Growth in Microculture of Single Tobacco Cells Infected with Tobacco Mosaic Virus

Abstract. *Single tobacco callus cells with or without tobacco mosaic virus inclusion bodies from systemically infected* Nicotiana tabacum *plants were grown in microcultures. The culture medium consisted of mineral salts and sucrose; it also contained coconut milk. Out of 100 inclusion-bearing cells and 150 inclusion-free cells, 10 and 70 cultured cells divided; eventually 5 and 65 cells, respectively, formed single cell clones. The 5 clones derived from inclusion-bearing cells, and all but 3 of 40 clones from inclusion-free cells, showed virus inclusions in some cells. The virus could not be detected in three inclusion-free clones by local lesion assay. The results suggest single-cell culture methods for differentiating virus-free plants from cells of pathogen-infected plants.*

Much progress has recently been made in culturing single cells of higher plants (*1*). Vasil and Hildebrandt produced tobacco plants from a single cell isolated in microculture in fresh medium (*2*). Earlier studies had indicated that not all tobacco cells from callus infected with tobacco mosaic virus (TMV) were infected (*3*). Infectivity assays of single cells from TMV-infected callus tissue showed that 40 percent contained virus. Similarly, some plants differentiated from the infected callus tissue were free of virus. Previously, TMV inclusion bodies were reported in tobacco tissue cultures, and homogenates of single cells containing virus inclusion bodies produced more lesions on local lesion host assay plants than homogenates of cells without inclusion bodies (*4*). In our new studies, attempts were made to culture single, completely isolated, inclusion-bearing and inclusion-free cells to measure the severity of infection in single cell clones and the perpetuation of infection in succeeding subcultures of the tissue.

The leaves of 3-week-old *Nicotiana tabacum* plants of variety Havana 38 (systemic host) were inoculated with a TMV extract. Stem sections were excised 6 weeks later from plants showing severe symptoms of tobacco mosaic virus, placed on agar medium containing coconut milk and 2,4-dichlorophenoxyacetic acid ("D" medium) (*5*), and incubated in a dark room maintained at 26°C and 30 percent relative humidity. The callus that proliferated from stem sections was subcultured on "D" medium in dark and in light. The callus tissue was transferred to liquid "C" medium (same as "D" medium but without 2,4-D) to permit dissociation into single cells. Liquid cultures were maintained on a reciprocating shaker. After 10 to 11 days, single cells were picked with micropipettes and transferred aseptically to microchambers (*6*). The microculture permitted periodic examination of living cells over a number of days.

The inclusion bodies of tobacco mosaic virus have been carefully studied in infected plants (*7*) and in tissue cultures (*4*). Such inclusion-bearing and inclusion-free cells were cultured for this study. Small cells with large nuclei and a large number of active cytoplasmic strands were selected and placed in the microchamber after they had been washed in fresh "C" medium.

Out of 100 inclusion-bearing and 150 inclusion-free cells thus cultured, 10 and 70 cells divided, and eventually 5 and 65, respectively, formed colonies. Some of the cells did not divide after the two- or four-celled stage. The pattern of division, in most cases, conformed to that described earlier for normal tobacco cells (*2*). There was an initial lag period of 6 to 48 hours. Rarely, a cell would remain dormant, excrete material which surrounded it, and then divide.

The TMV inclusions observed were hexagonal plates, needles, and vesicles (*4*). The hexagonal plates disappeared and reappeared in many cases. In 90 percent of the cultured cells with needles, hexagonal crystals appeared within 6 to 24 hours after the preparation of cultures. Rarely, however, did needles appear when hexagonal crystal-bearing cells were cultured.

The position of the inclusion body in the daughter cell depended upon the original position of the inclusion in the dividing cell. The virus inclusions appeared to go to one or both of the resulting cells. In the sequence illustrated, needles and, later, hexagonal crystals appeared in the daughter cells (Fig. 1, *A–C*). Needles were not always seen in the original cell isolated in microculture or in the resulting two daughter cells (Fig. 1*A*), but after 6 hours, needles (*n*) appeared (Fig. 1*B*). A TMV hexagonal crystal (*c*) developed in the top cell later (Fig. 1*C*). Cells with bundles of long needles and abundant crystals frequently failed to divide. In some cases there was evidence of failure of cell-wall formation following nuclear division, resulting in a bilobed nucleus or two nuclei appressed to each other. This was observed in healthy tobacco callus cells but was perhaps more frequent in infected cells. In some cases, inclusions (particularly long needles) appeared to interfere by acting as mechanical barriers to cell division. This reversion to interphase was particularly evident when the cell showed numerous cytoplasmic strands and a stage of "fixed tension." In other cells, cross walls were formed with half the needle in one cell and half in the other.

About 50 percent of the inclusion-free cells divided, as compared with 10 percent of the inclusion-bearing cells. Divisions after the first were in quick succession, and a colony of 60 to 100 cells resulted within a week (Fig. 1*D*). Many of the cells of the colony after a week in microculture entered the enlargement phase.

A peculiar type of cell division was observed in some large spherical cells, both healthy and TMV-infected, with active streaming and abundant cytoplasmic strands (Fig. 1, *E–G*). Such cells, after 4 or 5 days or more, lost the transvacuolar cytoplasmic strands, and cyclosis was restricted to the cell periphery. The nucleus, and most of the cytoplasm, came to occupy a place at the periphery of the cell, where it divided and a cell wall formed. Thus a small cell rich in cytoplasm was cut off. The large cell again produced cytoplasmic strands but did not divide further. The smaller cell, however, began a series of divisions, giving rise to a colony.

All five colonies from inclusion-bearing cells and 40 out of 65 from inclusion-free cells were transferred to agar "D" medium and established as single cell clones. These clones of tissue were examined for virus inclusions. All five clones from inclusion-bearing cells showed TMV inclusion bodies in some cells. It was interesting that 37 clones from inclusion-free cells also had TMV inclusions in some cells. Three clones, however, were free of virus inclusions.

All the clones were assayed for TMV infectivity. A piece of the clonal

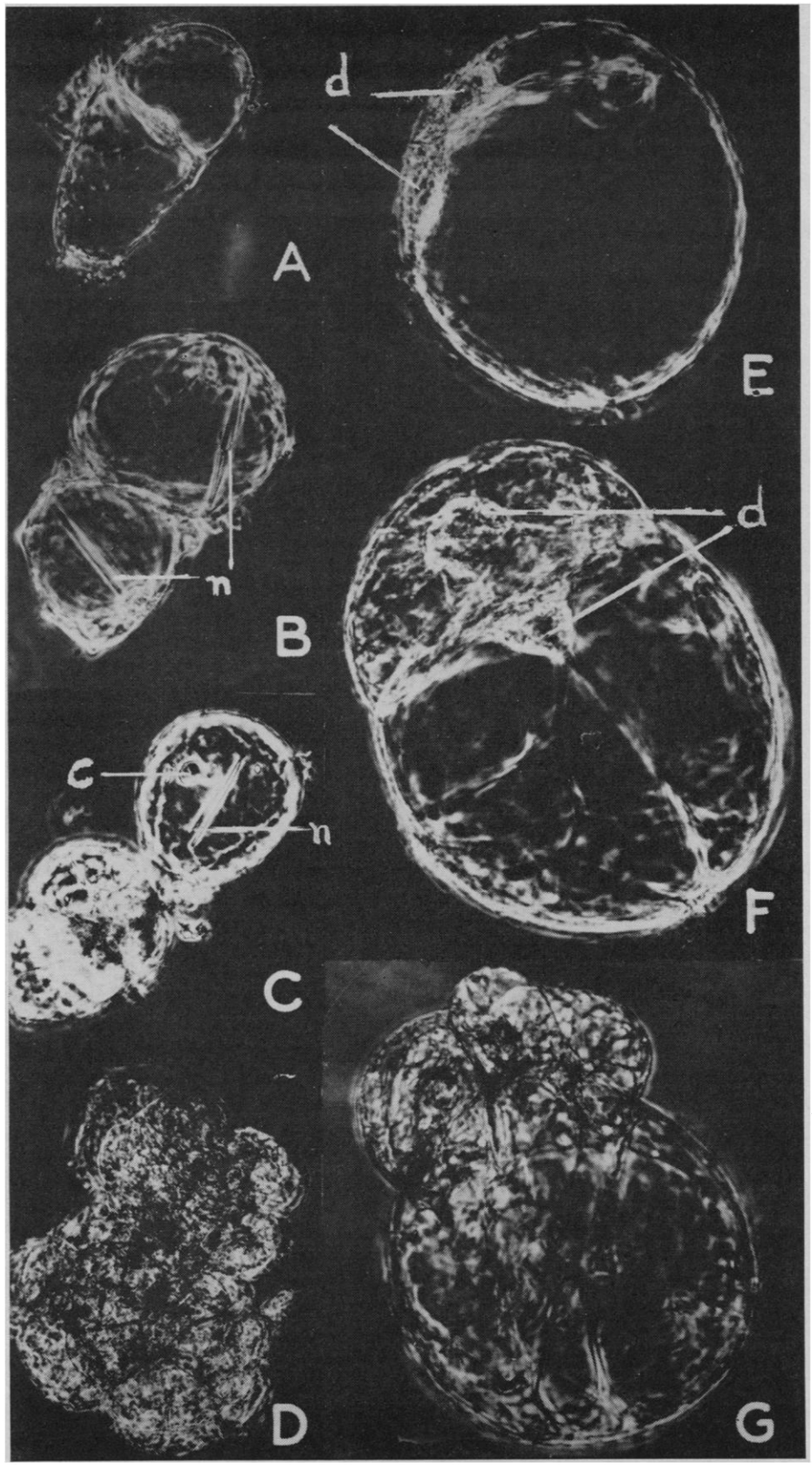

Fig. 1. (Left) Sequence of cell division and colony formation from a single tobacco cell growing in microculture: *A*, Two cells without TMV inclusions resulting from division of a single, isolated cell; *B*, the same cells showing TMV needles (*n*) that developed 6 hours after the first division; *C*, a hexagonal crystal (*c*) developed in the top cell; *D*, a colony of cells derived from *A*. Magnification about 600. (Right) Sequence of cell division and colony formation in some large spherical cells: *E*, A cell in division with two daughter nuclei (*d*) and without transvacuolar cytoplasmic strands; the small, richly cytoplasmic daughter cell at the top, *F*, subsequently divided to produce a mass of cells, *G*, while the large cell did not divide but showed renewed cytoplasmic activity. Magnification about 900.

tissue weighing approximately 50 mg was homogenized in phosphate buffer (*p*H 7.0), and the homogenate was applied to a Carborundum-dusted leaf of the local lesion host (*N. tabacum* × *N. glutinosa*) with a glass spatula. Extracts of the 37 clones with visible virus inclusions produced necrotic spots on assay plants, but the three clones without visible inclusions were free of TMV.

There are two possibilities as to the origin of the TMV-free cells: (i) that in the infected plant there are some cells in the stem which are free of the virus and continue to be propagated during subculturing, and (ii) that during subculturing some of the cells lose virus.

It is concluded that single TMV-infected cells are capable of division and colony formation in complete isolation and in fresh medium. Such meristematic cells are also capable of supporting TMV multiplication.

Currently meristem culture and heat therapy are often employed to eliminate virus from important horticultural crops. There are degrees of systemic involvement of tissues in virus diseases of plants. In some diseases, affected plants may yield healthy plants if short, stem-tip cuttings are rooted. In others, healthy plants can be obtained by growing smaller stem tips as scions on healthy seedlings until they are large enough to be rooted and established. In still others, healthy plants can be obtained only by differentiating plants in tissue culture from tiny blocks of apical meristem. Induced growth and differentiation from virus-free, single cell clones as obtained in this study and in others suggest another means to obtain virus-free plants not possible by other methods.

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Vitamin D_3-Induced Calcium-Binding Protein in Chick Intestinal Mucosa

Abstract. *The administration of vitamin D_3 to rachitic chicks induces in intestinal mucosal tissue the formation or elaboration of a calcium-binding factor which is found in the supernatant of the mucosal homogenate. The enhanced binding of Ca by the "vitamin D" supernatant (in contrast to "rachitic" supernatant) was indicated by a slower rate of diffusion of Ca^{45} across a cellophane dialyzing membrane and by a lesser amount of Ca^{45} being bound to an ion-exchange resin (Chelex-100) in the presence of vitamin D_3 supernatant. The binding activity was only associated with the protein fraction from a Sephadex G-25 column and was destroyed by trypsin digestion. This and other evidence suggest that the soluble factor is a protein. The vitamin D_3–enhanced duodenal absorption of Ca^{47} in rachitic chicks occurred almost simultaneously with the appearance of the vitamin D_3–induced factor, and there was good correlation between the concentration of binding factor and the rate of absorption of Ca^{47}.*

The intestinal absorption of Ca is generally depressed in vitamin D–deficient animals and is restored to normal by vitamin D supplementation. Despite extensive investigations, the mechnism by which this vitamin elicits its response is not known, although several theories have been proposed (*1*). Among these was the suggestion that vitamin D may act by stimulating the synthesis or operation of a "carrier" that would facilitate the uphill or downhill transepithelial movement of calcium. Such a carrier could possibly be proteinaceous in nature with the intrinsic ability to form an association with the substrate being transported, in this case, Ca. That vitamin D may be involved, directly or indirectly, in protein synthesis was supported by the observations that actinomycin D inhibited vitamin D–induced hypercalcemia in rats (*2*) and inhibited the vitamin D–stimulated absorption of Ca in rachitic chicks (*3*, *4*) and rats (*5*).

We reported evidence (*3*, *6*) suggesting that there is a factor in the supernatant of intestinal mucosa homogenates from vitamin D_3–treated chicks which depresses Ca^{45} uptake by homogenate debris. The factor was not present (or to a lesser extent) in the supernatant of mucosal homogenates from untreated rachitic chicks, and appeared to be a protein or closely associated with one. We now report that the vitamin D–dependent supernatant factor is indeed a protein, that it forms a soluble complex with Ca, and that there is a close relation, in time after vitamin D_3 administration, between the appearance of the Ca-binding protein and the enhanced absorption of Ca^{47}.

White Leghorn cockerels (1-day-old) were fed a rachitogenic chick diet (General Biochemical, Inc.). After 4 to 5 weeks on this diet, severe rickets was evident. The chicks were given 500 international units of crystalline vitamin D_3 orally in vegetable oil, or vegetable oil only, and decapitated 72 hours later. The duodenum was excised immediately, cooled to 4°C, slit open, rinsed with cold 0.12*M* NaCl, and blotted. The mucosal tissue was scraped from the underlying muscle layers with a glass slide, and the harvest was homogenized in tris buffer (20 percent weight/volume) with a Potter-Elvehjem homogenizer with Teflon pestle. The composition of the tris buffer was $1.37 \times 10^{-2}M$ tris hydrochloric acid, $0.119M$ sodium chloride, $4.74 \times 10^{-3}M$ potassium chloride, $9.85 \times 10^{-5}M$ glucose; *p*H 7.4. The homogenate was then centrifuged at 38,000*g* in a refrigerated centrifuge for 20 minutes, and the supernatant was recovered for subsequent use. The supernatant was heated at 60°C for 10 minutes to remove extraneous proteins; this treatment decreased the protein content without greatly affecting Ca-binding activity. Protein was analyzed by the procedure of Lowry *et al.* (*7*).

Calcium binding was determined in two ways. First, the rate of diffusion of Ca^{45} across a semipermeable membrane was measured in the presence of supernatant obtained from vitamin D–treated or rachitic chicks. The diffusion chamber consisted of a precursor-compartment containing 20 ml of supernatant from the mucosa of either rachitic or vitamin D–treated chicks, and the product compartment with 2.5 ml of "rachitic" supernatant only. The two compartments, separated by Visking cellophane dialysis membrane, were continuously mixed. After 6 μc of Ca^{45} was added to the precursor compartment, 0.1-ml samples were taken from the product compartment at 15-minute intervals over a period of

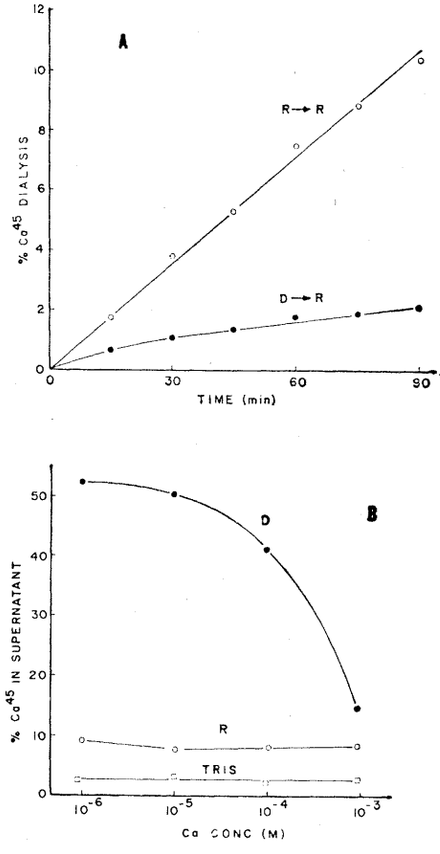

Fig. 1. (*A*) Diffusion of Ca^{45} across dialyzing membrane. $R \rightarrow R$ represents diffusion from rachitic supernatant to rachitic supernatant, and $D \rightarrow R$, from vitamin D supernatant to rachitic supernatant. Precursor volume = 20 ml; product volume = 2.5 ml. The protein concentrations of rachitic and vitamin D supernatants were 3.0 and 3.3 mg/ml. *B*, Influence of vitamin D supernatant on Ca^{45}-binding to Chelex-100. Each point represents the mean of three values. Each tube contained 0.1 ml of resin, 1 ml of vitamin D (*D*) or rachitic (*R*) supernatant or tris buffer, 0.1 ml Ca^{40} solution, and 0.1 ml Ca^{45} solution. After mixing and centrifuging, a portion of the supernatant was assessed for residual Ca^{45} activity. The protein concentrations of rachitic and vitamin D supernatants were 5.4 and 5.3 mg/ml.