Cell Growth on Liquid Microcarriers

Abstract. *Anchorage-dependent cell growth is demonstrated on microcarriers of fluorocarbon fluid formed by emulsification and stabilized with polylysine.*

In 1967 van Wezel (*1*) introduced microcarrier culture, in which anchorage-dependent cells are grown as monolayers on small spherical beads. Such a system facilitates mass culturing of cells by providing a relatively large amount of surface area for cell growth in a small volume, allowing efficient utilization of medium. Beads composed of DEAE-Sephadex were first used; recently, solid plastic beads have also been employed in growing a variety of cells (*2*). In this report we describe a novel system in which anchorage-dependent cell growth is demonstrated on microcarriers of fluorocarbon fluid formed by emulsification and stabilized with polylysine.

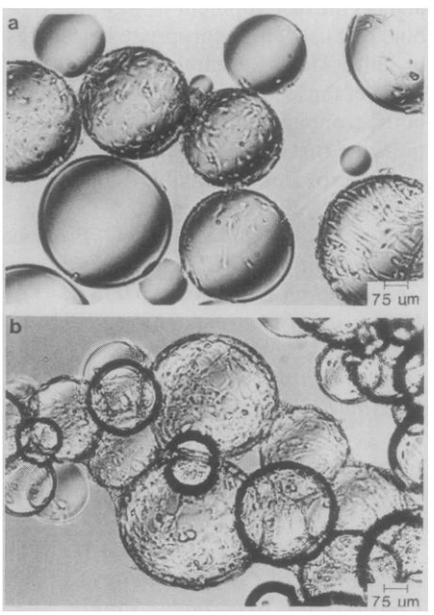

Fig. 1. Micrographs showing characteristic portions of an emulsion after 4 days incubation (Nomarski optics). Two milliliters of FC-70 fluorocarbon fluid was combined with 2.0 ml of poly-L-lysine (2 mg/ml; molecular weight 60,000; Sigma) in 0.01*N* KOH. The mixture was emulsified by rapid agitation in a vortex mixer for approximately 5 seconds to produce the desired droplet size. After 10 minutes, the continuous phase of the emulsion was replaced first with 0.15*M* NaCl and then with culture medium (90 percent Dulbecco's modified Eagle's medium with antibiotics and 10 percent newborn calf serum) by repeated rinsing (rinsing is easily accomplished as the fluorocarbon droplets quickly settle out, allowing the excess continuous phase to be drawn off without loss of the emulsion). A 0.1-ml portion of the emulsion was pipetted into 0.4-ml microwells, and then 0.1 ml of 3T3-L1 cell suspension in culture medium (2×10^5 cells per milliliter) was added to each well without mixing. The remaining volume was filled with culture medium and the inoculated emulsions were incubated at 37°C in a humidified 5 percent CO_2 atmosphere. A 0.2-ml portion of spent medium was withdrawn from each well daily and replaced with fresh medium.

The ability of cells to spread and grow on the surface of fluorocarbon fluids was reported by Rosenberg (*3*), who used the planar interface between a fluorocarbon and aqueous phase as a substrate. When cells are grown on such liquid substrates, as in solid-substrate cultures, they do not interact directly with the substrate but rather with the layer of denatured protein that adsorbs to the interface (*3*). In studying the behavior of cells at liquid-liquid interfaces, we found that the strength of the interfacial protein film can be increased by using polylysine to build a bimolecular protein layer (*4*). Interfaces stabilized with such a film were much better able to support cell growth than those with monomolecular layers of serum proteins.

Emulsions were prepared by combining the fluorocarbon fluid FC-70 or FC-72 (*5*) with a solution of polylysine in 0.01*N* KOH (to adsorb a complete monomolecular layer of polylysine it is advantageous to raise the solution *p*H considerably above the *p*K value of the amino groups of the side chains, resulting in a relatively uncharged polymer) (*6*). The two immiscible fluids were agitated and formed a stabilized emulsion in which the disperse phase consisted of droplets 100 to 500 μm in diameter. To form a second layer on the polylysine-coated droplets, the droplets were washed and exposed to culture medium containing serum. At physiological *p*H, a layer of serum proteins adsorbs to the charged polylysine. The final microcarrier system consists of liquid fluorocarbon droplets with a base layer of polylysine and an outermost layer of serum proteins dispersed in a continuous phase of complete culture medium.

We inoculated such emulsions with a variety of cells, including BALB-3T3, 3T3-L1, and SV-T2 murine cells and human newborn foreskin fibroblasts. All of these showed attachment and spreading on the protein-treated droplets; the best results in terms of cell growth to date have been with the anchorage-dependent 3T3-L1 cells and the transformed SV-T2 cells. To follow the growth of 3T3-L1 cells attached to the fluorocarbon droplets, we withdrew small amounts of emulsion at different times after inoculation for both microscopic examination (Fig. 1) and enumeration of cell nuclei (Fig. 2). During the log phase of growth, the measured generation time is approximately 18 hours, which compares well with that determined in conventional culturing of this cell line.

The cells can be harvested without the use of trypsin. The emulsion is broken into its component phases by centrifugation, and the cells, being intermediate in density between the fluorocarbon fluid and the culture medium, form a clearly visible layer at the phase boundary. We removed this cellular aggregate by pipetting and transferred it to a conventional treated-polystyrene substrate, where attachment and spreading were observed for most cells.

Both the polylysine and the polylysine–serum protein stabilized fluorocarbon emulsions could be stored for weeks with no appreciable change in droplet size. The molecular weight of the polylysine used was not critical; successful emulsions were prepared with polylysine ranging from 3000 to 240,000 daltons. In

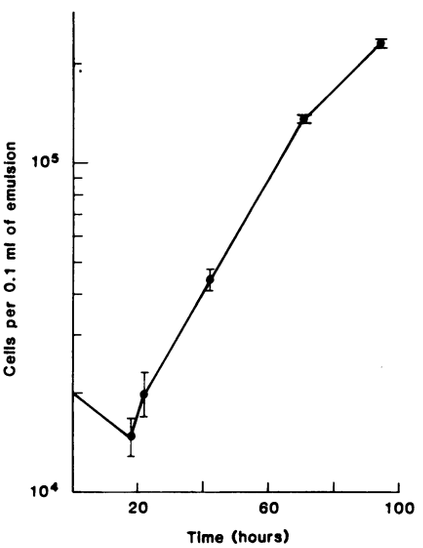

Fig. 2. Emulsions were prepared and inoculated as described in Fig. 1. At the times indicated, the entire contents of a well was transferred by pipette to a small tube where the excess continuous phase was aspirated. The emulsion was then combined with 0.4 ml of citric acid–crystal violet nuclear staining solution (*7*) for 1 hour with occasional vigorous agitation. Stained nuclei, released from the cells by the staining solution, were counted in a hemacytometer. This procedure does not count nuclei from cells attached to the surface of the microwells and eliminates most of those from cells which might have detached from the droplets at any point before removal of the excess continuous phase. Each point is the average for two separate emulsions whose individual values are indicated by the bars.

addition to serum proteins, we used gelatin as the outermost protein layer with little noticeable effect on cell growth.

Fluorocarbon emulsion could be stabilized with a single protein layer of, for example, serum proteins. Although some cell growth could be observed, this system did not, in general, produce a confluent cell layer on the microcarrier droplets under our experimental conditions.

Not all fluorocarbon fluids can be used for microcarrier systems by the methods we have presented. Emulsions of FC-77 could not be stabilized at the desired droplet size with polylysine and, following agitation, rapidly separated into two continuous phases. We have reported that cell spreading on planar polylysine-coated FC-77 surfaces is greatly reduced (*4*). These observations suggest that the adsorbed layer of polylysine which forms at this liquid-liquid interface has a relatively low tensile strength.

The system we have described could be significantly improved through the use of a perfusion or stirring arrangement to facilitate nutrient and waste exchange. Using other oils and adjusting the density of the beads by combining different oils might also be beneficial. In preliminary experiments we observed attachment and spreading of cells on emulsified silicone oil droplets; however, at present the fluorocarbon fluids seem superior both in supporting efficient cell growth and in producing stable emulsions with the desired droplet size.

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4. I. Giaever and C. R. Keese, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 219 (1983).
5. Fluorocarbon fluids are referred to by the manufacturer's designation (3M Co., St. Paul, Minn.). The fluids are blended by the manufacturer and are not chemically defined. The compositions of the particular lots used in this work were reported to be: FC-70, primarily 15-carbon perfluoro tertiary amines; FC-72, primarily C_6F_{14}; FC-77, primarily a mixture of C_8F_{18} and cyclic $C_8F_{16}O$.
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Proglumide: Selective Antagonism of Excitatory Effects of Cholecystokinin in Central Nervous System

Abstract. *Extracellular single-unit recording techniques were used to test the ability of proglumide to block cholecystokinin-induced excitation of rat midbrain dopaminergic neurons and dopamine-sensitive prefrontal cortex cells. Intravenous and iontophoretic proglumide administration consistently blocked cholecystokinin-induced excitations while having no effect on glutamic acid–induced increases in activity. This selective blockade of central cholecystokinin effects by proglumide suggests that this drug may be valuable for studying the possible role of cholecystokinin as a neurotransmitter or neuromodulator in the central nervous system.*

A gastrin-like peptide was first described in the mammalian brain by Vanderhaeghen *et al.* (*1*). Subsequent studies have shown that the carboxy-terminal octapeptide cholecystokinin (CCK) is probably the fragment of gastrin active in the central nervous system (*2*), and that this peptide is localized in vesicles and released by calcium-dependent mechanisms (*3*). By means of immunofluorescence histochemical methods it has been shown that CCK coexists with dopamine (DA) in a subpopulation of midbrain neurons (*4*). In addition, both systemic injection and local microiontophoretic application of CCK increase the neuronal activity of DA cells (*5*) in midbrain regions where CCK-immunoreactive DA neurons occur (*4*). Recently, the glutaramic acid derivative, proglumide, which has been used in the treatment of peptic ulcers in Europe (*6*), has been shown to be an antagonist for CCK receptors in the pancreas (*7*). Here we report that proglumide selectively blocks CCK-induced excitations of both DA neurons in the ventral tegmental area and non-DA neurons in the prefrontal cortex.

Male albino rats (Charles River, 250 to 370 g) were prepared in accordance with the guidelines of the American Physiological Society. All surgery was performed on rats under temporary halothane anesthesia. A tracheotomy was performed on each animal which was then mounted in a stereotaxic apparatus. A hole was bored in the skull over both the prefrontal cortex [anterior 9650 to

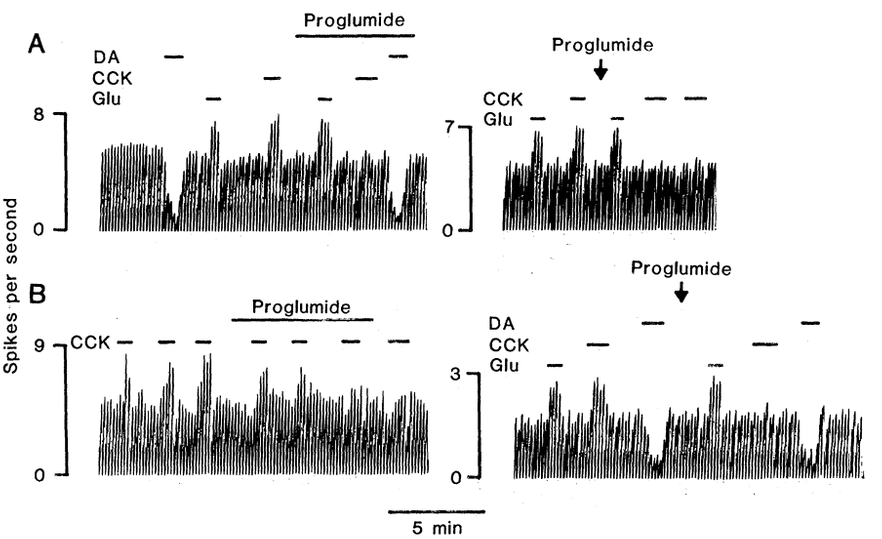

Fig. 1. Typical blocking effects of proglumide on CCK-induced changes in the activity of dopamine innervated prefrontal cortex neurons and midbrain dopaminergic neurons. (A) Cumulative rate histograms of the spontaneous activity of DA-sensitive prefrontal cortex neurons. (Left) Iontophoresis of CCK (10-nA ejection current) and glutamic acid (*Glu*, 2 nA) increased neuronal activity whereas DA (25 nA) inhibited cell activity. Local application of proglumide (30 nA) selectively antagonized the CCK-induced increases in firing rate. (Right) Iontophoretic application of CCK (15 nA) and glutamic acid (3 nA) increased cell activity. Only the CCK effect was blocked by the intravenous administration of proglumide (arrow, 0.2 mg/kg). (B) Cumulative rate histograms of the spontaneous activity of midbrain DA neurons. (Left) Iontophoretic application of CCK (30 nA) reproducibly increased neuronal discharge and the concurrent ejection of proglumide (50 nA) blocked this effect. (Right) Local application of CCK (10 nA) and glutamic acid (3 nA) excited neuronal activity whereas DA (20 nA) inhibited firing. Intravenously administered proglumide (arrow, 0.2 mg/kg) selectively blocked the excitatory effect of CCK. Horizontal bars represent the onset and duration of iontophoretic drug application.