Electron Microscopy of the Formation of Wound Tumor Virus in Abdominally Inoculated Insect Vectors

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Fifth-instar nymphs of *Agallia constricta* leafhoppers were injected abdominally with extracts from root tumors confining wound tumor virus (WTV). The insects were sacrificed at predetermined intervals, their internal organs dissected, fixed, embedded, sectioned, stained, and examined in a Siemens Elmiskop I. Sequential stages in virus development were reconstructed from consecutive samples of fatbody tissues. Changes resulting from the infection were: (i) a viroplasm, i.e., an accumulation of electron-dense aggregates; (ii) the appearance at the periphery of the viroplasm of a few fully formed virus particles recognized as virions; (iii) the formation of increasing numbers of individual virions, not only at the periphery but also in the viroplasm; (iv) the engulfing of virions within multimembranous structures; and (v) the formation of virus microcrystals either at the sites of former viroplasms, or at some distance. These morphological findings indicate that, following abdominal inoculation of WTV, the plant-pathogenic virus develops within the cytoplasmic matrix proper of insect vector cells. In addition to the fatbody tissues, WTV was detected in the epidermis, muscles, and trachea of abdominally inoculated insects, demonstrating the systemic invasion of the mechanically infected arthropod host. No virus was found in the gut tissues.

Electron microscopy of wound tumor virus (WTV) in thin sections of infected plants and orally infected insect vectors had previously revealed the presence of virions in the cell cytoplasm of plant and arthropod hosts of this virus (10, 12, 22, 24, 25, 26). In certain tissues and organs the virus was found only rarely and widely scattered while in others it formed several types of aggregates and even microcrystals (23). As our study of plant and insect sections progressed and over 4,000 electron micrographs were prepared and examined, it became apparent that large accumulations of virions do not necessarily indicate sites of virus assembly, but rather sites of virus storage or even virus destruction. A study was then undertaken to determine the specific sites of virus assembly in plant and insect cells of susceptible hosts. Subsequently, such assembly sites were detected in the cytoplasm of WTV-infected plants and in insect vectors infected orally (12, 25).

When infective insect vectors are confined to susceptible plants, the WTV may be introduced into the plants on the first day of confinement,

but more often several days are required for plant inoculation. Similarly, when virus-free insects are confined to WTV-diseased plants, they occasionally acquire the virus within a short feeding period, but oral acquisition is usually slow. Several days, or even weeks, may elapse before WTV is taken up by feeding insects. Since no precise timing of virus acquisition by the oral route is possible, it was decided to study the earliest stages of virus assembly by using insects that had acquired WTV by needle injection. Abdominal injections with WTV-containing extracts (17) define the exact day and hour of virus acquisition, and nearly 100% of the inoculated insects become infected. Recent modification of the injection technique (16) allowed rapid inoculations of many hundreds of insects per hour, thus facilitating the previously tedious procedure.

The principal aim of the study was to determine the sequential WTV formation in fatbody tissues and the sites of virus assembly. This paper describes the finding of viroplasms, assumed to be the "virus factories," in mechanically inoculated insect vectors. A note reporting these findings, without illustrations, has been published (18).

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MATERIALS AND METHODS

Details of the standard experimental procedures have been published previously (16, 26). Virus-free fifth-instar nymphs of the leafhopper *Agallia constricta* Van Duzee from a stock colony, maintained on alfalfa plants (*Medicago sativa* L.) in a greenhouse insectary, were injected with WTV-containing extracts from root tumors of sweet clover plants (*Melilotus alba* Desr.). Since highly purified WTV preparations are known to be only marginally infective, the virus inoculum was partially purified by centrifugation to remove plant cell debris, and used without further concentration. Insects were injected at room temperature after brief carbon dioxide anesthesia. Groups of 20 nymphs were placed in a specially constructed insect holder (16). Intra-abdominal inoculations were made by piercing the abdomens between the second or third segment with fine glass capillaries. A small amount of inoculum, sufficient to cause a barely visible swelling of the abdomen, was blown into the abdominal cavity. The injected insects were confined to alfalfa plants immune to WTV, and representatives were removed at intervals for dissection. Whole abdomens were usually severed, fixed, and embedded. The removal of internal organs, and the fixation and embedding have been described (16, 23, 25). Fixation was in 6% glutaraldehyde in insect Ringer solution at pH 7 for 1.5 hr, followed by three 30-min washings in insect Ringer solution. Postfixation was in 2% osmic acid for 1 hr, followed by dehydration in graded ethyl alcohol concentrations. The fixed insect fragments were embedded in epoxy resin. Four weeks after injection, a group of 25 insects was transferred from alfalfa to individual crimson clover (*Trifolium incarnatum* L.) seedlings for 10 days, to check whether the insects would become infective. These insects were sacrificed 38 days after injection. Crimson clover seedlings were employed because they are highly susceptible to WTV infection. In earlier experiments, orally infected insects were compared with virus-free controls from stock maintained on healthy plants. In none of these earlier controls did we detect viroplasms or particles similar to WTV. Present controls consisted of 100 stock insects, injected with extracts from roots of healthy *M. alba* plants, and dissected at the same intervals as the virus-inoculated individuals. The number of control insects observed for each of five intervals was 3, 5, 5, 3, and 3. In addition, groups of ten noninjected controls were also dissected at the same intervals and sections from two insects from each group observed. Although it was not likely that WTV-like particles would occur in control leafhoppers, nearly 800 sections from controls were carefully examined. The two types of controls were deemed important to establish whether any abnormalities occurred because of aging, as a result of injections, or as artifacts.

RESULTS

Three of 20 embedded insects that were sacrificed 3 days after virus inoculation were sectioned and carefully examined. No virus particles were detected in the cells of the fatbody, gut, Malpighian tubules, skin, or muscle. A repre-

sentative ultrathin section of the fatbody is pictured in Fig. 1. The tissue appeared normal, except for a single section in which WTV-like particles were detected. Figure 2, taken from the same individual as Fig. 1, shows three virus-like particles at a higher magnification. Their size, approximately 60 m μ in diameter, corresponds with that of WTV, and they could have been the remnants of the original inoculum. Smaller, 20 to 30 m μ , particles were observed in phagocytic structures in some of the sections. These are shown in Fig. 3. Their nature and significance remain obscure.

Abdomens of eight insects collected 7 days after inoculation were sectioned and examined. A representative section of the fatbody at that stage is shown in Fig. 4. No pronounced cytological changes were seen. Vacuolated cells were common, but vacuolation also occurred in occasional virus-free individuals, depending on their nutrition, age, and location of fatbody tissues within the insect. At that stage, virus particles were seen in many electron-dense structures of the cells, as shown under low- and high-power magnification in Fig. 5 and 6.

Ten days after inoculation, 6 of 15 embedded insects were examined. In several sections from different insects, remarkable cytopathic changes were commonly observed in a limited part of the fatbody. The cytoplasm was loose and abnormal in appearance, indicating deterioration of the fine structure (Fig. 7). Whenever this type of cytopathic change was detected, we were also able to find WTV in these or nearby cells. No changes of this type were ever found in fatbody cells of virus-free insects. Small accumulations of WTV particles were found in many fatbody cells within electron-dense structures (Fig. 8). Two types of these structures could be distinguished at this stage: one containing myelin-like membranous layers within the structures and surrounded by two membrane layers (Fig. 9), and the other having many myelin-like layers at the periphery (Fig. 10). Microtubules, not associated with the virus particles, were sometimes seen (Fig. 8). The WTV particles often accumulated in small masses within electron-dense structures, as can be seen in Fig. 10, where regularly arranged virus particles seem to have been engulfed. Ten days after the injection of WTV, only one part of the fatbody tissue contained a few viroplasmic matrixes. A typical viroplasmic matrix is shown in Fig. 11. On the margin of the matrix, numerous fully formed virions can be seen. In addition, virus shells, without the dense inner core, could be detected under higher magnification (Fig. 12). The proximity of ribosomes and polyribosomes was characteristic of the areas

FIG. 1. An ultrathin section of a fatbody cell of *Agallia constricta* 3 days after WTV injection. No WTV was detected in cells at this stage and the cytoplasm appeared normal. N = nucleus. $\times 10,000$.

FIG. 2. WTV particles encountered 3 days after virus injection in a fatbody cell of the same individual as in Fig. 1. The virus particles (arrows) might have been the original virus inoculum. $\times 60,000$.

FIG. 3. *Electron-dense particles of obscure origin, smaller in diameter than WTV, sometimes found in the cytoplasm of fatbody cells 3 days after virus inoculation. $\times 90,000$.*

FIG. 4. *Vacuoles (V) in the cytoplasm of a fatbody cell, 7 days after WTV injection. N = nucleus. $\times 3,000$.*

FIG. 5. Arrows point to WTV particles in electron-dense structures in the fatbody cell cytoplasm 7 days after virus injection. Note vacuoles (v). $\times 3,000$. WTV particle (upper arrow) is shown in the insert at $\times 15,000$.

FIG. 6. Single WTV particle engulfed in an electron-dense structure within the cytoplasm of a fatbody cell 7 days after virus injection. $\times 40,000$.

FIG. 7. Areas of cytopathic changes (x) in the fine structure of fatbody cell cytoplasm 10 days after virus injection. $\times 50,000$.

FIG. 8. Particles of WTV in structures of the fatbody cell cytoplasm (arrows) 10 days after virus injection. Note the microtubules (m) in the cytoplasm. $\times 40,000$.

FIG. 9. Higher magnification of a loose aggregation of WTV in a double-membrane surrounded structure in fatbody cell cytoplasm 10 days after virus injection. Note the membranous layers within the structure. $\times 80,000$.

FIG. 10. Dense aggregate of WTV in a multimembranous structure in the fatbody cell cytoplasm 10 days after virus injection. $\times 80,000$.

FIG. 11. Large viroplasm of WTV in fatbody cell cytoplasm 10 days after virus injection. In addition to ribosome accumulations, note polyribosomes in upper right corner. $\times 40,000$.

FIG. 12. Small viroplasm in fatbody cell 10 days after WTV injection. A few virus particles (arrows) seem devoid of the dense cores, characteristic of the fully formed virions. $\times 80,000$.

FIG. 13. Crystalline arrangement of WTV particles in a multimembranous electron-dense structure in the fatbody cell cytoplasm 10 days after virus injection. $\times 60,000$.

FIG. 14. Cytopathic changes (x) in the fine structure of fatbody cell cytoplasm 17 days after WTV injection. Contrary to the typical changes illustrated in Fig. 7, those shown here, although very frequently observed in WTV injected insects, were also occasionally found in older virus-free insects, as well as in controls injected with extracts from healthy plants. $\times 4,000$.

where viroplasms were encountered. No membrane structures were detected enclosing the viroplasms. The ribosomes and polyribosomes apparently come in contact with the viroplasmic matrix. In some fatbody cells WTV microcrystals were found within electron-dense structures surrounded by several myelin layers as seen in Fig. 13. Other parts of the fatbody of the same insect in which the viroplasms and virus microcrystals were detected appeared perfectly normal and indistinguishable from fatbody cells of control insects.

Seventeen days after virus inoculation, 5 of 15 embedded insects were examined. WTV particles were found in almost all fatbody cells and the cytoplasm of the cells was strongly vacuolated (Fig. 14, 15, and 19). Contrary to the typical cytopathic changes found only in viruliferous insects (Fig. 7), the vacuolation observed on the 17th day was not specific for WTV-infected insects. Occasionally it was also seen in controls injected with extracts from healthy plants, as well as in older, noninjected leafhoppers. Numerous viroplasms were found in fatbody cells at this time (Fig. 16). The electron-dense structures in the infected cells contained larger masses of WTV than at earlier stages of infection, and some of these masses formed microcrystals (Fig. 17, 18, 19, and 20). Surprisingly, no WTV particles were found in any of the other organs examined at this stage of infection. In the earlier stages of infection, only small microcrystals or aggregates of a few virions were seen in electron-dense structures, whereas the progressing assembly of WTV within the viroplasms apparently resulted in high concentrations of WTV and in the formation of large, often crystalline accumulations within such structures. Figure 16 shows a higher magnification of a viroplasm with fully formed WTV and with virus shells, devoid of the dense core. These might represent virus coats. The closely packed WTV particles, formed at the same time, can be seen in Fig. 17 in two electron-dense structures of the fatbody cell cytoplasm. However, these particle accumulations were not yet completely assembled crystals despite their regular arrangement. The spaces of occasionally missing particles within the regularly packed virions are illustrated at $\times 200,000$ in Fig. 18.

Since the formation of microcrystals of a plant-pathogenic virus in an insect host is not clearly understood, it might be worthwhile to show in two additional electron micrographs the details of microcrystal formation in an insect sacrificed 17 days after virus inoculation. The striking difference between a viroplasmic matrix (v) in the lower part of the micrograph, and three closely packed, but not yet perfectly arranged, micro-

crystals of WTV, is apparent in Fig. 19. The electron-dense structures become completely filled as seen in Fig. 20. The electron-dense structures are not at the same sites as the viroplasms, but in close proximity, in the cytoplasm of the same cell that actively assembles WTV virions.

Finally, observations were made on insects that acquired virus by injections 38 days before dissection. The cytoplasm of fatbody cells contained numerous inclusions and accumulations of WTV (Fig. 21). Interestingly, the insect whose fatbody cells are shown in Fig. 21 to 25 did not transmit the virus to plants during 10 days of testing. Obviously, it was a poor transmitter or a nontransmitter. The only individual that transmitted virus in the 35-day group was the insect that provided material for Fig. 27 to 30. However, as demonstrated earlier by Sinha and Reddy (28), WTV-injected insects may contain viral antigens irrespective of whether they can act as vectors or not. *A. constricta*, while a more efficient vector than *A. novella*, and more susceptible to WTV infection (10), nevertheless does not infect plants regularly, particularly when the insects are older, as was the case here.

Insects dissected after 38 days contained electron-dense structures in the cytoplasm, with very densely packed masses of WTV, in addition to frequently encountered smaller particles of obscure nature (Fig. 22). These smaller particles could have been the result of contamination of the inoculum with another, unidentified virus, or might have been the result of a spontaneous infection, but their possible association with WTV cannot be excluded entirely at this time.

Figure 23 presents a large accumulation of WTV in a vacuolated area of the fatbody cell cytoplasm, next to a small viroplasmic matrix at an advanced stage of virus assembly. Virions were not seen in vacuoles at earlier stages of infection. A higher magnification of the small viroplasm, and of the edge of the huge accumulation of WTV particles is given in Fig. 24.

Since earlier electron microscopy studies have been limited to insects that acquired WTV by feeding on diseased plants, it was of considerable interest to determine whether, in abdominally inoculated insects, the virus distribution parallels that of orally infected vectors. Irrespective of the mode of virus acquisition, the infection became systemic. In addition to the fatbody tissues, WTV particles were frequently encountered in other tissues and organs of mechanically inoculated insects. However, contrary to insects that acquired WTV by feeding, mechanically inoculated insects contained no virus in gut tissues. An almost perfect, densely packed WTV microcrystal is shown in Fig. 25 in an ultrathin section of a

FIG. 15. Highly vacuolated cytoplasm of a fatbody cell 17 days after WTV injection. Virions can be seen at the edge of two viroplasms (v). $\times 10,000$.

FIG. 16. Higher magnification of larger (upper) viroplasm from Fig. 15, showing virions and several incomplete particles or developmental stages (arrows) devoid of dense cores. $\times 60,000$.

FIG. 17. Electron-dense structures in the fatbody cell cytoplasm, with closely packed accumulations of WTV 17 days after virus injection. Mitochondria (m) are seen near the electron-dense structures in the endoplasmic reticulum. $\times 40,000$.

FIG. 18. Densely, but not completely, packed WTV, showing irregularities in the distances between particles. $\times 200,000$.

FIG. 19. Large microcrystal inclusions of WTV (top of micrograph) in the endoplasmic reticulum of the fatbody cell cytoplasm, and a viroplasm (v) 17 days after virus injection. $\times 12,500$.

FIG. 20. Higher magnification of virus inclusions, showing various stages of particle accumulation, from small aggregates to densely packed crystalline formations. $\times 50,000$.

FIG. 21. Large numbers of crystalline inclusions (arrows) found in the fatbody cell cytoplasm 38 days after WTV injection. $\times 5,000$.

FIG. 22. Two WTV microcrystals in an electron-dense structure 38 days after virus injection. Arrow points to smaller particles of obscure nature, encountered within the same electron-dense structure. $\times 80,000$.

FIG. 23. Loosely accumulated WTV in a vacuolated area in the fatbody cytoplasm 38 days after virus injection. A viroplasm is seen nearby (v). $\times 40,000$.

FIG. 24. Higher magnification of a section of Fig. 23, showing details of the viroplasm (v) and of the adjoining vacuole with accumulated WTV. $\times 75,000$.

FIG. 25. Hexagonal cross section of a WTV microcrystal, found in a muscle cell of *Agallia constricta* 38 days after virus injection. Arrow points to remnant of membrane that appears to have surrounded the structure while the microcrystal was forming. $\times 160,000$.

FIG. 26. Dense accumulation of WTV in a muscle cell surrounding the gut 38 days after virus injection. $\times 40,000$.

FIG. 27. Cross section of a tracheal cell of *Agallia constricta* 38 days after WTV injection, showing dense virus accumulations. $\times 40,000$.

FIG. 28. Arrows point to three microcrystal inclusions of WTV in a muscle cell of *Agallia constricta* 38 days after virus injection. $\times 10,000$.

FIG. 29. Large and small WTV accumulations (arrows) in the cytoplasm of a cell in the epidermis of *Agallia constricta* 38 days after virus injection. N = nucleus. $\times 12,500$.

FIG. 30. Accumulation of WTV in a structure in the cytoplasm of an epidermal cell of *Agallia constricta* 38 days after injection. In addition to WTV particles, smaller particles (arrow) are also present, probably engulfed within the electron-dense structure. $\times 80,000$.

FIG. 31. Section of a fatbody cell of *Agallia constricta* 10 days after injection of virus-free plant extract. No abnormalities were detected in the cells. $\times 3,000$.

muscle cell, 38 days after abdominal WTV injection. The hexagonal cross section of the microcrystal indicates that it had probably formed an icosahedral body in the muscle cell cytoplasm.

The microcrystal was formed within a structure, surrounded by a membrane, the remains of which can be seen in the upper part of the micrograph. Figure 26 illustrates a virus accumulation in the

gut muscle after 38 days. WTV accumulations appeared in the trachea of mechanically infected leafhoppers 38 days after inoculation (Fig. 27). Numerous perfect microcrystal inclusions were formed in muscle cells (Fig. 28).

Virus accumulations found in electron-dense structures of the epidermis (Fig. 29 and 30) gave the impression that the structures had engulfed and immobilized WTV virions. In addition to WTV, smaller particles were also observed in the electron-dense structures (Fig. 30).

No structures resembling viroplasms or particles resembling WTV were found in nearly 800 sections prepared from controls injected with extracts from healthy plants and from additional noninjected leafhoppers. The cytoplasm of fatbody cells appeared normal, as shown in Fig. 31, and no abnormalities were detected in the fine structure.

DISCUSSION

It has been known for several years that WTV is transmitted by *A. constricta* to susceptible plants, following multiplication of the virus in the body of the insect vector (1). The fact that the virus systemically infects various organs and tissues of the invertebrate host has been demonstrated by serological methods (27) and by direct electron microscopical investigation (22). Electron micrographs had earlier revealed the actual sites of virus assembly in orally infected insects, but no information was available on the fate of the virus acquired mechanically by abdominal injection. Since needle inoculation provides the only means for bioassay in this and several other circulative plant-pathogenic viruses, it seemed important to ascertain whether the mechanical infection by the abdominal route also results in systemic invasion comparable to that caused by oral infection of the insect. Furthermore, the abdominal inoculation provides a means for precise timing of virus acquisition, not available in the case of oral acquisition. It was hoped, therefore, that mechanically inoculated insects might give a clue to the sequential formation and development of viroplasms. The search for the latter was made in fatbody tissues, because they were easily available and in the past provided the best material for WTV electron microscopy in ultrathin sections of insect cells. We wish to stress, however, that the findings reported in this paper do not imply that the fatbody is the only, the first, or the main tissue in which WTV multiplies. Viroplasms were also observed in muscle, Malpighian tubules, trachea, salivary gland, and epidermal cells (25), and more recently in nerve (12) and in blood cells (Granados et al., *unpublished data*).

Recent studies on animal viruses have provided convincing evidence that viroplasms develop within cytoplasmic or nuclear matrixes (2, 4, 5, 6, 7, 8, 11, 19, 20). Lately, assembly sites of WTV have been detected in diseased plants and in insects which had acquired WTV by feeding on diseased plants (25). These findings have indicated that WTV is assembled in viroplasms formed within the cytoplasm of host cells.

The present study on the formation of WTV in abdominally inoculated insects resulted in the detection of a few WTV particles 3 and 7 days after injection. These virus particles were within electron-dense structures of the fatbody cell cytoplasm, and it was assumed that they represented either the original WTV inoculum or virus that had multiplied in other tissues of the insect. The first formation of fatbody cell viroplasms probably occurred less than 10 days but later than 7 days after injection, because, on the 10th day a few viroplasms were already seen with WTV particles at their margins, but most others had no discernible particles as yet. Remarkable cytopathic changes accompanied this formation. On the 17th day, numerous viroplasms were present in almost all parts of the fatbody of inoculated insects, and, in addition to virions at the periphery of the matrix, some seemed to be formed within the matrix. As the number of viroplasms gradually increased, the number of WTV particles presumably trapped in electron-dense structures also increased. Eventually, virions filled entire vacuoles as well as many electron-dense structures, finally forming crystalline virus inclusions. There was no evidence of WTV formation in, or penetration into, nuclei. Apparently, WTV develops within the cytoplasmic matrix proper, as do many typical animal cytoplasmic viruses. The findings also proved that abdominal inoculation of the virus resulted in systemic infection, somewhat similar to that which follows oral acquisition, except for the lack of virus in gut cells.

So far as could be ascertained from the literature, no one has previously demonstrated the sequential changes in assembly sites of a plant-pathogenic virus. There have been very few reports concerning insect-borne plant-pathogenic viruses where electron micrographs showed viroplasms, and where the respective areas have been recognized as viroplasms (9, 19, 29). The breakthrough was made possible by the truly remarkable resemblance between the structures encountered in increasing numbers in abdominally inoculated insects and those described from other laboratories from animal viruses grown in tissue culture. The whole process, when reconstructed from sequential stages, is strikingly reminiscent of

the assembly of cytoplasmic animal viruses. We tentatively suggest the following assembly cycle. After the original inoculum is introduced into the abdominal cavity of the susceptible insect, it is taken up by the hemolymph and transported to various organs and tissues, or taken up by some part of the fatbody cells. In the fatbody, the original inoculum apparently becomes phagocytized and engulfed in electron-dense structures as shown in Fig. 5 and 6. Subsequently, the virus genome, as in the case of reovirus (7), is released from the electron-dense structures, which enables it to reach the virus factories, that is, the viroplasmic matrixes. These earliest virus factories are soon followed by the formation of numerous others, while the newly assembled virions accumulate in vacuolated areas of the cell cytoplasm or in electron-dense structures. Gradually, virions become regularly packed, finally forming crystalline virus inclusions.

There is a certain amount of speculation in this interpretation of the observations and of the suggested sequence. Whether the interpretations are justified must await further study of insect cells infected *in vitro*. Recent advances in *A. constricta* tissue culture (3, 13, 14, 15) make such an approach possible.

The examination of the fatbody cells revealed some other interesting phenomena, particularly the apparent immobilization of virus particles and the deleterious cytopathic changes of the cells. The latter are of particular interest. Until now, oncogenic ribonucleic acid (RNA) viruses have not been found to have any cytotoxic effects on cells; they either multiply without any morphological effects, or they produce malignant transformation (21). The double-stranded RNA virus of wound tumor causes cytopathic changes that are reminiscent of those induced as a rule by all deoxyribonucleic acid viruses that produce experimental malignancies.

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