Response of Cultured Cells of Systemic and Local Lesion Tobacco Hosts to Microinjection with TMV

T. E. Russell and R. S. Halliwell

Assistant Plant Pathologist and Professor, respectively, Department of Plant Pathology, University of Arizona, Tucson 85721; and Department of Plant Sciences, Texas A & M University, College Station 77843. Present address of senior author: University of Arizona Experiment Station at Mesa, 1601 W. Main St., Mesa 85202.

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ABSTRACT

Cultured cell chains derived from two cultivars of *Nicotiana tabacum* 'Samsun' and 'Samsun NN' were microinjected with TMV to compare the effects of the virus on cultured cells of a systemic host (Samsun) with those of a local lesion host (Samsun NN). Eight Samsun NN cell chains were injected with infectious TMV; six became infected and contained inclusions, and two showed no response. Five Samsun NN chains injected with heat-treated TMV produced neither symptoms nor inclusions. Eight 'Samsun' chains injected with infectious TMV showed symptoms with inclusion formation, and five chains injected with heat-treated TMV were symptomless. Evidence of infection in the Samsun NN cells included rapid loss of transvacuolar streaming, apparent infection of some cells and not others in a cell chain, cytoplasmic vesiculation, and infrequent formation of small virus inclusions. Response of Samsun cells in chains was milder and more predictable than that in infected Samsun NN cells. Infected Samsun cells maintained their integrity longer, produced larger and more numerous inclusions, and symptoms appeared in all cells of each chain, indicating virus spread from cell to cell. Bioassay of inclusion-bearing cell chains of both cultivars verified that virus multiplication resulted from the injected TMV.

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The infection of tobacco leaves by tobacco mosaic virus (TMV) usually results in either systemic mosaic symptoms or the formation of local lesions. The local lesion response is often referred to as a "hypersensitive" reaction and is controlled by a dominant gene designated "NN." The usual source of this gene is *Nicotiana glutinosa* L. and cultivars of *N. tabacum* L. such as Samsun NN.

Tissue obtained from both systemic and local lesion hosts has been cultured on various media and the interaction with TMV studied. With few exceptions, most of these studies were carried out with tissue derived from *N. tabacum* varieties not containing the "NN" gene. Little is known about the necrotic response in cultured tissue and until this work and that of Beachy and Murakishi (3) the response had not been previously detected.

Wu et al. (12) inoculated liquid cell cultures of *N. tabacum* L. × *N. glutinosa* L. crosses with TMV and indicated that virus multiplication took place, but no comparisons were made to similarly treated tissue derived from a closely related systemic host.

Hirth and Lebeurier (8) inoculated clumps of cultured systemic and local lesion host tissue with TMV and TMV-RNA. Infections were achieved, but no differences were noted in the response of either of the cultures to infection and subsequent virus multiplication. Continuous observation at the cellular level was not made, but bioassays were used to trace the infection throughout the tissues.

Just recently, Beachy and Murakishi (3) were able to demonstrate local lesion response in cells cultured on a suitably modified and solidified Murashige and Skoog medium. No attempt was made to study this response at the cellular level.

Nims et al. (10) studied the response of cultured *N. tabacum* L. 'Samsun NN' cells to TMV infection at the cellular level. Two cell chains composed of four and two cells, respectively, and a single cell were microinjected with TMV and observed by light microscopy. Virus inclusion formation was described in the two cell groups and the single cell, but few other changes occurred which might be attributed to the necrotic response.

This study expands the work of Nims et al. (10) and compares the reaction of virus-infected cultured Samsun NN cells with injected cells not having the "NN" gene. A preliminary report has been made (11).

MATERIALS AND METHODS.—*Preparation of tissue cultures.*—A modified version of Murashige and Skoog's (MS) medium (9) was used. The modification included the addition of 150 ml of coconut milk, 0.1 mg of 2,4-dichlorophenoxyacetic .acid (2,4-D), 0.5 mg of naphthaleneacetic acid (NAA), 50 ml of culture filtrate from a mature tobacco tissue culture per liter. The pH of the medium was adjusted to 5.8 with NaOH. Slit-bottom Bellco flasks (150 ml) were filled with medium (50 ml), plugged with cotton, and capped with a stainless steel cap.

Leaf disks cut from 10- to 15-week-old *N. tabacum* Samsun and Samsun NN leaves with a No. 14 cork borer were rinsed in 95% ethyl alcohol for 60 seconds, placed in 10% Clorox (5.25% sodium hypochlorite): water solution, v/v for 10 minutes, and washed three times, each of 15-minute duration, with sterile distilled water.

Five disinfected leaf disks were placed in each flask and placed on a gyrorotary shaker operating at 80 cycles per minute. A 12-hour diurnal light regime was provided by a light bank having both incandescent and fluorescent light sources. The culture room temperature ranged from 22-26 C.

An incubation period of 10-15 days was required for free cell formation. At this time 20 ml of cell-laden medium was transferred to 70 ml of fresh medium. This transfer procedure was used throughout the experiment to produce third, fourth, etc., generations of cell chains.

Preparation of TMV inoculum.—Common TMV was purified from *N. tabacum* L. 'White Burley' plants according to the method described by Fraenkel-Conrat (6), differentially centrifuged five times, and then passed through a Sephadex 25 column. The final virus preparation was suspended in sterile distilled water and the final concentration of virus was determined to be 56 μg/ml by spectrophotometric methods. The virus preparation was then passed through a Sweeny syringe millipore filter aid containing a filter pad with a pore size of 0.30 μm.

Microculturing of cell chains.—A small amount of tissue culture medium (0.1 - 0.5 ml), containing cell chains, was added to 10-15 ml of the liquid decanted from the same culture. A droplet containing two cell chains of approximately the same size and cell number were removed with a heat-drawn capillary pipet, and the drop mounted on a sterile number 00 cover slip (30 × 35 mm). A short distance from this bubble, a drop of purified TMV was deposited. The droplets were separated by and covered with nonsterile paraffin oil (U.S. Pharmacopeia heavy mineral oil) (10). The cover slip with the cells, medium, virus and oil were inverted and placed on a Leitz microculture dish, 3 mm in height. All observations of injections and cell responses were made by phase microscopy, utilizing a Leitz Ortholux microscope with Vickers phase attachment.

Preparation and injection of cell chains.—Glass needles, used for injection, were fabricated from 0.78-mm capillary tubes with the aid of a Leitz needle puller. A de Fonbrune microforge was used to put a slight bend in the needles to facilitate injection.

Cell injection was carried out with the aid of a needle held in a Leitz micromanipulator. Needles were attached by tubing to an oil-filled hydraulic system regulated by a screw-type syringe. Positive and negative pressures could be exerted on the system for uptake and discharge of virus. One cell, usually located in the center of the chain, was injected.

Before injection, the needle was placed in the oil-coated droplet of infectious or heat-inactivated virus adjacent to the oil-coated droplet containing the cells and the virus preparation drawn into the needle. The needle was then placed in the vicinity of the cell chain to be injected. Bracing the cell chain with a micro-glass holding rod, held and manipulated by an additional micromanipulator, the needle tip was gently forced through the cell wall and plasmolemma into the cytoplasm, and the contents of the needle discharged. After injection, the needle was retained in place to allow formation of a callose plug (10). It was estimated that the average volume of virus suspension introduced into the cell was 2.5×10^{-6} μl. Measurements were taken from the photographs of the internal diameter of the needle and the distance from

needle tip to the bottom of the meniscus. The values were then applied to the formula for the volume of a cone:

$$V = \pi/3\ r^2h.$$

Each injected cell chain was accompanied by an

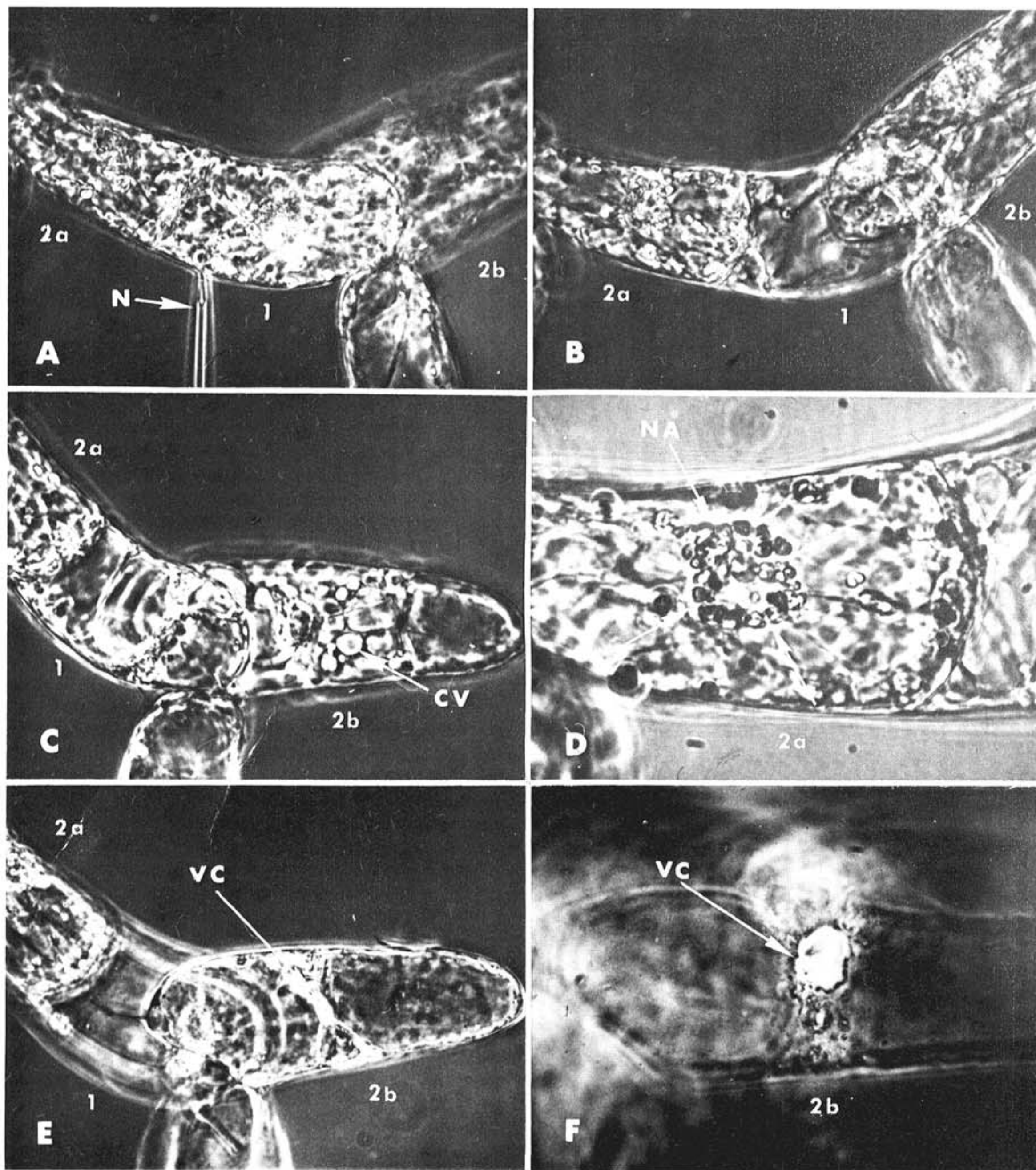

Fig. 1-(A-F). A TMV-injected Samsun NN cell chain showing response to infection of cytoplasmic vesiculation (CV), loss of cell integrity in infected cells, and TMV crystal (VC) formation. **A)** Cells 1, 2a, and 2b at 30 minutes postinjection time (PIT) with needle (N) still in place (× 360). **B)** Cells 1, 2a, and 2b at 9 hours PIT with cell 1 showing loss of integrity and with no apparent loss in cells 2a and 2b (× 360). **C)** Cells, 1, 2a, and 2b at 17 hours PIT with cell 1 showing almost complete vacuolation, cell 2b cytoplasmic vesiculation (CV), and cell 2a no apparent change (× 360). **D)** Cell 2a at 58 hours PIT showning no symptoms or sign of infection. The plastids are clumped normally around nucleus to form the nuclear area (NA) and transvacuolar streaming is unchanged from injection time (× 770). **E)** Cells 1, 2a, and 2b at 58 hours PIT. Cell 1 has lost all transvacuolar streaming and the cell has become vacuolate. Cell 2b has lost most of its transvacuolar streaming and a TMV crystal (VC) (side-view) is forming in a streaming cytoplasm (× 360). **F)** Cell 2b at 105 hours PIT. The crystal seen in illustration E has become larger and a view through the widest portion shows its hexagonal angularity (× 770).

uninjected cell chain for comparative purposes.

Only microcultures visibly free from microbial contamination were utilized.

Bioassay of injected cell chains.—Cell chains injected with heat-inactivated or infectious TMV were bioassayed for TMV. Each cell chain was removed from the nutrient drop and placed in 0.5 ml of 0.01 M phosphate buffer of pH 8.0. The cell chain was then triturated in the buffer and the mixture was applied by a glass rod to half of each of six leaves of *N. tabacum* L. Samsun NN previously

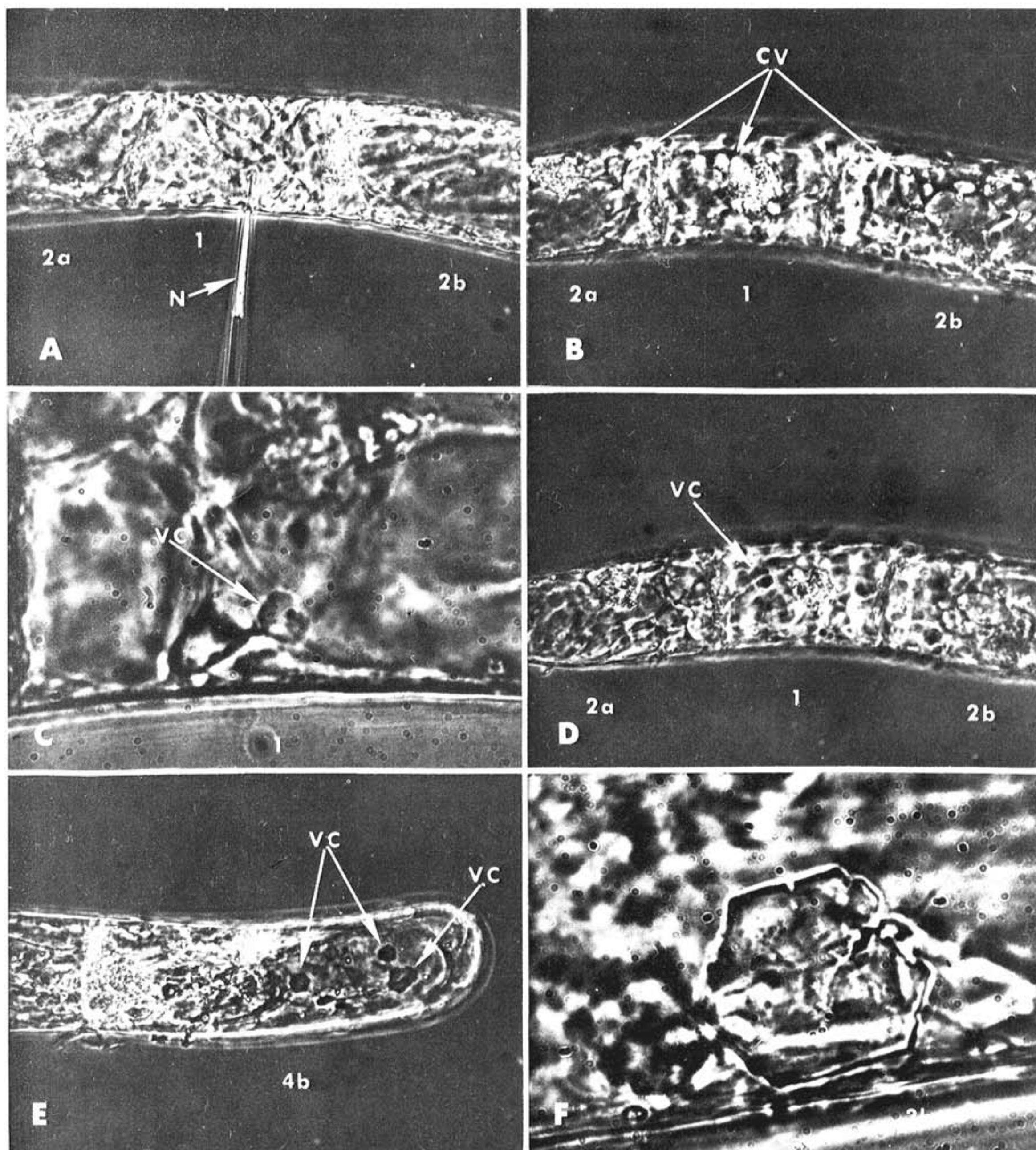

Fig. 2-(A-F). A TMV-injected *Nicotiana tabacum* 'Samsun' cell chain showing responses to infection of cytoplasmic vesiculation, crystal formation, and no apparent lack of cell integrity. **A)** Cells 1, 2a, and 2b at 20 minutes postinjection time (PIT) with needle (N) still in place (× 364). **B)** Cells 1, 2a, and 2b at 26 hours PIT showing cytoplasmic vesiculation (CV). CV was observed at 9 hours PIT in these cells (× 364). **C)** Cell 1 at 26 hours PIT with a TMV crystal (VC) starting to form in streaming cytoplasm (× 1508). **D)** Cells 1, 2a, and 2b 28 hours PIT. The TMV crystal (VC) in cell 1 is now visible at lower magnification, and no apparent loss of cell integrity can be seen in any of the three cells (× 364). **E)** Cell 4b at 49 hours PIT showing virus crystal (VC) formation. No apparent loss of cell integrity is visible (× 364). **F)** Cell 3b at 68 hours PIT showing a TMV virus crystal with typical hexagonal shape (× 1508).

dusted with Carborundum. Controls consisted of a different set of six leaves treated similarly, but with various concentrations of the TMV preparation used to inject the cells.

RESULTS.—*Observations on uninoculated microcultured Samsun NN and Samsun cells.*—Most cells in the cell chains observed were asymmetrical in shape with the major axis two to three times longer than the minor. The major axis of these cells ranged in length from 50 to 400 μm with an average length of about 150 μm. The short axis ranged from 50 to 120 μm in width with an average of about 65 μm.

Plastid-like bodies were observed in all cells. Their size ranged from small refractive spots, presumably starchy in nature to large green spheres 2.5 μm in diameter. Most of these plastid-like organelles were aggregated around and adhered to the nucleus and tended to obscure nuclear detail (Fig. 1-D).

Two other organelles, mitochondria and sphaerosomes, could be distinguished. The distinctions were made on the basis of Bald's (2) description. Mitochondria were oval to pear shaped in cells showing vigorous cyclosis. As the cells senesced, the mitochondria became filiform and often appeared to attach themselves to other mitochondria to form chains. The apparent disappearance of filiform mitochondria was noted 48-72 hours before cell death, in both normal and infected cells.

Most uninoculated cell chains of both Samsun and Samsun NN survived from 175-200 hours in microculture. Normal cell senescence was characterized by: (i) a gradual loss of transvacuolar streaming (TVS) and vacuolation until the only cyclosis noted was parietal, (ii) degeneration of plastid-like bodies, (iii) loss of plastid cover around the nucleus, (iv) total degeneration of the nucleus 12-24 hours before termination of cyclosis, and (v) eventual plasmolysis which occurred 12-24 hours following termination of cyclosis.

Cellular response of Samsun and Samsun NN to injection with heat-treated TMV.—The initial response to injection, both with infectious and heat-treated virus was basically the same. However, the reactions were not altogether the same as those described by Nims et al. (10), Benda (4, 5), and Hirai and Hirai (7). But these differences are minor and are probably due to biological variation in the systems utilized.

Puncture of the cell wall and plasmolemma by the needle elicited an immediate cell response. Within 30-60 seconds a bright refractive area appeared at the point of entry. Also, cytoplasm containing large quantities of oscillating mitochondria and sphaerosomes accumulated in the damaged area within 1-3 minutes. The accumulation of cytoplasm increase in volume for 30-60 minutes and transvacuolar strands appeared at the injury site. The refractive material, which Nims et al. (10) demonstrated to be callose, continued to accumulate along the penetrated surface of the needle, completely coating it. Needle removal at different intervals indicated that the callose plug was solid after 12 hours. A temporary increase in cyclosis was noted in the general vicinity of the puncture, but after 2-3 hours this activity returned to normal. No changes in cell activity which could be attributed to infection occurred during the rest of the life of the cells (cell chains).

Symptom and sign development in infected Samsun NN cells.—Eight cell chains of Samsun NN leaf tissue were injected with infectious TMV. Five cell chains produced both symptoms and a sign of infection, one cell chain behaved similarly to infected Samsun cells and two cell chains did not produce any visible symptoms or sign of infection.

In five Samsun NN cell chains injected with infectious TMV, two symptoms and a sign were associated with infected cells; (i) formation of cytoplasmic vesicles (CV) bordered by dense cytoplasm, (ii) premature loss of transvacuolar streaming, and (iii) appearance of TMV crystalline inclusions.

Cytoplasmic vesiculation occurred in the injected cell within 10 hours post-injection time and preceded loss of TVS. Cytoplasmic vesicles were not observed in the injected cell in the cell chain shown in Fig. 1-(A to F) due to the rapid loss of TVS and of cell integrity, but were observed preceding loss of TVS in an adjacent cell (Fig. 1-C). In general, CV appeared in adjacent cells that showed other symptoms and virus crystals within 14 hours and in cells further removed within 24 hours.

Loss of TVS always followed CV and usually occurred in the injected cell within 5-10 hours (Fig. 1-B). In those cells adjacent to the injected cell which showed CV, loss of TVS could be detected within 16-24 hours (Fig. 1-E) and in further removed cells within 25 hours. It must be noted that a similar loss of TVS or cell integrity accompanies normal cell senescence and usually occurs 2-3 hours before cell death. This loss though was generally not noticed in uninjected cells and those injected with heat-inactivated TMV until after 190 hours.

Virus inclusion formation was first apparent in cells as denser areas in the cytoplasm, which quickly began to show angularity. The crystals were the typical hexagonal TMV crystals so often described and pictured in the literature. When these crystalline inclusions appeared in cells, this was considered to be evidence of infection.

TMV inclusions were identifiable in 18 out of 27 cells showing CV and loss of TVS (67%). In most injected cells, virus inclusions appeared as soon as 19 hours and as late as 48 hours post-injection time. Inclusion formation in cells adjacent to the inoculated cell, occurred at about 40-60 hours (Fig. 1-E) and about 45-60 hours for the remainder of the cells in the chain.

The spread of virus and subsequent infection, from injected cells to serially located cells in the chain, varied from chain to chain. In one chain, only the injected cell produced symptoms and a sign of infection. In the cell chain shown in Fig. 1-(A to F), symptoms and virus inclusion formation appeared only in the injected and one adjacent cell, while the other adjacent cell apparently remained uninfected (Fig. 1-D). In two cases, all cells in the chain gave previously noted responses to infection. All cells showing symptoms and signs of TMV infection succumbed within an average time of 69 hours from injection (avg. 27 cells—range from 120 to 15). Cells not showing symptoms or signs of infection succumbed within an average of 150 hours. The average time of death for uninjected controls was 160 hours.

A sixth Samsun NN cell chain that was inoculated with infectious TMV failed to produce the symptoms of premature loss of TVS or cell integrity as a result of

infection, that was observed in the previously mentioned cell chains. CV and virus crystal formation was noted in four of the six cells comprising the chain. The other two, which were adjacent to a symptom- and sign-producing cell were apparently unaffected. Except for apparent infection failure of these two cells, this chain responded to injection with infectious TMV, in the same manner as did all Samsun chains.

The death of this chain occurred at the same time as the uninjected control chain (144 hours).

Two Samsun NN cell chains were injected with infectious virus with no apparent disease development. One cell chain survived for 240 hours in culture, as did the control, and the other survived for 160 hours compared to 260 hours for the control. For all practical purposes, these cells appeared to be as normal as injected or uninjected control cells.

Control cells injected with heat-treated virus produced no symptoms or sign of TMV infection. Injected control cells survived for an average of 180 hours, while the corresponding uninjected control chains succumbed after an average of 200 hours.

Bioassay of five cell chains injected with heat-inactivated TMV and five cell chains injected with infectious TMV and removed immediately after injection gave no local lesions on Samsun NN host. Bioassay for two cell chains showing TMV inclusions yielded an average of 14 and 20.5 lesions per half leaf.

Symptom and sign development in Samsun tissue.—Two basic responses were noted in Samsun cell chains injected with infectious TMV: (i) cytoplasmic vesiculation, and (ii) virus inclusion formation. Eight cell chains, comprised of a total of 59 cells, were injected with infectious TMV. All 59 cells showed the symptom of cytoplasmic vesiculation (100%). Forty-four of these cells produced identifiable virus crystals (74.5%).

Cytoplasmic vesiculation (Fig. 2-B) appeared in the injected cells (cell 1) of the eight cell chains at a postinjection time span of 7-16 hours and always preceded inclusion formation. Ten hours was the average. Post injection occurrence of this symptom in adjacent cells, was after 15-26 hours with an average of 20 hours. Cells further removed showed this symptom after 15-30 hours postinjection time, with an average of 22 hours. The appearance of vesiculation in cells two or more removed from the injected cells was not always serially formed and a number of cells exhibited vesiculation simultaneously.

Inclusion formation in injected cells occurred within 23-50 hours with an average of 37 hours [Fig. 2-(C,D)]. Appearance of inclusion in cells immediately adjacent to the injected cells occurred from 29-84 hours after injection with an average of 46.2 hours. In cells further removed from the injected cell, inclusions were formed 38-84 hours after injection of cell 1 with an average time of formation of 51 hours [Fig. 2-(E,F)].

With few exceptions, all cells in any given cell chain terminated cyclosis at approximately the same time, and in all cases the injected cell chain lived as long if not longer than the accompanying uninjected control chain. The average injected cell chain lived for 145 hours and uninjected controls for 131 hours.

Five cell chains were injected with heat-treated virus. No cytoplasmic vesiculation or virus inclusion formation was noted in any of the cells comprising these chains. The average longevity of cells in these chains was 176 hours, with the uninjected controls surviving for an average of 162 hours.

Bioassay of five cell chains injected with heat-inactivated virus and five with infectious virus and incubated for 12 hours produced no local lesions on assay host, but two cell chains showing inclusion formation and assayed after 120 hours gave an average of 40 and 52.5 lesions per half-leaf.

DISCUSSION.—It is believed that previous to this report the local lesion response or hypersensitive reaction had not been observed at the cellular level in a living cell with a known time of virus infection.

The conclusion that the hypersensitive reaction was demonstrated is based on the markedly different response exhibited by cultured Samsun NN and Samsun cells to infection by TMV. The rapid loss of cell integrity and apparent failure of disease development in attached cells adjacent to infected ones, as exhibited by cultured Samsun NN cells, parallel the in vivo response in whole plants; i.e., the rapid death of cells in local lesions and the apparent self-limiting nature of lesions by apparent prevention of virus spread, or on some cells not being susceptible. It has been suggested that this loss of integrity and ultimately cell death in injected Samsun NN cells may be due to nutrient depletion and/or staling, and not the hypersensitive response. But in light of the failure of apparently uninfected cells in the same chains and accompanying control chains to show symptoms it is felt that the response was to TMV infection. The failure of any Samsun cells to show this response only reinforces this conclusion.

In cultured Samsun cells, the response to TMV infection was quite different from that observed in the Samsun NN cells and probably quite close to that found in uncultured tissue. In TMV infected Samsun cells, there was little cell degradation after the introduction of TMV into the cell and a uniform spread of virus with symptom development in all and sign development in almost all cells of all cell chains. It appeared that Samsun cells produced more virus than did Samsun NN cells, as evidenced from the size and number of TMV inclusions found in Samsun cells. The failure of two Samsun NN cell chains to show any symptoms or sign of infection after inoculation probably indicates some form of resistance. Successful inoculations in both cultivars established inoculum infectivity. Evidence for apparent resistance in individual cells in mature tissue of *Nicotiana tabacum* L. cultivar White Burley has been reported (1). But these resistant cells only became evident after infection was noted in adjacent cells, so it cannot be determined whether these cells were resistant initially or became so as a result of interaction with infected cells. Several references in the literature indicate that young meristematic tissue may be resistant to virus infection. Whether it is an escape phenomenon or a true resistance is not known, but that response may be related to that observed in this study.

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