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Cell Biology

Cell growth on liquid interfaces: Role of surface active compounds

(protein adsorption at interfaces/fluorocarbon and silicone fluid substrates/liquid microcarriers)

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ABSTRACT **Anchorage-dependent fibroblasts can be cultured by using as a substrate the protein layer that spontaneously forms at the liquid–liquid interface between fluorocarbon fluids and tissue culture medium. For this novel substrate to be effective in supporting confluent cell layers, the protein monolayer must support the stresses exerted by spreading fibroblasts. The composition of the fluorocarbon fluid has a significant effect on the strength of the protein layer and, thus, on the patterns of cell growth. Evidence is presented demonstrating that a protein film, sufficiently strong to support cell growth, does not occur on purified fluorocarbon fluids but requires the presence of trace amounts of polar, surface active compounds. By the addition of small quantities of pentafluorobenzoyl chloride to alumina-treated fluorocarbon fluids, excellent interfacial substrates can be produced. We have applied this understanding to produce a liquid microcarrier system capable of general use with a variety of cells, including human fibroblasts. A microcarrier in which the fluorocarbon is replaced with polydimethyldiphenyl siloxane is also described.**

Cell–substrate interactions have been extensively studied in tissue culture by using a wide variety of different materials as substrates. (For review see ref. 1.) Although these materials have almost exclusively been solids, it is possible to grow anchorage-dependent cells at the phase boundary between fluorocarbon fluids and tissue culture medium (2–4). With both solid and fluid surfaces, the cells do not interact directly with the substrate but with a monolayer of denatured protein that spontaneously adsorbs to the interface. Unless steps are taken to first coat a surface with a specific protein, this monolayer will consist of a collection of those proteins present in the tissue culture medium, usually serum proteins.

Patterns of cell growth observed on fluid interfaces often differ from those seen on solids and are dependent upon the cell line studied. For example, when the mouse fibroblast 3T3-L1 cell line was grown on a suitable fluorocarbon, "lake-like" openings were observed in an otherwise confluent monolayer of cells. On the same fluorocarbon the transformed mouse fibroblast SV-T2 line, which on solid substrates forms a confluent cell layer, tended to clump into isolated aggregates of cells. It has been demonstrated that these patterns are a consequence of cell-mediated fracturing of the absorbed protein monolayer. In experiments in which the tensile strength of the adsorbed protein film was increased in two independent manners, these patterns were essentially replaced by normal, confluent cell growth (3).

The composition of the fluorocarbon fluid used for interfacial culturing was also observed to influence the pattern of cell growth. For example, 3T3-L1 cells grown on a commercial grade of either 15-carbon perfluorotertiary amines or perfluorohexanes exhibited excellent growth and normal fibroblast morphology. However, the same cells on a commercial grade of a mixture of perfluorooctanes and eight-carbon perfluoroethers exhibited poor growth and resembled epithelial cells in morphology (3). In this paper evidence is presented that the different behavior of cells on the various fluorocarbon fluids is due to the presence of a trace amount of surface active compounds and not due to the bulk composition of the oils.

MATERIALS AND METHODS

Materials. Fluorocarbon fluids were gifts from 3M (St. Paul, MN) (see Table 1); pentafluorobenzoyl chloride (F_5BzCl) was purchased from PCR Research Chemicals (Gainesville, FL); polydimethyldiphenyl siloxane (SF1154) was obtained from General Electric Silicone Products Department (Waterford, NY). In some cases the fluorocarbon fluids were passed through columns of loosely packed alumina (neutral, Brockman activity 1, 80–200 mesh; Fisher). The volume of fluorocarbon treated in this manner usually did not exceed ≈3 times the bed volume of the column.

Cells. The fibroblast lines 3T3-L1 and MRC-5 were obtained from the American Type Culture Collection. The human female fibroblast line was established from amniocentesis (from D. Wilson, Rensselaer Polytechnic Institute). 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% fetal bovine serum for the human lines or 10% newborn bovine serum for the murine 3T3-L1 line.

Preparation of Planar Fluorocarbon Interfaces. Planar interfaces were prepared as described (3). Fluorocarbon fluid (0.1 ml) was placed in a cylindrical well of a 96-well microtest plate (inside diameter 7 mm), and 0.2 ml of complete culture medium was carefully pipetted over the fluorocarbon, forming a two-phase system. A cell suspension (0.1 ml; 3×10^5 cells per ml) was added to the upper phase, and after ≈18 hr the interface was observed.

Preparation of Fluorocarbon Microcarriers. Fluorocarbon fluids were combined with F_5BzCl (see individual experiments for concentration). The fluorocarbon was forced by using air pressure through a drawn out a Pasteur pipet (terminal bore, ≈100 μm) into a culture tube containing tissue culture medium with 10% bovine serum. The volume of the culture medium was usually 5–10 times the volume of fluorocarbon to be emulsified. The air pressure could be regulated up to a maximum of 15 psi (1 psi = 6.89 kPa). With a little experience it was possible to select a specific pressure that produced approximately uniform droplets with 200-μm diameters.

Immediately after the emulsification procedure, the tube containing both the droplets and the excess culture medium was gently agitated for 10 min to prevent aggregation of the

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Abbreviation: F_5BzCl, pentafluorobenzoyl chloride.

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droplets. The droplets were then allowed to settle, the excess medium was aspirated, and fresh medium was added to the tube. After at least 1 hr of gentle agitation, the excess medium was again replaced and the emulsion stored at 4°C.

Preparation of Silicone Oil Microcarriers. Two milliliters of polydimethyldiphenyl siloxane containing F_5BzCl was combined with 4 ml of complete culture medium (10% bovine serum). The mixture was emulsified by rapid agitation in a vortex mixer for ≈5 sec to produce the desired droplet size. Additional culture medium (6 ml) was added and the emulsion was gently agitated. From this point on, the emulsion was handled in a manner identical to the fluorocarbon fluid microcarrier described above.

Microcarrier Inoculation and Cell Counting. A 0.1-ml portion of emulsion was pipetted into 0.4-ml microwells with a large-bore pipet, and 0.1 ml of a cell suspension in culture medium was added to each well without mixing. The remaining volume was filled with culture medium and the inoculated wells were incubated. To feed the cultures, a 0.2-ml portion of spent medium was withdrawn from each well and replaced with fresh medium.

To determine cell numbers the entire content of a well was transferred by pipet 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 (5) for 1 hr at 37°C with occasional vigorous agitation. Stained nuclei were counted in a hemocytometer. This procedure does not count nuclei from cells attached to the surfaces of the microwell and eliminates most of those from cells that might have detached from the droplets at any point before removal of the excess continuous phase.

RESULTS

Table 1 lists the fluorocarbon fluids we have attempted to use as interfacial substrates for growth of 3T3-L1 fibroblasts. The ability of the fluids to form acceptable substrates was clearly not related to their bulk composition. One striking confirmation of this was that two of the fluids (FC-72 and L-2345), both analyzed to be primarily perfluorohexanes, elicited very different cellular behavior when employed as substrates. These results suggested that the variability in the fluids was due to contaminating compounds that either spoiled or facilitated cell spreading and growth. These contaminants could be present in only trace amounts, as analysis of the fluids by gas chromatography/mass spectrometry and infrared spectrometry revealed only the bulk constituent(s) (data not shown).

Table 1. Cell behavior on fluorocarbon fluid substrates

Composition of fluorocarbon fluids	3M designation	Interfacial cell growth*
C_6F_{14}	FC-72	F
C_6F_{14}	L-2345	R
C_8F_{18}	L-1041	R
$C_8F_{16}O$	L-2577	R
$N(C_4F_9)_3$	FC-43	F
15-Carbon perfluoro-tertiary amines	FC-70	F
C_8F_{18}, $C_8F_{16}O$	FC-77	E

The primary composition of each fluorocarbon fluid is listed as determined by gas chromatography/mass spectrometry. Planar interfaces were prepared and inoculated with 3T3-L1 cells. After 18 hr the interfaces were examined.

* F, cells well spread with fibroblastic morphology. E, cells spread but with epithelial morphology. R, no cell spreading observed; cells remain rounded.

When any of the fluorocarbons tested was passed through neutral alumina columns, its ability to serve as a substrate for cell growth was lost, and the cells remain rounded at the interface. Silica gel adsorption was also investigated, but this procedure had no discernible effect on the various fluorocarbons (data not shown).

Suspecting that polar, surface active contaminants of the fluorocarbon fluids facilitated cell spreading and growth, we added different fluoronated amphipathic compounds to various alumina-treated fluorocarbon fluids and observed their effect on cell behavior at the interfacial substrate. The most effective of a limited number of compounds tested in promoting interfacial cell growth was F_5BzCl. Without this additive, 3T3-L1 cells settled to the interface and eventually drifted together to form isolated clusters of rounded cells (Fig. 1*A*). At low F_5BzCl concentrations, the cells appeared epithelial in morphology (Fig. 1*B*), whereas, when present in small quantities of a few micrograms per milliliter, excellent cell growth was obtained at the fluorocarbon interface, resulting in a confluent cell layer (Fig. 1*C*).

Best growth resulted when the concentration of F_5BzCl was ≈2 μg/ml or equivalent to about 120 ng/cm^2 of interfacial area (the actual surface concentration is not known as we have not studied the partitioning of this compound between the two phases with the adsorbed protein layer in place). If the F_5BzCl concentration was elevated to 16 μg/ml, cell growth was reduced (Fig. 1*D*), and at a high concentration (250 μg/ml), no cell spreading could be observed and the cells remained in a dispersed pattern (Fig. 1*E*). Also, at high levels of F_5BzCl, the adsorbed protein film was clearly not monomolecular as it could sometimes be seen to wrinkle or tear under Nomarski optics. At even higher levels of the additive (>500 μg/ml), the interface appeared grainy, presumably the result of spontaneous emulsification.

We have obtained results similar to those reported above where the fluorocarbon phase has been replaced with silicone oil. By using polydimethyldiphenyl siloxane with F_5BzCl, patterns similar to those in Fig. 1 were obtained for corresponding concentrations of the acid chloride (data not shown).

The reduction of cell spreading and growth observed at high concentrations of F_5BzCl under the experimental conditions of Fig. 1 *D* and *E* is due to toxic effects of the acid chloride. When 3T3-L1 cells were introduced 24 hr after the preparation of the interfaces, the fibroblasts grew with as high as 250 μg of F_5BzCl per ml in the fluorocarbon phase. A better experiment involved inoculating an interface twice. After the first inoculation the cells remained rounded. The interface was then inoculated 24 hr later, and the newly added cells were observed to spread and grow. Presumably during the time between inoculations, a sufficient amount of F_5BzCl reacted with the protein at the surface or in the medium and was no longer toxic to the fibroblasts.

Another means of converting alumina-treated fluorocarbons into fluids that form good phase-boundary substrates for cell growth was discovered in experiments involving attempts to extract the endogenous surface active materials by sonication of the fluorocarbon fluids with polar solvents. Quite unexpectedly, when water was used in this procedure, the unsuitable, alumina-treated fluorocarbons were converted into excellent substrates. This change was reversed by alumina adsorption. The behavior of 3T3-L1 cells on fluorocarbon fluid that was sonicated with water for varying times is shown in Fig. 2; the effect of sonication with water completely mimics the addition of F_5BzCl in terms of cell behavior. Effective substrates could also be generated by sonication of alumina-treated fluorocarbons with ethylene glycol but not with methanol (data not shown). The changes in the fluorocarbon fluids upon sonication

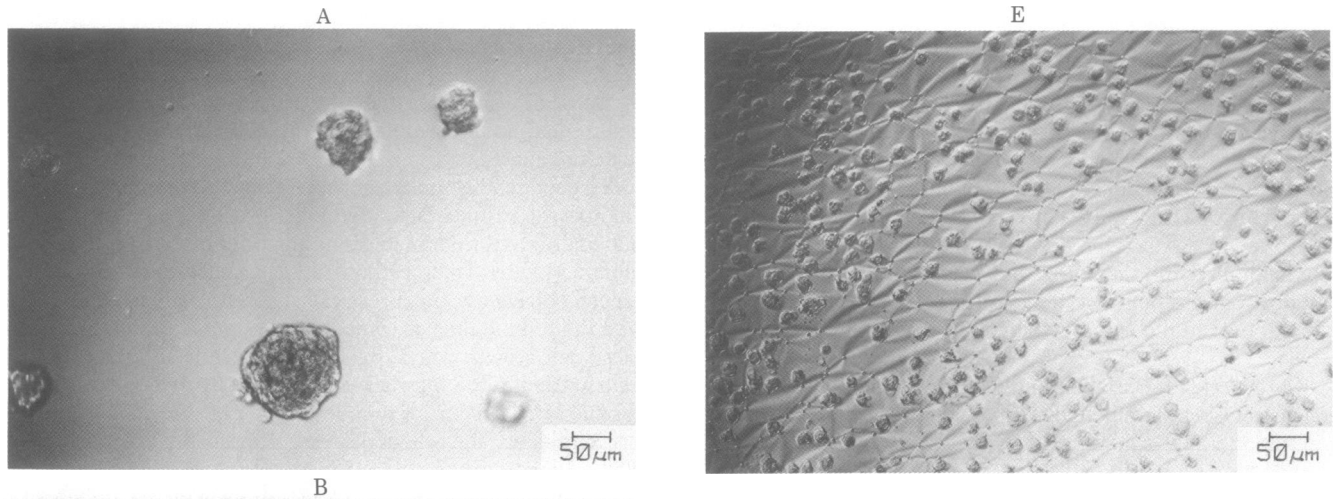

FIG. 1. Cell behavior on interfaces prepared with F_5BzCl-containing fluorocarbon fluids. Planar interfaces were prepared by using alumina-treated L-2577 containing different concentrations of F_5BzCl. Eighteen hours following inoculation with 3T3-L1 cells, the interfaces were photographed (Nomarski optics). Concentration of F_5BzCl (μg/ml): (*A*) 0, (*B*) 0.2, (*C*) 4, (*D*) 16, (*E*) 250.

with water or ethylene glycol probably involve the formation of surface active compounds from reactions driven by localized heating; however, the chemistry has not been elucidated.

We have applied this understanding of interfacial substrates in redesigning a liquid microcarrier system. In previous work

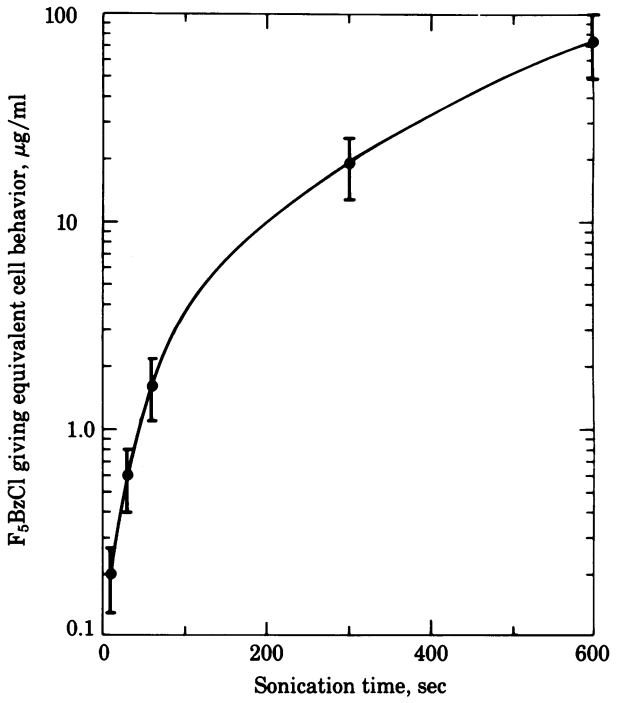

FIG. 2. Effect of sonication of fluorocarbon fluids with water. L-2577 (0.25 ml) and distilled water (0.50 ml) were combined in separate 1.5-ml micro test tubes. The mixtures were sonicated for different times by using a microtip source (output, ≈30 W). The fluorocarbon fluids were recovered by centrifugation, and 1:2 serial dilutions of the fluids in nonsonicated L-2577 were prepared as was a 1:2 serial dilution of F_5BzCl in L-2577. Planar interfaces were prepared and inoculated with 3T3-L1 cells. After 18 hr of incubation, the degree of spreading and type of cellular morphology were noted, and each sonicated fluorocarbon fluid series was correlated with the concentration of F_5BzCl that yielded a similar response. The uncertainty in this determination is represented by the bar for each time.

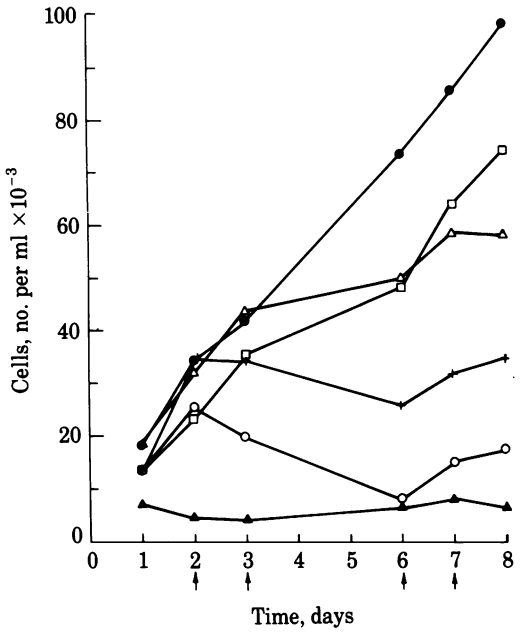

FIG. 3. Growth of fibroblasts on fluorocarbon microcarriers containing different concentrations of F_5BzCl. Microcarriers were prepared with L-1041 fluorocarbon fluid containing from 0–500 μg of F_5BzCl per ml. Each 0.1 ml of emulsion was inoculated with 30×10^3 human female fibroblasts, and growth was followed for several days. Arrows indicate times of cell feeding. Concentration of F_5BzCl (μg/ml): ▲, 0; ○, 8; +, 32; △, 125; ●, 500; □, 2,000.

we had found that fluorocarbon fluids that served as acceptable interfacial substrates in a planar configuration were unable to support cell growth when dispersed into small droplets (100–500 μm in diameter) and stabilized with only a serum protein monolayer. To obtain logarithmic cell growth on such droplets, it was necessary to first adsorb a layer of poly-L-lysine at alkaline pH, followed by a second layer of serum proteins (4).

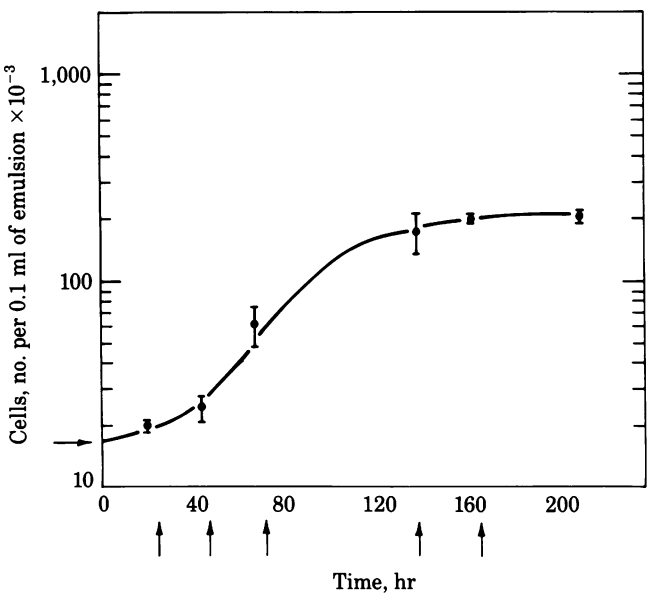

FIG. 4. Growth of MRC-5 cells on a fluorocarbon fluid microcarrier. A microcarrier was prepared by using L-1041 containing 500 μg of F_5BzCl per ml and inoculated with MRC-5 cells (passage number 22, 17×10^3 cells per 0.1 ml of emulsion). Vertical arrows indicate times of cell feeding. Each point is the average of three separate emulsions; bars indicate the range of values obtained.

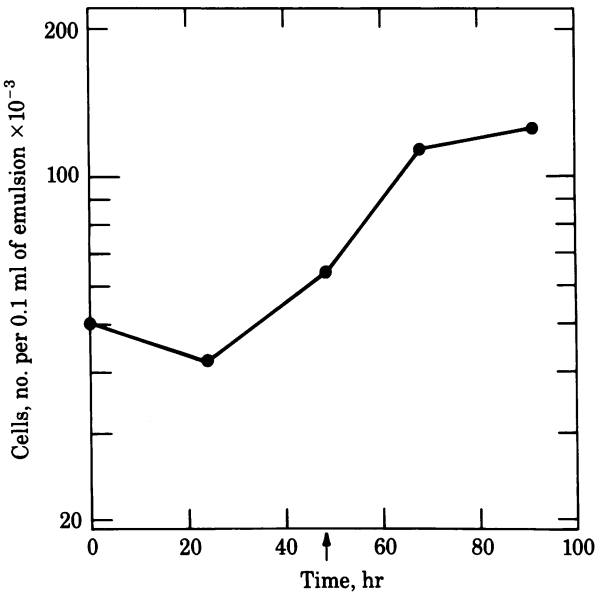

FIG. 5. Cell growth on a silicone fluid microcarrier. Microcarriers were formed by emulsification of polydimethyldiphenyl siloxane containing 250 μg of F_5BzCl per ml and were inoculated with 3T3-L1 cells (50×10^3 cells per 0.1 ml of emulsion). The cells were fed 48 hr after inoculation.

This double-layered protein coat was able to support growth of 3T3-L1 cells but not of normal human fibroblasts. Previously we were unable to explain why interfaces coated only with serum protein served as appropriate cell substrates in a planar configuration but not in the spherical configuration of the small droplets. However, in light of the results presented in this paper, the explanation is clear. The surface area-to-volume ratio of the planar arrangement was ≈4 cm^{-1} compared with 300 cm^{-1} for a sphere 200 μm in diameter. Thus, in the spherical configuration, the beneficial endogenous contaminant(s) of the fluorocarbon, required for interfacial growth, was not present at the interface in sufficient concentration.

To produce a liquid microcarrier stabilized only by serum proteins, F_5BzCl was added to the fluorocarbon fluid in varying concentrations prior to dispersing the liquid into droplets. As expected based on area-to-volume considerations, when the concentration of F_5BzCl was ≈100 times the appropriate level for cell growth on planar interfaces, the microcarriers were capable of supporting growth of diploid human fibroblasts (Fig. 3). Microcarriers formed of alumina-treated perfluorooctane containing 500 μg of F_5BzCl per ml were used to obtain logarithmic growth of MRC-5 cells (Fig. 4). A silicone oil microcarrier has also been used to obtain logarithmic growth of 3T3-L1 cells (Fig. 5).

CONCLUSION

It has been suggested that an acceptable substrate for anchorage-dependent fibroblasts must be sufficiently rigid to withstand the forces exerted on it by the cytoskeletons of the spreading cells (6). The results presented in this paper suggest that interfaces between *pure* fluorocarbon fluids and tissue culture medium are *not* capable of supporting the growth of normal fibroblasts. Although surface tension measurements in this laboratory have indicated that proteins adsorb to alumina-treated fluorocarbon interfaces (data not shown), when this monolayer is used as a substrate, it quickly yields to the stresses exerted by the cells, presumably because the interactions between the individual protein molecules are weak, and spreading is not

possible. By adding appropriate surface active compounds such as F_5BzCl to the fluorocarbon phase one can affect the tensile strength of the adsorbed protein layer(s). We speculate that when sufficient amounts of F_5BzCl are added to the fluorocarbon phase, these amphipathic molecules become concentrated at the interface where the acid chloride reacts with water to produce pentafluorobenzoic acid that dissociates to produce a negatively charged layer or the F_5BzCl reacts directly with amino and other susceptible groups on the adsorbed proteins (or both). In this situation we believe the proteins become denatured to the point that the interactions between them form a rigid network that can support stresses generated by the spreading cells. Previous successful results of growth of anchorage-dependent cells on commercial grade fluorocarbon fluids were undoubtedly due to the presence of trace amounts of a surface active contaminant(s) that most likely mediated the adsorption of a rigid protein film at the interface.

It should be noted here that in contrast to anchorage-dependent cells, the transformed cells we have tried are capable of multiplying on a purified fluorocarbon fluid. The reason for this is that transformed cells can sustain growth in a rounded state; this is presumably the reason why transformed cells also can grow dispersed in soft agar.

By utilizing fluorocarbon fluids containing acid chloride, a liquid microcarrier system has been developed capable of use with normal human fibroblasts. In this arrangement the minimal concentration of F_5BzCl required for cell growth was that predicted based on the increased area-to-volume ratio of this configuration. A microcarrier based on silicone oil has been used to culture mouse fibroblasts. The silicone oil used in these studies has the advantage of having a specific gravity (1.05) suitable for stirring arrangements that are often employed in mass culturing cells using microcarriers. As we have previously indicated (4), such liquid microcarriers are unique in that cell harvesting can be carried out by mechanical disruption of the emulsion. When this is performed, the cellular mass collects at the phase boundary between the two continuous phases.

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