(major fraction), and 0.31 to 0.4, respectively. Biological activity was noted in the major component.

Principal absorptions in infrared analysis were almost identical for each of the three fractions. The major component had the following values: 3380 (broad); 2920, 2860; 1712; 1670 and 970; 1440; 1380 and 1365 $cm^{-1}$. Treatment of the material with sodium borohydride in isopropanol (*5*) eliminated the absorption peak at 1712 $cm^{-1}$, and it indicated the presence of a C=O function. Spraying the material with 2,4-dinitrophenylhydrazine on thin-layer chromatography plates gave a positive response for a carbonyl function (*6*). Bromination in ether completely eliminated the infrared absorption peaks at 1670 and 970 $cm^{-1}$, and there was a concomitant shift in the peak at 1712 $cm^{-1}$ to 1725 $cm^{-1}$. The infrared pattern of the major component remained unchanged on treatment with diazomethane.

The ultraviolet spectrum of the tobacco isolate showed $\gamma_{max}^{EtOH}$ 208 nm (strong $\varepsilon_{max}$), and analysis of the material by combined gas chromatography and mass spectrometry suggests that the compound has a molecular ion at *m/e* 288.

Infrared studies demonstrate that the major isolate from tobacco contains OH, C=O, and *trans* double bond functions, and that there is no indication for aromaticity. Nor does the spectrum match that of abscisin. Furthermore, the ultraviolet absorption for abscisin is $\gamma_{max}^{EtOH}$ 253 and it has a molecular weight of 264. I conclude that the growth inhibitor isolated from expanding tobacco leaves is not abscisin, but rather, a new growth-inhibiting substance.

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## Microcloning and Replica Plating of Mammalian Cells

Abstract. *A technique for cloning single mammalian cells has been developed by means of a microculture method (0.010 milliliters per culture). A technique of replica plating has also been developed for use with clones of single cells in microcultures; this technique permits the screening of desired properties of many clones simultaneously before the growth of stock cultures. With these techniques an investigator can with limited budget, space, and personnel perform research using tissue culture systems.*

I have recently described a technique of cloning and replica plating for animal virus in microculture (*1*). This report describes the development of analogous methods for culturing and replica plating mammalian cells in microculture.

The cell line used was CV-1, a line derived from African green monkey kidney cells (*2*). The culture media used were the following: 3E, Eagle's minimum medium supplemented to threefold concentrations of the amino acids, glucose, and vitamins (NIH medium unit); 3F, powdered nutrient medium F-12 without $NaHCO_3$ (Schwarz BioResearch, Orangeburg, N.J.) supplemented to threefold concentrations of amino acids, glucose, and vitamins; and F—nutrient medium F-12 with no additional amino acids, glucose, or vitamins. All three media contained penicillin (100 unit/ml), streptomycin (100 $\mu$g/ml), Mycostatin (10 unit/ml), and fetal bovine serum (9 percent) (Flow Laboratories, Rockville, Md.); they were buffered at *p*H 7.5 with 0.05*M* *N*-tris(hydroxymethyl) methylglycine (tricine, Calbiochem) (*3*). All incubations were carried out in humidified incubators (Wedco, Inc., Silver Spring, Md.) without $CO_2$. The replica plating device was described previously (*1*).

Cloning efficiencies for the cell line in each of the media were determined by the conventional petri dish method and the microcloning method (Table 1). Equal numbers of cells were grown in each of the media in 30-ml plastic flasks (Falcon). Growth rates in the media were similar. When the monolayers were about 75 percent of confluency, suspensions of single cells were made by dissociating the cells with trypsin [0.10 percent trypsin crystallized once (Worthington Biochemical Corp., Freehold, N.J.) in Puck's saline A (*4*)] at 26°C for 15 minutes and then gently pipetting them. The cells were washed once with phosphate buffered saline (PBS), centrifuged at low speed, and resuspended in their respective media. Two sets of dilutions were made from each of the original cell suspensions, one for microcloning in Microtest plates (2 by 3 inches, Falcon) and one for cloning in 32-mm plastic petri dishes (Falcon). The F medium was more suitable for cloning CV-1 cells than either 3F or 3E (Table 1). Since the microenvironment of cells in petri plates is probably different from that of cells in Microtest wells, it is difficult to evaluate the significance of the differences between the cloning efficiencies for the two sets of cultures. However, the efficiencies appear to be in reasonable agreement and are parallel for the three media.

There is currently no convenient technique for establishing that any individual "clone" of cells has been derived from a single cell. The following method was developed for this purpose. A suspension of single cells was made with F medium; each well in five Microtest plates (300 wells) received 0.001 ml of cell suspension with an average of 0.87 cell per well. Immediately after plating, the number of cells in each well was recorded by bright-field microscopy. (It was easy to count all the cells present in 0.001 ml of medium in the bottom of the well while they were rounded up and refractile prior to attachment.) The fact that the observed number of wells containing a given number of cells was quite close to that expected from the Poisson distribution indicates that the cell suspension was of single cells. After the number of cells per well was recorded, 0.010 ml of F medium was placed in each well, 0.25 ml of sterile PBS was placed along the sides of each plate, and the plates were tightly sealed in plastic bags and incubated at 38°C. After 18 days, the plates were fixed in absolute methanol and stained with Giemsa stain. The cloning efficiency with 0.87 cell per well was 21

percent; of all the wells that contained a single cell, 22 percent developed monolayers. (No monolayers formed when 0.005 ml of medium was added instead of 0.010 ml after the number of cells per well was counted.)

The main disadvantage of the microcloning system is that a small number of single cell clones are obtained per Microtest plate (between 2 and 20 depending upon the cloning efficiency). A system that could screen the clones for desired properties before the time-consuming growth of stock cultures would greatly offset the small number of clones obtained. The procedure of replica plating developed for animal viruses (*1*) was adapted so that one could screen the clones for specified properties using only the cells present in the original well. Selected clones can then be grown into stocks for further analysis.

Table 1. Cloning efficiency of CV-1 cells at 38°C in three media for techniques of microcloning and conventional cloning. For microcloning, three dilutions were made with each of the three media, and 0.010 ml of the appropriate cell suspension was placed into each well of the Microtest plate (*1*). Three plates (180 wells) were made for each cell dilution (5, 10, or 50 cells per well) in each medium. After 0.25 ml of sterile PBS was placed along the sides of each plate to prevent dehydration, the plates were tightly sealed in plastic bags and incubated at 38°C for 18 days. For conventional cloning, three cell dilutions were made so that about 100, 500, or 1000 cells were present in 2.0 ml of medium in duplicate 32-mm petri dishes for each medium; the cultures were then incubated at 38°C for 18 days. The Microtest plates and petri dishes were fixed with absolute methanol and stained with Giemsa. The cloning efficiency for the petri dish method was calculated by counting the number of clones per plates, dividing by the number of cells plated, and averaging the duplicate values. The number of cells capable of cloning per milliliter from the microcloning method was calculated by counting the number of wells that had not developed monolayers and by applying the Poisson distribution (*5*). The cloning efficiency was then calculated by dividing the number of clonable cells per milliliter by the number of cells in the original suspension and averaging the triplicate values. CE, cloning efficiency; AWC, all wells cloned; TM, too many clones to count.

| Microcloning | | Conventional cloning | |
|---|---|---|---|
| Cells per well (No.) | CE (%) | Cells per plate (No.) | CE (%) |
| | *Medium 3E* | | |
| 50 | AWC | 1100 | 5.4 |
| 10 | 0.55 | 550 | 0 |
| 5 | 0 | 110 | 0 |
| | *Medium 3F* | | |
| 50 | AWC | 900 | TM |
| 10 | 9.3 | 450 | 12 |
| 5 | 2.7 | 90 | 8.0 |
| | *Medium 4* | | |
| 50 | AWC | 1000 | TM |
| 10 | AWC | 500 | TM |
| 5 | 49 | 100 | 44 |

Once the single cell clones formed monolayers, they were treated with trypsin and replica plated in the following manner. The wells to be replica plated, or recovered for stocks, were carefully aspirated with low suction and a sterile Pasteur pipette. (About 0.002 ml of medium or PBS should be left covering the cells or they will be killed by dehydration in a matter of seconds.) After aspiration, 0.010 ml of PBS was placed into each well. After another aspiration, 0.005 ml of trypsin solution was added to each well. The plates were then incubated at 38°C for about 20 minutes until the cells had fully detached from the surface of the wells. Finally, 0.010 ml of F medium was added to each well. The recipient plates were prepared by placing 0.010 ml of F medium in each well. The replicator was placed in absolute ethanol, flamed, cooled in air for 3 minutes, and placed in a Microtest plate containing 0.010 ml of PBS per well in order to fill the grooves in the bolts and dilute any remaining ethanol to nontoxic levels. The replicator was placed into the master plate containing the dissociated cells and rocked several times to suspend the cells in the medium and then placed into the recipient wells and again rocked several times to insure adequate release of cells into the recipient wells. Inoculation of the recipient plate was performed as many times as was desired to insure that an adequate number of cells was transferred. After one master plate was replica plated, the replicator was dipped in glass-distilled water to remove cells and medium and then placed in absolute ethanol until the next master plate was replica plated. After replica plating, the master plates were incubated at 35°C as stock plates. (The fetal bovine serum inactivated the trypsin in each well to a level that allowed remaining cells to attach and divide to form complete monolayers.) Two inoculations were sufficient for the production of complete monolayers in 100 percent of those recipient wells receiving cells after 14 days at 38°C. For cell lines with high cloning efficiencies, a single inoculation might be sufficient to establish monolayers. The number of cells transferred was very dependent upon the completeness of cell detachment, but was about 1 percent.

When the clones were to be grown into large stocks, the following procedure was found to be simple, reliable, and without a significant incidence of contaminating infections. After cell detachment with trypsin, a 1.0-ml sterile disposable tuberculin syringe (JELCO Labs, Raritan, N.J.) with a sterile disposable 22-gauge needle was filled with 0.10 ml of medium and used to suspend and remove the cells from the well. The cell suspension was placed into a 32-mm plastic tissue-culture petri dish containing 2.0 ml of medium. Two of these dishes were placed in an 85-mm plastic petri dish containing 1.0 ml of sterile PBS, tightly sealed in plastic bags, and incubated at 38°C for 14 to 21 days. They were then subcultured into larger containers.

The techniques described in this report are widely applicable to (i) the microculturing of mammalian cells, (ii) the isolation of cell clones unequivocally derived from a single cell, and (iii) the rapid screening of such clones for desired properties by replica plating into suitable test media. A similar technique of microcloning and replica plating has been described by Goldsby and Zipser (*6*), who used larger plates and ten times the volume of medium per culture. Reasonable areas of application include screening for cell mutants; establishing growth requirements for specific cells; screening and quantitating mutagenic, carcinogenic, pharmacologic, and toxic effects of drugs on cells and/or viruses; measuring virus production in a single cell; and screening single cells or clones for production of specific molecules.

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