each step of the procedure are described. Enzyme markers from plasma membranes are purified 10–20-fold relative to whole cell homogenates while internal membrane markers are depleted 10–20-fold.

Introduction

Recently, a number of techniques have been introduced for the isolation of plasma membranes based upon the principle of adherence of whole cells to solid supports, followed by a shearing away of internal components. Most of these methods involve the interaction of the negatively charged cell surface [1] with a positively charged surface. In earlier procedures, glass [2] or polyacrylamide [3] beads were derivatized with poly(L-lysine) to place the positive charge on the beads. The derivitization of these beads is both time-consuming and expensive.

A more recent method made use of the commercially available cationic bead DEAE-Sephadex [4]. This procedure compared favorably with earlier methods, and had the advantage of being less costly and time-consuming. The problem of internal components sticking to these beads after lysis of the attached cells still existed. Neutralization of the bare regions of the beads has been accomplished by the use of polyacrylate [5].

In the present paper, commercially available polycationic beads specifically designed to carry cells are used as the solid support. The neutralization of the bare regions on the beads is accomplished merely by shifting the pH from that of the optimum for cell attachment (pH 5) to pH 8. Recoveries of plasma membrane markers and depletion of internal markers (both enzymatic and chemical) compare favorably with other similar methods.

Materials and Methods

Reagents. Biocarrier microcarrier culture beads (Lot 20622) were a gift of the Biorad Corporation. Cytodex-1 (positively charged Sephadex) was a

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Abbreviation: FMH, fat head minnow cells.

gift of the Pharmacia Corporation. All other materials were obtained from Sigma Chemical Co., St. Louis, MO. Beads were prepared for use as outlined by the manufacturers and stored in phosphate-buffered saline (pH 7.4) at 4°C with 1% antibiotic/antimycotic.

Cell culture. LM cells (American Type Culture Collection No. CCL1.2) were a gift of Dr. Richard Pagano, and were maintained in Earle's minimum essential medium supplemented with 5% fetal calf serum (Reheis) and 1% antibiotic/antimycotic. Fat Head Minnow cells (FHM) (American Type Culture Collection No. CCL42) were maintained in Leibowitz's L-15 medium supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic [6]. Teratocarcinoma-derived cell lines Nulli-SCCl, PYS-2, TerC and TerJ were maintained in minimum essential medium with 5% fetal calf serum and 1% antibiotic/antimycotic. F9 cells were grown in minimum essential medium with 10% fetal calf serum and 1% antibiotic/antimycotic. Cells were routinely harvested with a mixture of trypsin (2 mg/ml), collagenase (0.02 mg/ml), and 2.5% chick serum in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution.

Attachment of cells to beads. All attachment procedures were performed on ice and follow closely those described earlier [2–4]. Cells and beads were washed three times in attachment buffer consisting of 7 vol. 310 mM sucrose and 3 vol. of a 310 mosM solution of one of the following buffers at various pH values (ranges indicated in parenthesis): acetate (pH 3.0–5.6), citrate (pH 3.6–6.4), citrate-phosphate (pH 4.0–6.4), acetate-phosphate (pH 4.0–7.4), phosphate (pH 5.6–8.0), Tris-HCl (pH 7.0–9.0), carbonate-bicarbonate (pH 9.0–10.6) or glycine-NaOH (pH 8.6–11.0). Attachment of cells to beads was monitored by removing aliquots of the mixed beads and cells and counting unbound cells with a hemacytometer. The optimal ratio of cells to beads was determined to be that ratio which gave the highest fraction of bound cells, since large numbers of cultured cells are not always readily available (as opposed to erythrocytes). Viability was determined by the ability of the cells to exclude trypan blue (0.4% in phosphate-buffered saline, pH 7.4).

Washed cells and beads were mixed in a volume of 20 ml in a 50 ml conical vial and swirled occasionally for 15 min. Attachment was monitored as described above at various times.

Isolation of membranes on beads. Jacobson [5] has used polyanions to neutralize or block exposed surfaces of the beads so that internal components are less likely to bind upon lysis of the cells. In an effort to minimize this effect we have used a shift in the pH of the lysing medium. Low ionic strength solutions of HCl or NaOH were used to lyse cells, with pH values ranging from 5.0 to 11.0. Beads with isolated membranes were assayed for various markers (both internal and external).

The cells were lysed in cold hypotonic solution and washed twice in cold 10 mM Tris-HCl (pH 8.0). The beads were agitated on a vortex mixer for 10 s, washed twice and sonicated at 1.5 A in an MSE Ultrasonicator for 10 s and washed twice more.

Removal of membranes from beads. Membranes were removed from beads by treating the beads with a solution consisting of 0.2 M borate buffer and 0.2 M NaCl (pH 9.8) for 10 min. Beads were then assayed for membrane components and the supernatant concentrated over an Amicon PM 10 filter for analysis of recovery.

Enzymatic and chemical assays. Protein was measured by the method of Lowry et al. [7]. Phospholipid was determined by extracting lipid according to the modification of Makino et al. [8] of the method of Bligh and Dyer [9], and assaying phosphorous by the method of Rouser et al. [10]. DNA was assyed according to Burton [11] and cholesterol was determined by the method of Allain et al. [12].

Succinate dehydrogenase was used as a mitochondrial marker [13] and assayed by the method of Pennington [14]. Glucose-6-phosphatase was used as a marker for endoplasmic reticulum [15] and assayed according to DeDuve et al. [16]. Cytochrome *c* reductase was also used as a marker for endoplasmic reticulum [15] and was assayed by the method of Mahler [17]. Acid phosphatase was used as a lysosomal marker [18] and was assayed by the method of Hubscher and West [19].

Leucine aminopeptidase was used as a plasma membrane marker [20] and assayed according to Berger and Broida [21]. 5'-Nucleotidase was also used as a plasma membrane marker [22] and was assayed as described by Reis [23]. Ouabain-

sensitive ($Na^+ + K^+$)-ATPase was also used as a plasma membrane marker [24], was was assayed by the method of Fujita et al. [25]. For the ATPase, glucose-6-phosphatase, and 5′-nucleotidase assays phosphorous was determined by the method of Chen et al. [26]. In all cases, activities were controlled for nonspecific enzymatic activity and further controlled for possible interference due tot he presence of beads.

Though some enzyme markers were determined on two or three different batches of membranes a complete set of markers, together with DNA and phospholipid content, were only assayed for one fractionation of each cell type used, on each type of beads, total eight complete sets of assays.

Results

Attachment of cells to beads was complete after a 15-min incubation, and remained at a constant level after this time. Attachment kinetics were not affected by temperature shifts, and all subsequent experiments were performed with attachment taking place on ice. The optimum number ratio of cells to beads was judged to be 100:1. At ratios greater than this a significant fraction of the cells remained unbound (Fig. 1). Viability of attached cells after 30 min was greater than 90%.

The optimum pH for attachment appears to be 5.0–5.5, with acetate or acetate-phosphate buffers giving the maximum levels of attachment. At pH values less than 4.5 or greater than 7.0 attachment decreased to much lower levels (Fig. 2). All subsequent experiments were performed with attachment taking place in a pH 5.2 acetate buffer.

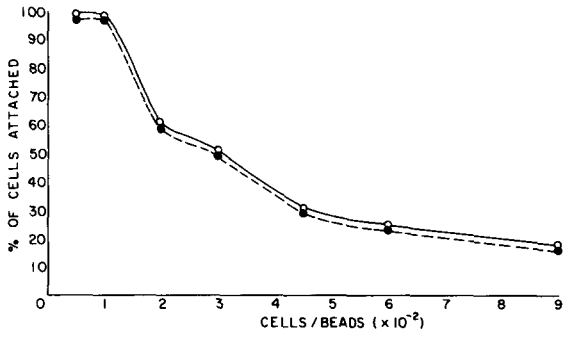

Fig. 1. Effect of varying the ratio of LM (○) or FHM (●) cells to Cytodex upon the fraction of cells attached, following attachment procedures described in the text. Similar results were obtained for Biocarriers.

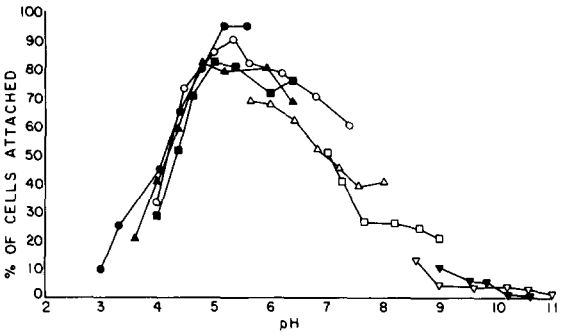

Fig. 2. Effect of attachment buffer pH and composition on attachment of LM cells to Cytodex. Attachment procedures are as described in the Materials and Methods section. Similar results were obtained for FHM cell attachment to Cytodex and for LM and FHM cell attachment to Biocarriers. Buffers are: acetate (●), citrate (▲), citrate-phosphate (■), acetate-phosphate (○), phosphate (△), Tris-HCl (□), carbonate-bicarbonate (▼), glycine-NaOH (▽). Ratio of cells to beads was 100:1.

After binding at pH 5.2, attached cells were lysed and the membrane-bearing beads washed in solutions with pH values ranging from 5.0 to 10.0. At pH values greater than 8.0 sufficient charge sites on the beads were neutralized to give decreased binding of intracellular contaminants DNA, succinate dehydrogenase (mitochondrial marker), and cytochrome *c* reductase (endoplasmic reticulum marker) (Fig. 3).

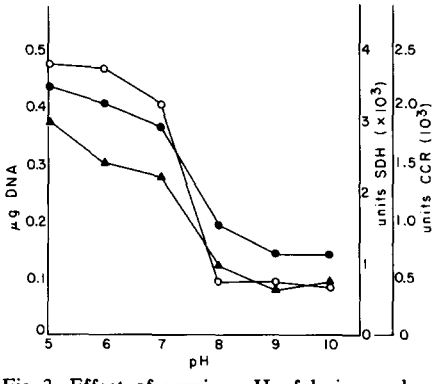

Fig. 3. Effect of varying pH of lysing and washing medium upon binding of intracellular contaminants to Cytodex. About $5 \cdot 10^7$ LM cells were attached to approximately $5 \cdot 10^5$ beads and membranes isolated as outlined in the text. Contaminants measured were DNA (○), succinate dehydrogenase (SDH) (●), and cytochrome *c* reductase (CCR) (▲).

TABLE I

PURIFICATION OF LM CELL PLASMA MEMBRANES ON CYTODEX

	Whole cell homogenate [a]	Membranes on beads [a]	Purification factor	% recovery on beads
Enzyme				
Leucine aminopeptidase	1.7	39	23	24
5′-Nucleotidase	8.8	157	18	20
$(Na^+ + K^+)$-ATPase	3.1	69	22	21
Glucose-6-phosphatase	1.2	0.19	0.16	0.1
Cytochrome *c* reductase	0.26	0.029	0.11	0.1
Succinate dehydrogenase	6.1	0.18	0.03	0.06
Acid phosphatase	0.016	0.0024	0.15	0.2
DNA (μg/mg protein)	85	6	0.07	0.1
Phospholipid (μmol/mg protein)	0.38	4.04	11	10
Protein	–	–	–	0.6

[a] Specific activity, expressed as μmol of product formed per min per mg protein.

TABLE II

PURIFICATION OF LM CELL PLASMA MEMBRANES ON BIOCARRIERS

	Whole cell homogenate [a]	Membranes on beads [a]	Purification factor	% recovery on beads
Enzyme				
Leucine aminopeptidase	1.7	37	22	21
5′-Nucleotidase	8.8	168	19	22
$(Na^+ + K^+)$-ATPase	3.1	71	23	20
Glucose-6-phosphatase	1.2	0.22	0.19	0.2
Cytochrome *c* reductase	0.26	0.02	0.08	0.1
Succinate dehydrogenase	6.1	0.13	0.02	0.02
Acid phosphatase	0.016	0.022	0.14	0.2
DNA (μg/mg protein)	85	7	0.08	0.1
Phospholipid (μmol/mg protein)	0.38	4.13	11	10
Protein	–	–	–	0.5

[a] Specific activity, expressed as μmol of product formed per min per mg protein.

TABLE III

PURIFICATION OF FHM CELL PLASMA MEMBRANES ON CYTODEX

	Whole cell homogenate [a]	Membranes on beads [a]	Purification factor	% recovery on beads
Enzyme				
Leucine aminopeptidase	0.53	12	23	21
5′-Nucleotidase	0.13	2.6	20	23
$(Na^+ + K^+)$-ATPase	0.30	6.1	20	19
Glucose-6-phosphatase	0.64	0.051	0.08	0.11
Cytochrome *c* reductase	0.25	0.025	0.10	0.09
Succinate dehydrogenase	0.30	0.003	0.01	0.01
Acid phosphatase	0.013	0.002	0.15	0.23
DNA (μg/mg protein)	83	5.9	0.02	0.15
Phospholipid (μmol/mg protein)	0.305	1.45	4.8	8.1
Cholesterol (μmol/mg protein)	0.083	1.21	15	14
Cholesterol/phospholipid	0.27	0.83	3.1	–
Protein	–	–	–	0.35

[a] Specific activity, expressed as μmol of product formed per min per mg protein.

TABLE IV

PURIFICATION OF FHM CELL PLASMA MEMBRANES ON BIOCARRIERS

	Whole cells homogenate [a]	Membranes on beads [a]	Purification factor	% recovery on beads
Enzyme				
Leucine aminopeptidase	0.53	13	24	21
5'-Nucleotidase	0.13	2.8	22	24
$(Na^+ + K^+)$-ATPase	0.30	6.6	22	19
Glucose-6-phosphatase	0.64	0.04	0.06	0.03
Cytochrome *c* reductase	0.25	0.03	0.12	0.11
Succinate dehydrogenase	0.30	0.0013	0.004	0.02
Acid phosphatase	0.013	0.0026	0.2	0.15
DNA (μg/mg protein)	83	6.2	0.07	0.13
Phospholipid (μmol/mg protein)	0.305	1.52	5	7.5
Cholesterol (μmol/mg protein)	0.083	1.25	15	13
Cholesterol/phospholipid	0.27	0.82	3.0	–
Protein	–	–	–	0.4

[a] Specific activity, expressed as μmol of product formed per min per mg protein.

A variety of membrane-associated enzymes were assayed following isolation of membranes on beads. In order to control for the effects of beads on enzyme activities or interference with assays, whole cell homogenates were assayed in the presence or absence of beads and the final values corrected for any deviation (which in no case exceeded 25%). A 20-fold enrichment in the plasma membrane markers, leucine aminopeptidase, 5'-nucleotidase, and ouabain-sensitive $(Na^+ + K^+)$-ATPase was achieved. Internal membranes were depleted 10–20-fold (Tables I–IV). About one-quarter of the membrane-associated activities were recovered on the beads, compared with less than 0.5% of the internal membrane markers. For FHM cells a 3-fold increase in the cholesterol to phospholipid molar ratio was obtained, in accordance with what would be expected for enrichment in plasma membranes. Similar results were obtained for the tetracarcinoma-derived cell lines (Tables V and VI).

The use of the high ionic strength pH 10 buffer to remove membranes washed from beads was found to remove 60–70% of the bead, associated phospholipid, leucine aminopeptidase and 5'-nucleotidase. Total recovery of enzyme activities of beads and wash solutions was 90%.

TABLE V

PURIFICATION FACTOR OF TERATOCARCINOMA-DERIVED CELL LINE PLASMA MEMBRANES ON CYTODEX

Cell line	Leucine amino-peptidase	Succinate dehydrogenase	Cyto-chrome *c* reductase
F9	18	0.05	0.13
Nulli-SCCl	16	0.036	0.11
PYS-2	19	0.093	0.05
TerC	17	0.11	0.12
TerJ	18	0.063	0.10

TABLE VI

PURIFICATION FACTOR OF TERATOCARCINOMA-DERIVED CELL LINE PLASMA MEMBRANES ON BIOCARRIERS

Cell line	Leucine amino-peptidase	Succinate dehydrogenase	Cyto-chrome *c* reductase
F9	18	0.031	0.11
Nulli-SCCL	19	0.08	0.07
PYS-2	21	0.063	0.11
TerC	21	0.09	0.082
TerJ	17	0.084	0.10

Discussion

The use of solid supports as a means of isolating plasma membranes provides an attractive alternative to conventional methods. Purification of plasma membranes is excellent and contamination from intracellular membranes is very low. Using beads for isolating membranes is both easier and more rapid than using sedimentation techniques. The prior need for derivitization of beads has been eliminated by using commercially available polycationic beads [4].

The use of various methods to prevent internal components from binding exists because bare patches on the beads are present between the areas where the cells attach. While these techniques are effective it seems that it should be possible, particularly with microcarrier culture beads, to grow cells on the beads until a monolayer of cells, with no spaces between them, exists. At this stage the membranes could be isolated by the techniques outlined here and elsewhere [2–4].

The method appears to be general, having been used on a number of other cells with similar success [3,27]. The possibility that portions of the membrane may be 'selected' during the attachment or isolation procedures would seem to be unlikely in light of the rather uniform recoveries of each of the three plasma membrane markers used in this study.

The optimum conditions for the isolation of membranes on these beads appears to be attachment in a pH 5.2 acetate buffer at a cell to bead ratio of 100:1 and lysis of attached cells at a pH of 8.0. The rapidity with which very pure preparations of membranes may be obtained (less than 1 h) should make this type of method useful in studies of plasma membranes.

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