has been the necessity of initiating treatment early during the incubation phase of a viral infection. It is apparent that any form of therapy which is effective only when utilized prior to onset of disease has a limited clinical potential. In the next experiment, treatment of mice infected with EMC virus was delayed until the animals developed symptoms of infection. The pathogenesis of EMC virus infection in mice is characterized by a viremia within 24 hours of infection, with seeding of target organs and the central nervous system by 24 to 48 hours. The amount of virus in the brain reached a maximum by 72 to 120 hours. The development of ruffled fur, hunched posture, and paralysis may be correlated with involvement of target organs and was observed at days 4 and 5 after infection. Treatment with interferon-producing macrophages was delayed until day 5 of infection when maximum titers of virus were already present in the brain and all animals were either paralyzed or had evidence of ruffled fur and hunched posture. Infected animals were divided into paralyzed, or sick but not paralyzed, groups and then randomly distributed into three subgroups each containing equal numbers of paralyzed animals and sick but not paralyzed animals. Treatment consisted of daily intraperitoneal inoculations of interferon-producing macrophages or control preparations for five consecutive days (Fig. 3). The combined results from both experiments were 10 survivors out of 26 animals in the macrophage treatment group compared with only 2 of 30 and 3 of 27 survivors in the control groups receiving sham injections or cell-free supernatant fluid, respectively.

These results demonstrate that the outcome of virus infection may be altered after involvement of target organs and onset of clinical symptoms. The efficacy of the technique of transferring virus-infected macrophages may (i) be a function of interferon production by these cells in recipient animals; (ii) reflect a capacity of these cells to reach the site of an inflammatory reaction; or (iii) be a result of the delivery of an interferon-inducer to other host cells. My data do not permit definition of the mechanism by which transferred macrophages affect the course of infection. Although no evidence of replication of CV in macrophage cultures in vitro could be found, the possibility exists that virus did multiply in vivo, thus inducing interferon production or interfering with SFV or EMC. This would seem unlikely because of the induction of only low levels of interferon by inoculation of the unadsorbed CV in the control preparations and because there was no evidence of the development of disease in any of the control animals receiving the supernatant or sonified preparations (*6*). The recovery of recipient animals from an established viral infection suggests that transfer of interferon-producing white blood cells may offer another approach to therapy for viral infections.

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A Growth Inhibitor from Young Expanding Tobacco Leaves

Abstract. *Extracts of expanding tobacco leaves inhibit the growth of wheat coleoptiles. Infrared, ultraviolet, and mass spectroscopy show that the active component is neither abscisin nor an aromatic structure.*

A general characteristic of young tobacco plants is that they have exceedingly small axillary shoots and that only when the plants mature, or when apical dominance is destroyed, do they begin rapid growth. Flue-cured tobacco (*Nicotiana tabacum* var. Hicks) is no exception to this general observation. Consequently, from studies of other genera, one would predict the presence of an inhibitor of axillary shoot growth either in the buds or in leaves adjacent to these organs (*1*). Recently, Steadman and Sequira reported an acidic ether-soluble substance in tobacco tissues and indicated that it was possibly abscisic acid, or inhibitor-β (*2*).

The isolation of a growth inhibitor from tobacco leaves for potential control of axillary bud development is of very practical value. In normal tobacco culture the developing inflorescence and uppermost expanding leaves are pruned. Consequently, the axillary shoots begin rapid growth and they must be removed if the main leaves are to attain maximum size. The lack of manual labor to perform this task has led to the use of synthetic inhibitors of axillary bud growth. Certain of these produce undesirable effects in the leaves, such as thickening, so that a natural inhibitor derived from the plant may yield a more desirable form of axillary bud control.

Expanding leaves were harvested from Hicks tobacco plants, immediately frozen in solid carbon dioxide, and ground in cold 80 percent methanol. After 24 hours at 0°C, the brei was filtered, and the filtrate was evaporated to a viscous liquid, under vacuum, at 45°C. The resultant aqueous phase was adjusted to *p*H 3 with HCl and extracted thrice with peroxide-free diethyl ether. The ether was removed under vacuum after the sample was dried over anhydrous sodium sulfate, and the residue was assayed by the *Triticum* (var. Wakeland) coleoptile bioassay (*3*) in citrate-phosphate buffer solutions containing 2 percent sucrose (*4*). A portion of this crude extract, equivalent to 100 mg (dry weight) of leaf tissue, inhibited coleoptile segments 54 percent relative to the controls ($P < .01$). Chromatography of the extract on Whatman No. 1 paper developed in isopropanol, ammonia, and water (10 : 1 : 1, by volume) and subsequent bioassay of 1-cm segments revealed that the growth inhibitor had migrated to the solvent front and demonstrated that this system was inadequate to resolve the inhibitor. Authentic abscisin had an R_F of 0.6 to 0.7 in this system.

When the crude extract was subjected to silica gel (Merck) column chromatography, with ethyl acetate–benzene (55 : 45, by volume) as the eluting solvent, one major and two minor components were separated. The three compounds were analyzed in silica gel (Gelman) thin-layer chromatography, with the same solvent, and were detected with iodine vapors. The R_F values were 0.04 to 0.11, 0.22 to 0.3

(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 γ_{max}^{EtOH} 208 nm (strong ε_{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 γ_{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 μg/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