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Cell Biology

# Behavior of cells at fluid interfaces

(protein adsorption at interfaces/fluorocarbon fluid substrates/cellular growth patterns)

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ABSTRACT **We have cultured the murine cell lines 3T3-L1 and SV-T2 using as a substrate the layer of denatured protein that forms at the phase boundary between culture medium and fluorocarbon fluids. The growth patterns observed on these interfaces differ from those seen on conventional solid substrates. Depending on the cell strain and the composition of the fluorocarbon fluid, cells will tend to clump into isolated aggregates or form nearly confluent cell monolayers containing "lake-like" openings. We demonstrate that these growth patterns can be attributed to the ability of cultured cells to stress and break the protein monolayer on which they grow.**

The behavior of cultured cells on different substrates has been extensively studied (1). In general, the substrates chosen have been solid materials, including glass, metals, and a wide variety of both natural and synthetic polymers. In such studies, the attachment, spreading, and growth of the cells do not occur directly on the substrate but on a monolayer of protein that adsorbs to the substrate's surface. Unless steps are taken to first coat a substrate with a specific protein, the protein monolayer that the cells encounter will be a collection of those from the serum present in the tissue culture medium.

In 1964 Rosenberg introduced the use of a fluid substrate for the growth of both transformed and anchorage-dependent cells (2, 3). In this method a cell suspension is introduced over an inert hydrophobic liquid having a density greater than that of the aqueous medium, and cells are observed to spread and divide on the liquid–liquid interface between the two immiscible phases. As is the case for solid substrates, the cells do not interact directly with the interface but rather with proteins that adsorb to the interfacial junction. These proteins presumably denature as their polypeptide chains unfold to achieve a low energy orientation with most of the hydrophillic portions exposed to the aqueous phase and the hydrophobic portions in the inert nonaqueous phase (4). This denatured film of protein forms a somewhat rigid skin to which the cells attach and spread. The hydrophobic fluids used for this purpose included both fluorocarbon and silicone fluids.

Since the original work was published, there seems to have been relatively little interest in the growth of cells on fluid substrates in spite of some advantages not afforded by solid substrates in studying cell behavior in culture. We have found the fluorocarbon interface to be well suited to provide an inert, nontoxic, hydrophobic substrate for cell growth. It has the advantage of being exceptionally homogeneous and reproducible when compared with hydrophobic solid surfaces which, in general, have polar molecular inhomogeneities (3, 5). In addition, cells grown on such substrates can be transferred from one fluid interface to another or from a fluid to a solid substrate by simply pipetting the cell layer. Such a procedure is of particular interest in that it obviates the use of trypsin or other proteolytic enzymes or chelating agents to passage cultured cells and avoids the uncertain effects of such treatments.

In this study we describe characteristic growth patterns observed of cultured cells on the fluorocarbon–aqueous interface. These patterns are highly dependent upon both cell type and the composition of the fluorocarbon fluid. Furthermore, we present evidence that these patterns are the result of the stressing and fracturing of the interfacial protein film by the cells.

## MATERIALS AND METHODS

**Cells.** The murine fibroblast lines 3T3-L1 and SV-T2 [a transformed BALB(3T3)] were obtained from the American Type Culture Collection. All culturing was under standard conditions of 37°C and 5% $CO_2$ in air with medium consisting of 90% Dulbecco's modified Eagle's medium with antibiotics and 10% newborn calf serum (GIBCO).

**Fluorocarbon Fluids.** Fluorocarbon fluids were gifts from 3M (St. Paul, MN). The fluids used in these studies are blended by the manufacturer to achieve specific physical properties and are not defined chemically. The compositions of the particular lots used in this work were analyzed to be: FC-70, primarily 15 carbon perfluorotertiary amines; FC-77, primarily a mixture of $C_8F_{18}$ and cyclic $C_8F_{16}O$; FC-72, primarily $C_6F_{14}$. Throughout this work these fluids will be referred to by their FC designations.

**Preparation of Fluorocarbon Interfaces.** Degassed fluorocarbon fluid (0.1 ml) was placed in a cylindrical well (7-mm inside diameter), and 0.2 ml of complete culture medium was carefully pipetted over the fluorocarbon forming a two-phase system separated by a layer of adsorbed serum proteins.

To treat the interface with glutaraldehyde, a monolayer of serum proteins was first adsorbed to the phase boundary as described above; generally, a time of 10 min was allowed to assure a complete protein monolayer. The culture medium above the fluorocarbon fluid was then carefully replaced with sterile saline by repeated rinsing. (In this and all procedures described below, one must be careful not to mechanically disrupt the interface or allow it to become exposed to air.) Approximately 0.1 ml of saline was left above the fluorocarbon phase; this was adjusted to 0.8% glutaraldehyde to crosslink the protein layer, and after 15 min the glutaraldehyde was removed from the aqueous phase by repeated rinsing with sterile saline. Finally, the saline aqueous phase was replaced with culture medium by repeated rinsing.

To form a polylysine-based interfacial protein layer, after the addition of fluorocarbon to the well, 0.2 ml of a polylysine solution [poly-L-lysine ($M_r$ = 90,000) at 2 mg/ml in 0.01 M KOH] was pipetted over the fluid and incubated for 10 min. The KOH and unadsorbed polylysine of the aqueous phase were removed from the well by repeated rinsing with small quantities of sterile saline. The same rinsing procedure was then repeated by using

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complete culture medium, resulting in a bimolecular layer at the interface with a base layer of polylysine and an upper layer of serum proteins (6).

## RESULTS

We have studied the growth of cultured cells on the liquid–liquid interface between the aqueous culture medium and various fluorocarbon liquids. Cells used have included the murine fibroblast lines 3T3-L1, BALB(3T3), and SV-T2 [a transformed BALB(3T3)] and human newborn foreskin fibroblasts. All of these cells exhibited quite different growth patterns when grown on the various fluorocarbons. These differences are best illustrated with the 3T3-L1 and SV-T2 cell lines.

3T3-L1 cells were observed to attach, spread, and move about on the FC-70 liquid interface. Although cell division was less frequent when compared with a treated-polystyrene solid substrate, the cells nevertheless divided and eventually formed a nearly confluent sheet of cells. However, the sheet was seldom seen to be totally confluent, as small "lake-like" openings were normally present where it appeared the cell sheet had been fractured, and the cells had retracted elastically (Fig. 1 *Upper*). A similar pattern has been described for 3T3 cells growing on solid cellulose acetate substrates (7).

On the other hand, the SV-T2 fibroblasts had a quite different growth pattern on the FC-70 fluorocarbon liquid. As with the 3T3-L1 cells, the generation time was considerably longer when compared with growth on solid substrates, but the SV-T2 cells were unable to form a confluent arrangement of cells. Instead, the cells spread and divided to first form a sparse network, which eventually collapsed to form aggregates of cells that increased in size over a period of days (Fig. 2 and Fig. 3 *Top*). If these aggregates were pipetted from the interface and placed on a conventional polystyrene substrate, the cells at the periphery of the cluster were observed to spread and migrate outward from the main cellular mass. In time, all the cells of the original aggregate seemed to have attached and spread on the substrate.

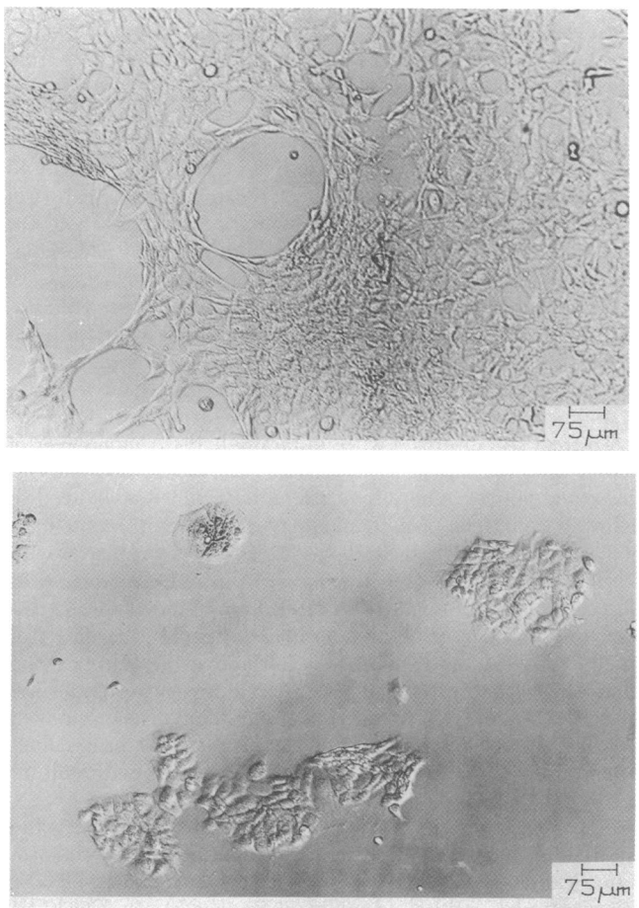


FIG. 1. Living 3T3-L1 cells on liquid–liquid interfaces. A 3T3-L1 cell suspension (0.1 ml; $10 \times 10^4$ cells/ml) was pipetted over a fluorocarbon interface. (*Upper*) FC-70 fluorocarbon fluid; 5 days of incubation. (*Lower*) FC-77 fluorocarbon fluid; 3 days of incubation. (Nomarski optics.)

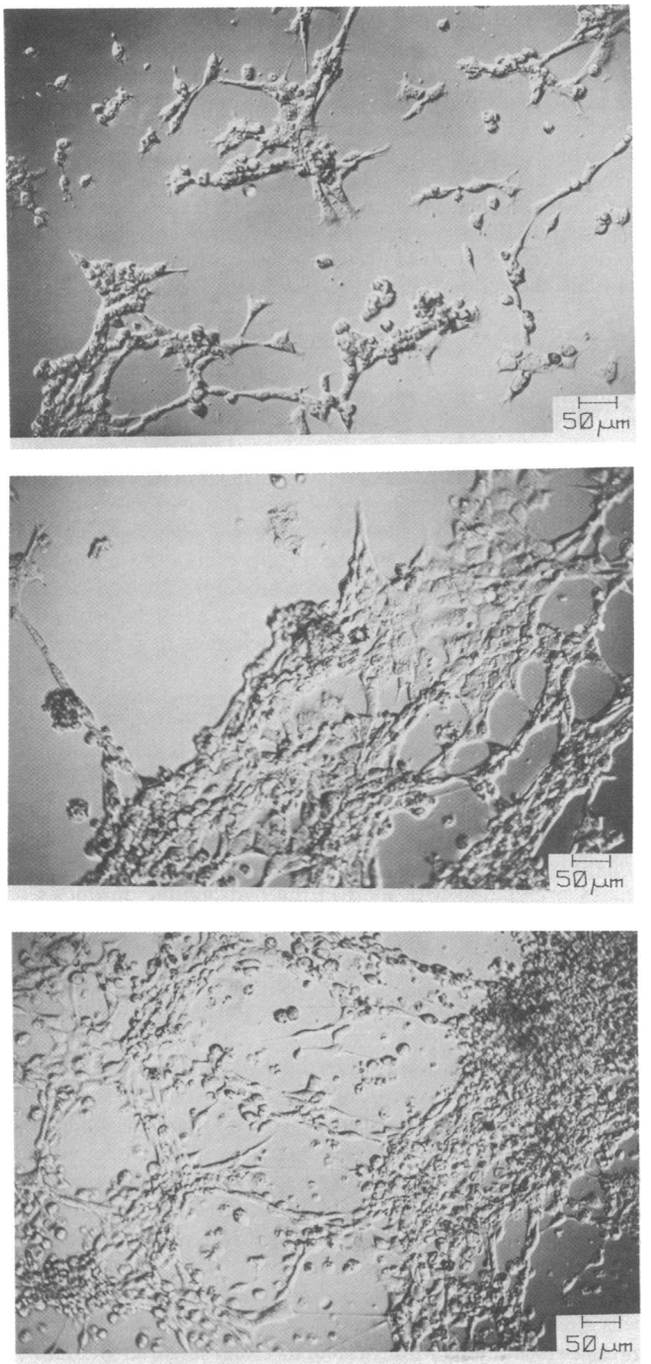


FIG. 2. Living SV-T2 cells on a liquid–liquid interface. An SV-T2 cell suspension (0.1 ml; $6 \times 10^4$ cells/ml) was pipetted over an FC-70 fluorocarbon fluid interface and photographed after 1 day (*Top*), 2 days (*Middle*), and 5 days (*Bottom*) of incubation. (Nomarski optics.)

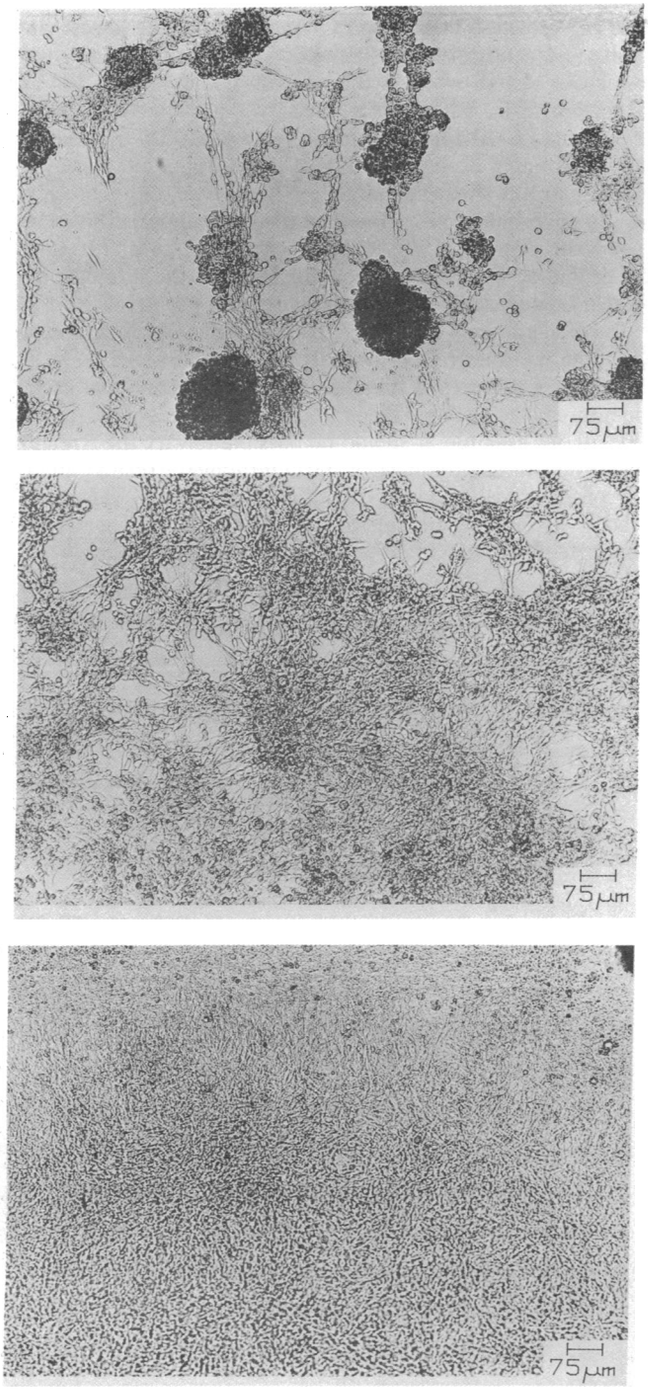


FIG. 3. SV-72 growth on treated interfacial protein films on FC-70 fluorocarbon fluid. Protein films were treated with glutaraldehyde or double layers of protein were formed by using polylysine as described. An SV-72 cell suspension (0.1 ml; $10 \times 10^4$ cells/ml) was pipetted over each interface and was incubated for 5 days. (*Top*) Untreated protein layer; (*Middle*) glutaraldehyde-treated layer; (*Bottom*) polylysine-mediated bimolecular protein layer.

Interfaces with FC-72 supported more rapid growth of cells, although the growth patterns observed for 3T3-L1 and SV-T2 cells were approximately the same as on the FC-70 fluid. Cell growth on FC-77 was greatly decreased when compared with that on FC-70. In addition, the pattern of cell growth was altered. The 3T3-L1 cells showed poor spreading and an apparent lack of motility, resulting in packed colonies of cells appearing epithelial in morphology (Fig. 1 *Lower*). Little or no spreading was seen with the SV-T2 cells on this fluorocarbon but rather small aggregates formed and grew in size.

If the adsorbed serum proteins at the interface are crosslinked by using glutaraldehyde or if a bimolecular layer of proteins is formed by using polylysine as a base coat (6), the patterns of cell growth can be altered significantly. Both of these procedures nearly totally blocked the lake-like formation and aggregation of 3T3-L1 and SV-T2 cells, respectively, when FC-70 was the fluorocarbon fluid; Fig. 3 illustrates this for SV-T2 cells. When these procedures were used with the FC-77 fluid we were unable to alter significantly the behavior of the SV-T2 cells by using polylysine; however, crosslinking the interfacial proteins with glutaraldehyde allowed these cells to form a confluent sheet of cells (data not shown).

## DISCUSSION

We believe all of the observations presented above are explained by the ability of cells to break physically the monolayer of protein with which they interact at these fluid interfaces. The different patterns of growth observed will depend on the relative magnitude of the forces exerted on the film by the cells and on the strength of the protein film. As the cells attach and spread they tension the monomolecular protein layer. After cell division the daughter cells spread, and the tension increases with cell number until a point is reached at which the combined forces exceed the tensile strength of the film; at this point the film fractures, releasing the stress. The cells, still attached to the protein film, move with the broken monolayer to form lake-like openings in nearly confluent sheets or aggregates on less populated films. The FC-70 interfacial film is sufficiently strong to support nearly confluent growth of 3T3-L1 cells; however, the SV-T2 cells exert greater stress on the protein layer, resulting in its fracture forming the initial aggregates of cells. The further growth of these aggregates is presumably due, at least in part, to the ability of transformed cells to grow without spreading as in soft agar cultures. The lack of spreading exhibited by the SV-T2 cells and the poor spreading and lack of motility of the 3T3-L1 cell on the FC-77 fluorocarbon when compared with FC-70 and FC-72 suggest that the adsorbed protein layer has a tensile strength so low that the tension exerted by a single cell can lead to its rupture.

The lake-like openings also can be produced mechanically by carefully poking a thin glass fiber through a nearly confluent cell layer. By observing this process in a microscope, the cells can be seen to contract, and thus the stresses are relieved. Eventually the cells will crawl out and fill in the lake-like regions.

Both crosslinking the protein layer with glutaraldehyde and the formation of a double protein layer with polylysine should increase the tensile strength of the protein film. The fact that FC-70 interfaces treated in either of these manners prevent lake-like openings and cell aggregation lends strong support to the suggestion that these patterns result from protein film breakage.

The inability of polylysine to prevent clumping of the SV-T2 cells grown on FC-77 fluorocarbon can be explained by the relatively poor ability of this interface to adsorb protein. This is supported by other studies we have carried out with emulsions of fluorocarbon dispersed in an aqueous phase by using protein as a stabilizing agent. Whereas both serum proteins and polylysine can be used to stabilize coarse emulsions of FC-70 and FC-72, emulsions prepared in the same manner with FC-77 rapidly break down (unpublished data). This suggests that the adsorbed film of protein about the FC-77 fluorocarbon droplets is easily desorbed or disrupted, allowing them to coalesce. In

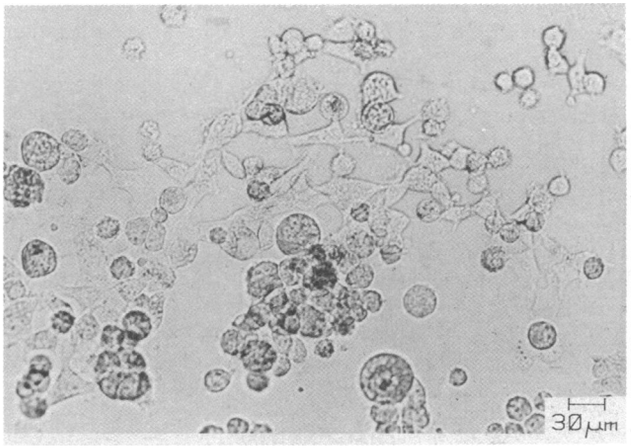


FIG. 4. Spreading of 3T3-L1 cells at the air–liquid interface of a hanging drop culture. A drop of 3T3-L1 cell suspension was placed on a glass coverslip that then was inverted and placed over a 3-mm-deep well of a microconcavity slide. The well had been wet previously with a small quantity of culture medium and had been flushed with 5% $CO_2$ in air to maintain proper conditions for cell growth. The junction between the coverslip and the slide was made airtight with silicone grease. Incubation was for 2 hr at 37°C. (Nomarski optics.)

the experiments described in this paper, the initial polylysine base coat on FC-77 may be sparse or so easily desorbed that an effective bimolecular layer cannot be formed. However, the heterogeneous serum proteins that attach to the same interface are adsorbed sufficiently well that fixation with glutaraldehyde results in a substrate strong enough to support the tension of a confluent SV-T2 cell sheet.

Harris has studied the forces exerted by cells on silicone fluid substrates and reported that the greater these forces are, the more viscous must be the substratum to support cell spreading (8). Our results do not concur with these findings in that the best cell growth occurred on FC-72, which has the lowest viscosity (0.4 centistoke; 1 centistoke = 0.01 $m^2/s$) of the fluorocarbons tested, whereas the poorest growth was on FC-77 (0.8 centistoke), which has a viscosity intermediate between FC-72 and FC-70 (13.4 centistoke). We suggest that the relative tensile strength of the protein film is the important consideration, and this should be largely independent of the viscosity of the underlying fluid. One example that supports this independence is given in Fig. 4, in which 3T3-L1 cells are shown spreading on the protein layer that forms at the interface between tissue culture medium and air.

Several attempts have been made to demonstrate and measure the tension exerted by cells on their substrates (5, 8, 9). The use of mixtures of liquid fluorocarbons may prove useful in future investigations. By using different blends of FC-77 and FC-70, for example, it should be possible to vary the strength of the denatured protein layer and determine the relative ability of cells to spread and crawl about on the interface. This ability should be inversely related to the contractile forces generated by the cells.

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