Letter to the Editor

Studies on the Mechanism of Viral Cross Protection

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Plants infected with a virus usually are resistant to superinfection by other strains of the same virus. Since the discovery of this phenomenon by McKinney in 1929 (5) cross protection has been found useful for establishing relationships among viruses and, in recent years, for controlling viruses such as tobacco mosaic virus in glasshouse-grown tomatoes (8). The mechanism whereby one strain of a virus prevents establishment of another strain of the same virus is not known. Several theories have been advanced; these were reviewed by Matthews (4). Among them are the theory of limited sites (the protecting virus occupies all of the sits in the cell that are required for multiplication of the challenging strain); the precursor exhaustion theory (the first strain uses all of the metabolites required for synthesis of the challenging strain); and recombination, wherein the challenging strain becomes lost in the huge genetic pool of the protecting strain (2). DeZoeten and Fulton (3) advanced the theory that the RNA of the challenging strain becomes encapsidated with coat protein of the protecting strain thereby becoming sequestered and unable to replicate.

All of the theories cited above are based on the premise that for a particular cell to be resistant to superinfection it must be infected with the protecting strain. Evidence is presented here that this may not necessarily be true. The following experiments indicate that a leaf may be completely immune to superinfection even though the large majority of cells are not infected with the protecting strain.

One leaf on each of 14 Turkish tobacco plants (*Nicotiana tabacum* L.) was inoculated with the common strain of TMV (U1) when the plants were in the six-leaf stage. Purified inoculum was suspended at a concentration of 3 mg/ml in 0.05 M sodium phosphate buffer, pH 6.8. The leaf was dusted with corundum and the inoculum was applied with a cotton swab. At various time intervals after inoculation with U1 the same leaf was redusted with corundum and challenge-inoculated with a 3 mg/ml suspension of U5 TMV (1). Immediately thereafter, the terminal half of the leaf was cut off to determine the level of infection with U1 at the time of challenge by fluorescent antibody staining (6). The plant bearing the basal half of the challenged leaf was allowed to incubate for 10-14 days at room temperature.

The severed part of the leaf was cut into thin strips and macerated in a 0.1% solution of R-10 Macerozyme (Yakult) in 0.7 M mannitol. Separated mesophyll and palisade cells were stained with fluorescein isothiocyanate-labeled antibody, reactive with both strains (U1 and U5) of TMV at dilutions of up to 1/64. Stained cells were examined with a Zeiss GFL research microscope equipped with a 100-W halogen UV source. The number of positively stained cells (FA-positive) was determined from a field of 100 randomly selected cells.

To determine whether U5 was able to multiply, the challenged basal half-leaf was removed from the plant (after incubation), ground with a mortar and pestle, and assayed for infectivity on leaves of *Nicotiana sylvestris* Sped. & Comes (strain U1 becomes systemic in this host whereas U5 only produces local lesions). Before being ground, the leaf was dipped in a hot detergent solution for 30 sec to destroy residual U5 inoculum. At the time the challenged leaf was removed for assay, a small terminal leaf exhibiting systemic symptoms of U1 was removed for assay on *N. sylvestris* for the presence of U5.

This experiment was repeated four times and in most experiments each challenge was duplicated on two plants.

The results are shown in Fig. 1 and 2. In directly challenged leaves (Fig. 1) the time at which leaves become completely protected against infection by U5 (as indicated by no lesions being produced on *N. sylvestris*) varied greatly from one experiment to another. In one experiment (experiment 4) protection occurred as early as 32 hr after inoculation with U1. In another (experiment 3) leaves continued to be susceptible to infection with U5 as long as 120 hr after inoculation with U1. The percentage of infected cells at the time of challenge (as indicated by fluorescent antibody staining) also varied greatly among the different experiments. In one (experiment 4) complete protection against U5 infection occurred when only 2% of the cells were rated FA-positive. In experiment 1 protection did not occur until 61% of the cells contained enough U1 to give a positive staining reaction. It is significant that in two of the experiments (experiments 2 and 4) leaves became completely protected even though a very small proportion of the cells stained positively at the time of challenge.

In one experiment (experiment 2, Fig. 2) U5 was prevented from moving into the terminal leaf when the challenged leaf contained no FA-positive cells. In another (experiment 1, Fig. 2) U5 did not move into the young leaf when 2% of the cells stained positively at the time of challenge. This was unexpected, since the challenged leaf itself did not become protected until the time (55 hr) when 61% of the cells were stained FA-positive (experiment 1, Fig. 1). Conversely, in experiment 4 strain U5 was prevented from becoming systemic at a time (48 hr, Fig. 2) when the challenged leaf had 26% of its cells FA-positive, whereas the challenged leaf itself was completely protected 16 hr earlier at a time (32 hr, Fig. 1) when only 2% of its cells were FA-positive.

On the basis of these studies it is tentatively concluded that a leaf may be rendered completely resistant to super-

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infection by U5 even though a large majority of the mesophyll and palisade cells are not infected with the protecting strain. Such a conclusion is based on the assumption that cells which do not become stained with fluorescent antibody are not infected. It is possible that many cells could be in early stages of infection but have

TABLE 1. Tobacco leaf cells stained with fluorescent antibody following incubation under conditions unfavorable for cell-to-cell movement of the common strain of TMV (U1)

Incubation conditions	Time after inoculation[a] (hr)	Positively stained cells: Before incubation[b] (%)	After incubation (%)	Change (%)
0.7 M Mannitol	24	1.5	1	− 0.5
	29	22	37	+ 15
	41	28	42	+ 14
No mannitol	24	0.4	26	+ 25
	29	23	41	+ 18
	41	26	68	+ 42

[a]Leaves inoculated with U1 at 3 mg/ml.
[b]Inoculated leaf strips were incubated on protoplast incubation medium either with or without mannitol for 72 hr.

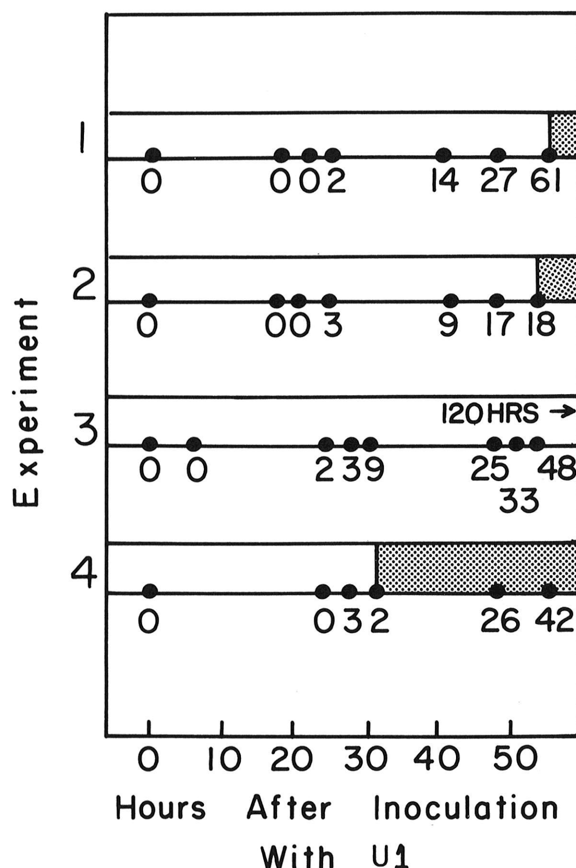

Fig. 1. Cross protection by the common strain of TMV (U1) in tobacco leaves against strain U5 based on assay of the directly challenged leaf on *Nicotiana sylvestris*. Shaded part of bars represents the time after inoculation that U5 was unable to super-infect and multiply. Dots represent the times after inoculation that the leaves were challenged with U5. Numbers under the dots are the percentage of fluorescent antibody-positive cells in the leaves at the time of challenge.

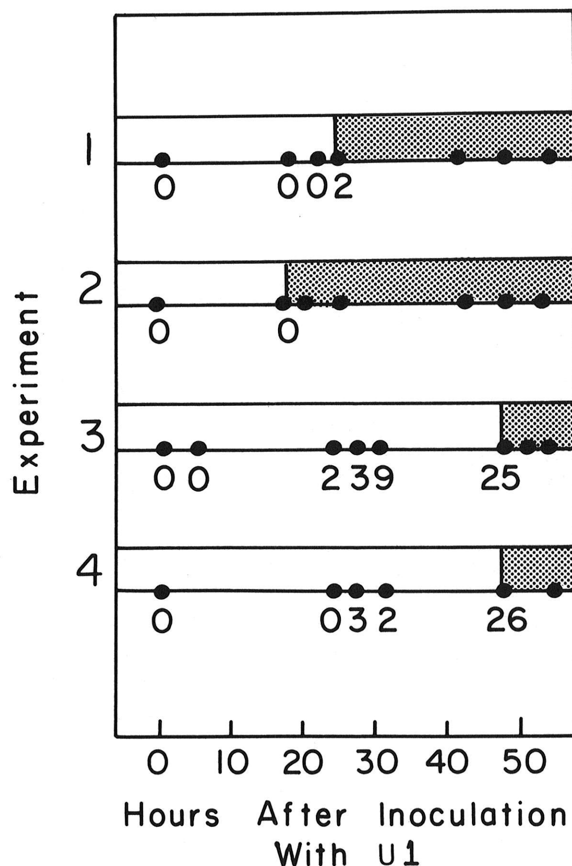

Fig. 2. Cross protection by the common strain of TMV (U1) against strain U5 based on assay of nonchallenged terminal leaves on *Nicotiana sylvestris*. Shaded part of the bars represents the time after inoculation that U5 was unable to multiply in the challenged leaf and become systemic in the plant. Dots are times after inoculation that leaves were challenged with U5. Numbers under dots are the percentage of fluorescent antibody-positive cells in the leaves at the time of challenge.

not accumulated sufficiently large amounts of viral antigen to be detected by staining. The following experiment was designed to test this possibility.

Tobacco leaves were inoculated as usual with strain U1. After 24, 29, and 41 hr the leaves were thinly sliced and subjected to one of the following treatments: (i) vacuum infiltration with protoplast incubation medium containing 0.7 M mannitol (7); (ii) vacuum infiltration with protoplast incubation medium with no mannitol; and (iii) maceration and preparation of cells for fluorescent antibody staining and determination of percent FA-positive cells. Treatment (i) was to provide conditions favorable for virus multiplication but unfavorable for cell-to-cell movement of virus due to plasmolysis and disruption of plasmodesmata. Treatment (ii) was a control in which conditions were favorable for virus multiplication but also favorable for cell-to-cell movement of virus. The leaf pieces then were incubated in the incubation media for 72 hr at room temperature. Following incubation they were macerated and stained with fluorescent antibody. Samples of leaf tissue also were embedded in epoxy resin for preparation of thin sections for electron microscopy. These confirmed that the protoplasts were highly plasmolyzed and no plasmodesmata could be found. Furthermore, on the basis of ultrastructure, the protoplasts appeared to have been living prior to fixation for electron microscopy.

Results are shown in Table 1. In the presence of mannitol there was no increase in the percent of FA-positive cells when the tissue was placed in the mannitol solution 24 hr after inoculation with U1. When placed in mannitol 29 and 41 hr after inoculation there was an increase in FA-positive cells of 15 and 14%, respectively. There was a substantial increase in all cases in which there was no mannitol in the incubation medium, probably owning to spread of virus and an actual increase in the number of infected cells. From this it is concluded that on staining with fluorescent antibody only a maximum of about 15% of the infected cells may go undetected because of subdetectable levels of antigen. Thus, in leaves completely protected against superinfection with U5 the large majority of palisade and parenchyma cells are uninfected in some instances.

Although the foregoing has shown that the protection mechanism is operational when many palisade and parenchyma cells are uninfected with U1, this may be of questionable significance since protection may take place in the epidermis, presumably the initial site of infection. To assess the level of infection in epidermal cells under protective conditions, epidermal strips were removed from the dorsal surface of leaves infected with U1, mounted under coverslips in water, and examined with phase-contrast optics for the presence of crystalline inclusions. As late as 96 hr after inoculation, long after the time when the leaf was completely protected against superinfection, only 20% of the epidermal cells contained crystalline inclusions. Assuming that all cells which become infected with U1 TMV develop crystalline inclusions, it can be concluded that the large majority of epidermal cells are not infected with U1 although the leaf is immune to superinfection.

Based on counts we estimate that the upper epidermis of a tobacco leaf contains approximately 3×10^6 ordinary epidermal cells, 1×10^6 guard cells, and 6×10^3 trichomes. Thus, there are over 4×10^6 potentially infectable cells on the upper surface of a tobacco leaf. If only 20% of these are infected with U1 at the time of challenge there still remain over 3×10^6 noninfected cells, and yet the entire leaf is immune to superinfection with U5.

On the basis of these observations we believe that a particular cell does not have to be infected with the protecting strain in order to be resistant to infection with the challenging strain. We propose that there is some substance formed in response to infection which moves into cells surrounding the initial U1 infection centers which somehow inhibits replication of either U1 or U5 when either virus enters those cells but which does not interfere with the replication of other unrelated viruses.

Another explanation for a protected leaf to have a large proportion of its cells uninfected is that only a small proportion of the epidermal cells may be potentially capable of being infected by mechanical inoculation, either by the protecting or challenging strain. If this were so then it could still be argued that a particular cell must be infected with the protecting strain in order to be immune to superinfection. Nevertheless, the evidence presented here raises the possibility of an alternative mechanism of cross protection.

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