PREPARATION OF IMMOBILIZED ANIMAL CELLS

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1. Introduction

In the last years there has been considerable interest in the immobilization of cells particularly those of microbial origin [1,2]. More recently the area has been extended by the reported immobilization of living plant cells in suspension [3].

Here we report on the immobilization of animal cells by adsorption of anchorage-dependent cell cultures to microcarriers such as gelatin beads (including 'magnetic' beads) and chitosan beads. An alternative convenient procedure for harvesting the cells is described. Some results obtained from gel entrapment of various animal cell types will also be given.

2. Materials and methods

2.1. *Materials*

Chitosan, agarose (type VII), alginic acid (sodium salt, type IV), collagenase (type I, 190 U/mg) were obtained from Sigma. Glutaraldehyde (25%) was from Merck AG. Cytodex and Sepharose CL-6B were products of Pharmacia, Sweden, and the magnetic particles, Fe_3O_4 (5 μm), were a gift from Höganäs, Sweden. Arlacel 83 was obtained from Atlas Chemical Co., dispase (grade II, 0.5 U/mg) was from Boehringer. Gelatin (commercial grade) was from Kebo, Sweden.

2.2. *Cell types*

The following cells were kindly provided by Professor O. Sjögren: the established cell lines HeLa and K562 (erythroleukemic), and the primary cell cultures S 157N (human skin fibroblasts), S 158A (human kidney carcinoma), DMH W49 (rat colon carcinoma) and DMH W1073 (rat colon carcinoma). All cells except K 562 were grown in Waymouth media supplemented with 20% foetal calf serum. K 562 were grown in RPMI medium with 10% foetal calf serum. All media were supplemented with gentamicin (50 mg/l).

2.3. *Gelatin microcarriers*

A gelatin solution (10 ml; 20% (w/v)) obtained by heating to 50°C was dispersed under vigorous stirring in 100 ml of a mixture of toluene:chloroform (73/27, v/v) containing 2% (w/v) of Arlacel 83 at room temperature. After 10 min the mixture was filtered through a 100 μm nylon net whence the collected microcarriers were transferred to acetone. After careful washing with acetone the microcarriers were evaporated to dryness. In order to obtain beads which would resist higher temperatures cross-linking with glutaraldehyde was carried out. Dry beads (1.5 g) were reswollen in 100 ml water followed by the addition of 20 ml 25% glutaraldehyde. After gentle stirring for 30 min the beads were collected and washed with 0.15 M NaCl on a 100 μm nylon net. The beads were dispersed in 0.15 M NaCl and autoclaved at 120°C for 15 min. In order to get a suitable size distribution of the beads they were first filtered through a 250 μm nylon net and the beads in the filtrate were then collected on a 100 μm net. They were then transferred to 0.15 M NaCl and sterilized through autoclaving at 120°C for 15 min. Magnetic microcarriers were obtained by the same procedure except that in addition to 9 ml 20% (w/v) gelatin solution, 1 g Fe_3O_4 particles were added prior to dispersion in the organic phase.

2.4. *Chitosan microcarriers*

A 2% (w/v) solution of chitosan in 1% formic acid was obtained by mixing chitosan with 1% formic acid and stirring overnight followed by filtration of any undissolved material through a 250 μm nylon net. The chitosan solution (18 ml) was mixed with 2 ml 25% glutaraldehyde and dispersed under vigorous stirring in the same organic phase as described for

gelatin microcarriers. After 15 min the mixture was transferred to methanol. The beads were then washed on a glass filter with methanol and subsequently with 0.15 M NaCl. After autoclaving at 120°C for 15 min (to obtain more rigid beads) the preparation was first filtered through a 250 μm nylon net after which the beads in the filtrate were collected on a 100 μm nylon net. The beads were transferred to 0.15 M NaCl and sterilized through autoclaving at 120°C for 15 min.

2.5. *Test for cell growth*

Before use all microcarriers were washed with 10 vol. medium. Gelatin and chitosan microcarriers (0.25 g wet wt) were mixed with 2.5×10^5 cells (S 157N, S 158A, DMH W49 and DMH W1073, respectively) in 5 ml media in 10 ml siliconized test tubes. They were placed in a CO_2-incubator (5%) at 37°C and mixed in an end-over-end shaker. The cultures were checked daily and the medium was replaced if necessary. The gelatin microcarriers containing Fe_3O_4 were tested with DMH W49. Sepharose CL-6B beads activated with different amounts of CNBr were placed in 20 mm Petri dishes and mixed with 2.5×10^5 DMH W49 cells in 3 ml medium. The cells S 157N and DMH W1073 were allowed to grow on gelatin microcarriers (1 g beads, wet wt) suspended in 50 ml spinners. The starting cell concentrations were 35 000/ml and 22 000/ml, respectively; 60% of the medium volume was replaced each day. For determination of the cell number, duplicate samples of 1 ml suspension were taken. After washing with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, the beads were incubated with trypsin (0.1% in PBS with 0.02% EDTA and without Ca^{2+} and Mg^{2+}) for 15 min at 37°C and the relased cells counted in a Bürker chamber.

2.6. *An alternative method for harvesting of cells*

After 6 days the spinner culture of DMH W1073 was divided into 3 equal fractions after washing as above. They were incubated with 3 ml enzyme solution, trypsin as described above and collagenase (2 mg/ml) in PBS with Ca^{2+} but without Mg^{2+} and dispase (4 mg/ml) in Waymouth medium at room temperature. Samples were taken at intervals and the cell concentration determined.

2.7. *Alginate immobilization*

Alginate (1 part 4% (w/v)) dissolved in 25 mM Hepes, 125 mM NaCl (pH 7.4) was mixed with 1 part cell suspension in medium supplemented with twice the concentration of serum. The cell alginate suspension was extruded through a 0.8 mm nozzle into a solution of 25 mM Hepes, 50 mM $CaCl_2$, 75 mM NaCl (pH 7.4) whereby beads with av. diam. 2 mm were formed. Immobilization was carried out at room temperature. After 5 min the beads were washed with medium and transferred to a spinner flask, which was placed in a CO_2-incubator at 37°C. At intervals beads were withdrawn and dissolved in 0.1 M EDTA (pH 7.4) and the cells were counted.

2.8. *Agarose immobilization*

Agarose (1 part 4% (w/v)) dissolved in 25 mM Hepes, 125 mM NaCl (pH 7.4) placed in a waterbath at 37°C was mixed with 1 part cell suspension in medium supplemented with twice the concentration of serum. This solution was made into beads by moulding it in a form made of Teflon. Solution was poured over a Teflon plate tightly covered with 3 mm holes. Another plate was used as support and the two were held together by clamps. Before moulding, the form was warmed to 37°C degrees. After the agarose had solidified the form was taken apart and the 'cylindrical beads' were taken out. The beads were put in medium in a spinner flask and placed in a CO_2-incubator at 37°C. At intervals beads were taken out and dissolved by heating to 70°C and the cells were counted.

2.9. *Preparation of entrapped β-cells of islets of Langerhans*

Ninety isolated islets of Langerhans (av. diam. ~0.1 mm) were prepared from rat [4] pancreas and washed with Krebs-Ringer buffer lacking phosphate [4]. The cells were suspended in 0.125 ml buffer and mixed with 0.5 ml 2.5% (w/v) sodium alginate in the same buffer. The cell alginate suspension was extruded into a solution of the buffer containing 50 mM $CaCl_2$. After 15 min the beads were washed 3×10 ml with the buffer. Visual inspection showed that the beads contained an average of one islet/bead.

The scintillation vials were each filled with 10 beads and 2 ml buffer, gassed for 1 min with carbogen (95% O_2, 5% CO_2) and preincubated for 30 min at 37°C in a waterbath with shaking. After addition of 1 mg glucose to the vials, they were gassed and 0.1 ml samples taken after 0, 30, 60 and 120 min. The samples were frozen and stored until analyzed for insulin using radioimmunoassay. Visual inspection showed no

release of the islets into the medium.

2.10. *Preparation of entrapped adipocytes*

Rat adipocytes were prepared according to [5]. For entrapment in alginate the cells were transferred to 20 mM Hepes, 130 mM NaCl, 2% (w/v) albumin (pH 7.4) mixed with an equal volume of 2% (w/v) alginate (autoclaved for 15 min at 120°C) and extruded into a solution of 25 mM Hepes, 50 mM $CaCl_2$, 75 mM NaCl (pH 7.4). After 5 min the beads were washed with Krebs-Ringer buffer containing 24 mM Hepes, 0.55 mM glucose and 1% (w/v) albumin (storage medium).

With entrapment in agarose the cells were transferred to the above storage medium containing 2% (w/v) albumin and mixed with an equal volume of 6% (w/v) agarose. Beads were prepared as in section 2.8. After gelling the beads were transferred to storage medium.

3. Results

3.1. *Cell attachment and growth*

In a preliminary test various cells were tested for their capacity to attach to different microcarriers (table 1). All cells could attach to the various beads. Studies on cell growth were subsequently carried out with the most promising microcarrier, gelatin beads with the cells S 157N and DMH W1073. A typical gelatin bead with attached cells is shown in fig.1. As seen from fig.2 no indication of cell death occurred and good growth was observed.

3.2. *Harvesting of cells*

In fig.3 the results from three different enzymic procedures leading to cell detachment from gelatin microcarriers are given. As seen, both collagenase and dispase give rise to a far higher total cell concentration than normally applied trypsin treatment.

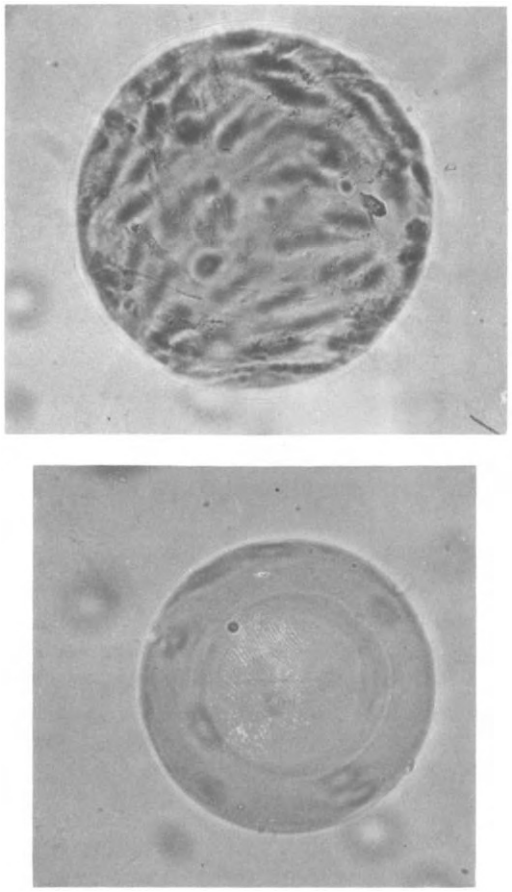

Fig.1. Photographs show: (a) a representative gelatin bead taken from a culture of DMH W49 after 48 h; (b) a gelatin bead prior to inoculation.

3.3. *Cell entrapment*

I. *Isolated cells:* In a preliminary study to test whether animal cells would survive entrapment in

Table 1
Attachment of primary cell cultures to different microcarriers

	S 157N	S 158A	DMH W49	DMH W1073
Gelatin	+	+	+	+
Gelatin (magnetic)	n.t.	n.t.	+	n.t.
Chitosan	+	+	+	+
CNBr-activated Sepharose CL-6B	n.t.	n.t.	+	n.t.

n.t., not tested

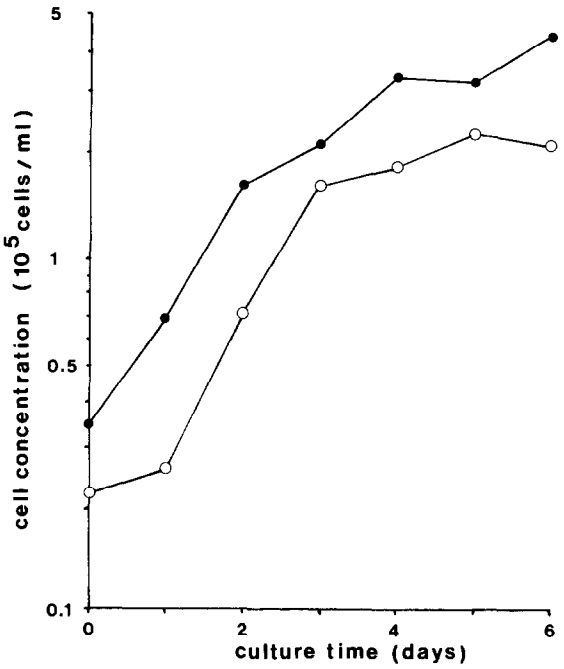

Fig.2. The growth of two primary cell cultures, S 157N (●——●) and DMH W1073 (○——○), on gelatin beads in spinner. The figures given represent the mean of 2 cell counts.

a gel matrix hepatocytes from rats were isolated [6] and entrapped in calcium alginate. The obtained beads were subsequently incubated in perfusion buffer and water, respectively. Release of lactate dehydrogenase was monitored. About 10-times lower leakage of the enzyme was found in the sample kept in the perfusion buffer compared to the sample in water. Subsequent incubation in water of the beads previously kept in perfusion buffer however led to considerable leakage of active lactate dehydrogenase. Subsequently islets of Langerhans containing β-cells were isolated and entrapped following the same procedure to check whether the latter were capable of insulin production/secretion in the immobilized state. Over 0, 30, 60 and 120 min samples were withdrawn from the medium and assayed for insulin content using radioimmunoassay analysis. The amount of insulin secreted was 1, 23, 40 and 78 immunoreactive insulin units (μU/ml), respectively.

Finally adipocytes were isolated and entrapped in both Ca-alginate as well as agarose. They were subsequently tested for their capability to incorporate [3-^{3}H]glucose into lipids [7] following insulin stimulation as well as for their ability to release free fatty acids by noradrenaline stimulation [8]. It was found that, at the outset, the immobilized adipocyte preparation showed a higher basal incorporation than free cells which was more pronounced with cells immobilized in Ca^{2+}-alginate. On stimulation with insulin both preparations showed an increase in glucose incorporation (when higher concentrations of alginate were used, the cells did not respond to insulin in the glucose test). The free fatty acid release was only studied with agarose immobilized adipocytes as the alginate beads were too soft. The immobilized cells also showed a response to added noradrenaline by releasing free fatty acids after about twice the time required for free cells, and at ~1/3rd the rate for free cells.

II. *Cell cultures:* A number of cell types, i.e., fibroblasts, HeLa, DMH W49 and K 562, the latter growing in suspension, were entrapped in gels to test whether they would grow within a gel-matrix or remain viable. The gels prepared were beads of: (a) 2% alginate; (b) 2% agarose; (c) a mixture of 2% agarose and 0.5% alginate; and (d) 2% agarose containing simultaneously entrapped Cytodex particles to which cells already had been attached. In all cases serum was entrapped simultaneously with the cells as in-diffusion of some of the components of serum was likely to be hindered. No proliferation of the cells tested was observed

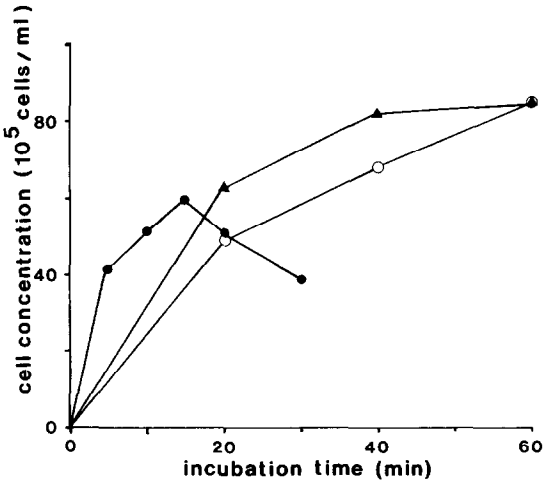

Fig.3. Release of microcarrier-bound cells using trypsin (●——●), collagenase (○——○) and dispase (▲——▲), respectively.

under the conditions used; however on average, 10–30% of the cells remained viable as judged by the trypan blue exclusion test, after 1 week of incubation. In controls run with HeLa cells non-entrapped but kept free in solution in siliconized tubes they had disappeared after 1 day.

4. Discussion

The use of microcarriers for culture of mammalian anchorage-dependent cells in suspension has been given increasing attention in the last years. Following the original experiments using DEAE–Sephadex [9], microcarrier beads are now commercially available based on related supports as well as those based on polyacrylamide carrying positively charged dimethylaminopropyl groups. Although the above carriers have proven most valuable there seemed room for improvement to obtain cheaper supports and from which cells, if required, could be removed more easily.

In the course of our studies to obtain a suitable gel matrix for entrapment of animal cells we found, in analogy to [10], that collagen substrate adhered to cells. On further investigation a simple technique leading to the preparation of solid beads, 100–250 μm, of the closely related gelatin (the product obtained on boiling collagen) was developed. All the cells tested attached and proliferated. In this context 'magnetic' gelatin beads were also prepared and as expected no adverse effect on cell growth was observed. Potentially, such supports would permit facile recovery when used in media of viscous or particulate nature. Furthermore, it is conceivable to suspend the beads by applying an outer magnetic field thereby minimizing the need for vigorous agitation when using carriers of high specific weight.

Beads of the ubiquitous chitosan, a partially deacetylated product of the polysaccharide chitin, were also prepared using a similar procedure and found to lead to attachment of cells. Their properties as supports for cell growth will be tested further. In our investigations to find supports for cell growth of anchorage-dependent cells we coupled polylysine covalently to CNBr-activated agarose beads. Although nothing definite can be said at this point on the attachment capacity of such preparations for the cell types tested it was found that agarose alone activated with high concentrations of CNBr did lead to attachment and growth of cells, probably due to the positive charge introduced on the matrix.

In our experiments involving detachment of cells we attempted an alternative approach as there was the possibility with the new microcarriers used to enzymically dissolve the support directly. Both dispase and collagenase dissolved the beads leading in the case of dispase to a clear solution. In contrast to trypsin treatment which involves destruction of the cell surface to accomplish cell removal the approach taken here should leave the entire cells intact and viable which is an advantage, e.g., when the cells are used for immunization or when analysis of surface antigens will be done. Summarizing, as gelatin is inexpensive and represents a more 'natural' surface for cell attachment than those commercially available, it appears that such microcarriers will turn out to be valuable supports for cell growth as also strongly indicated by the spinner culture experiments reported here. The ease of cell release adds to the merits of such microcarriers.

The studies reported here on the entrapment of animal cells in gels (detailed in [11]) were initiated because such immobilized preparations should offer the same advantages as those observed with microorganisms or plant cells. For instance, isolated cells when immobilized in the entrapped state would allow convenient assay in analytical metabolic perfusion studies or could be used for the enzymic conversion of certain metabolites by analogy to steroid transformation using entrapped bacteria [12] or be utilized for, e.g., hormone production. These data demonstrate that cell entrapment leaves at least the major part, if not all, of their metabolic machinery intact. We applied particularly one of the more recently developed entrapping techniques, i.e., inclusion in calcium alginate [13–15].

It could be demonstrated with isolated rat hepatocytes that the cells remained, at least partially, intact on immobilization, as disruption of the cell membrane would have lead to leakage of the enzyme, lactate dehydrogenase. Subsequently islets of Langerhans from mouse pancreas were immobilized by the same procedure giving preparations still capable of insulin production/secretion [16]. We also showed that adipocyts, entrapped in either calcium alginate or agarose, were still capable of metabolizing added radioactive glucose to fatty acids and could be stimulated further on addition of insulin [17].

A drawback of the calcium alginate procedure is

the requirement for Ca^{2+} or similar ions to keep the network intact and following this the necessity to operate in phosphate-free media. Entrapment in agarose particles, as described here and applied in the studies of free fatty acid release of adipocytes, may offer an alternative approach.

With regards to anchorage-dependent cells, good microcarriers including those described here are at hand, whereas for suspension cultures entrapment appears the best alternative for immobilization. Furthermore, anchorage-dependent cells might profit from entrapment within a three-dimensional network while adsorbed to their normal carrier as this would give some protection against, e.g., shear force. It may even be that anchorage-dependent cells for which no suitable microcarrier has yet been found may accept the polymer network as recipient attachment surface or at least remain viable as demonstrated here using trypan dye exclusion tests. Although no clear cell proliferation has yet been observed with the various entrapped cell types they remain viable for a considerable time and permit metabolic studies, enzymic transformations or syntheses to be carried out.

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