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Morphology and intracellular localization of bacilliform virus particles associated with the clover enation disease

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Abstract

In ultrathin sections of tumorous tissues of white clover plants artificially infected by grafting with the clover enation virus nuclei and nucleoli were very much enlarged. Such nuclei contained massive amounts of tubular particles in crystalline array. In spaces between the nuclear membrane lamellae such particles had an additional coat and usually occurred in irregular accumulations. These bacilliform particles measured about 200 × 80 mμ. This virus was not found in normal tissues.

Two other viruses with elongated particles of 475 and 665 mμ were also observed in non-neoplastic tissues. They were easily detected in negatively stained chop preparations, rapidly transmitted by sap, and considered to be mere contaminants.

Enation virus diseases are heterogeneous in epidemiology and etiology. Several have recently been found associated with dense accumulations of big spherical or polyhedral virus particles. The virus detected here closely resembles several bacilliform viruses described in recent years. These viruses are quite different in symptomatology and epidemiology.

Introduction

A peculiar virus disease of white clover (*Trifolium repens* L.) found in northern Italy has been described recently by Bos and Grancini (1963, 1968). The external abnormalities mainly consist of a few histoid enations on the underside of the main veins, whereas the gross morphology of the plants is not appreciably affected. Out of 12 leguminous species tested, *Medicago lupulina, Trifolium incarnatum, T. pratense, T. repens* and *Vicia faba* were found to be susceptible to infection by grafting. In *V. faba* vein swelling was more general and usually preceded by a slight vein clearing. On stems of this species conspicuous chains of bead-like protrusions were formed. They were studied for their anatomy and often contained spindle-shaped tumours.

The symptoms were found to be almost identical to those of beet curly top, citrus vein enation, Fiji disease of sugarcane, maize rough dwarf, rice black-streaked dwarf, tobacco leafcurl and wound tumour in several of their hosts. These diseases are caused by viruses which are usually not transmissible mechanically but by various vectors such as leafhoppers, white flies, or aphids in a persistent way. Recently some of these diseases were found to be associated with dense accumulations of rather big spherical particles (for literature see the discussion).

Neither Bos and Grancini (1968) nor the present authors succeeded in mechanically transmitting the clover enation virus. We have now tried to discover the causal agent

by studying ultrathin sections of neoplastic tissues in the electron microscope to further identify the virus.

Materials and methods

White clover plants infected artificially by grafting were grown, maintained, and propagated vegetatively in the greenhouse. Enations from the underside of the midrib and parenchymatic tissue of leaves from such plants were cut into small pieces of about 1 mm. They were fixed in 2% osmium tetroxide in Palade's Veronal acetate buffer for 2 hours at 4°C, saturated with uranyl acetate, kept overnight in a 70% acetone solution, dehydrated in a graded acetone series, and embedded in Durcupan. Ultrathin sections were made with an LKB Ultrotome with glass knives and expanded with chloroform. The sections were then mounted on carbon-stabilized Formvar-coated grids, stained with lead citrate, and examined in a Siemens Elmiskop I electron microscope.

For negative staining pieces of leaf veins of white clover and broad bean were chopped with a razor blade in some drops of 2% phosphotungstic acid pH 6.5 on a microscope slide. The juice thus obtained was then transferred to a coated grid and removed after $\frac{1}{2}$ min. To introduce an internal standard for magnification the material was cut together with about 4 mm^2 of 'White Burley' leaf with tobacco mosaic virus.

Results

In micrographs of ultrathin sections of tumorous tissues groups of cells near the vascular elements readily drew attention by having great numbers of bacilliform particles. In addition, nuclei and nucleoli of such cells were three to four times bigger than those of normal cells. The groups of deviating cells were surrounded by apparently normal ones not containing the particles just-mentioned.

The bacilliform particles were usually located in the perinuclear spaces between the two nuclear membrane lamellae (Fig. 1). They often occurred in irregular array. Some abnormal nuclei contained vesicles, presumably invaginations or tangentially cut perinuclear accumulations, full of similar irregularly arranged particles.

The bacilliform particles conisted of an inner tubular core surrounded by an outer membrane or envelope. In cross-section these particles showed a predominantly circular profile clearly differentiating the inner core and the envelope (Fig. 2). In longitudinal section both were rounded at their extremities. The inner tube sometimes showed a striate pattern or transverse banding. On the surface of the envelopes no projections were observed.

Measuring of these particles was difficult since they occurred at various angles in the embedding material. An orientational estimation revealed two main sizes, viz. 390 mμ long and 80 mμ wide and 200 mμ long and 80 mμ wide. In some of the longest particles a septum of narrowing in the middle was observed (Fig. 3 and 5).

In some of the nuclei the nucleoplasm around the nucleolus contained abundant amounts of tubular particles generally occurring in regular (crystalline) array (Fig. 4 and 5). Morphology and size of these particles corresponded to those of the internal cores of the bacilliform particles described above. In some cases such tubular particles were observed, which had apparently been fixed at the moment of migration from the

Fig. 1. Part of an infected cell showing part of the nucleus (nu) and nucleolus (no) and virus particles in spaces between the nuclear membrane lamellae (perinuclear spaces); im = inner membrane lamella, om = outer membrane lamella; × 27,000; the framed area has been enlarged in Fig. 6.

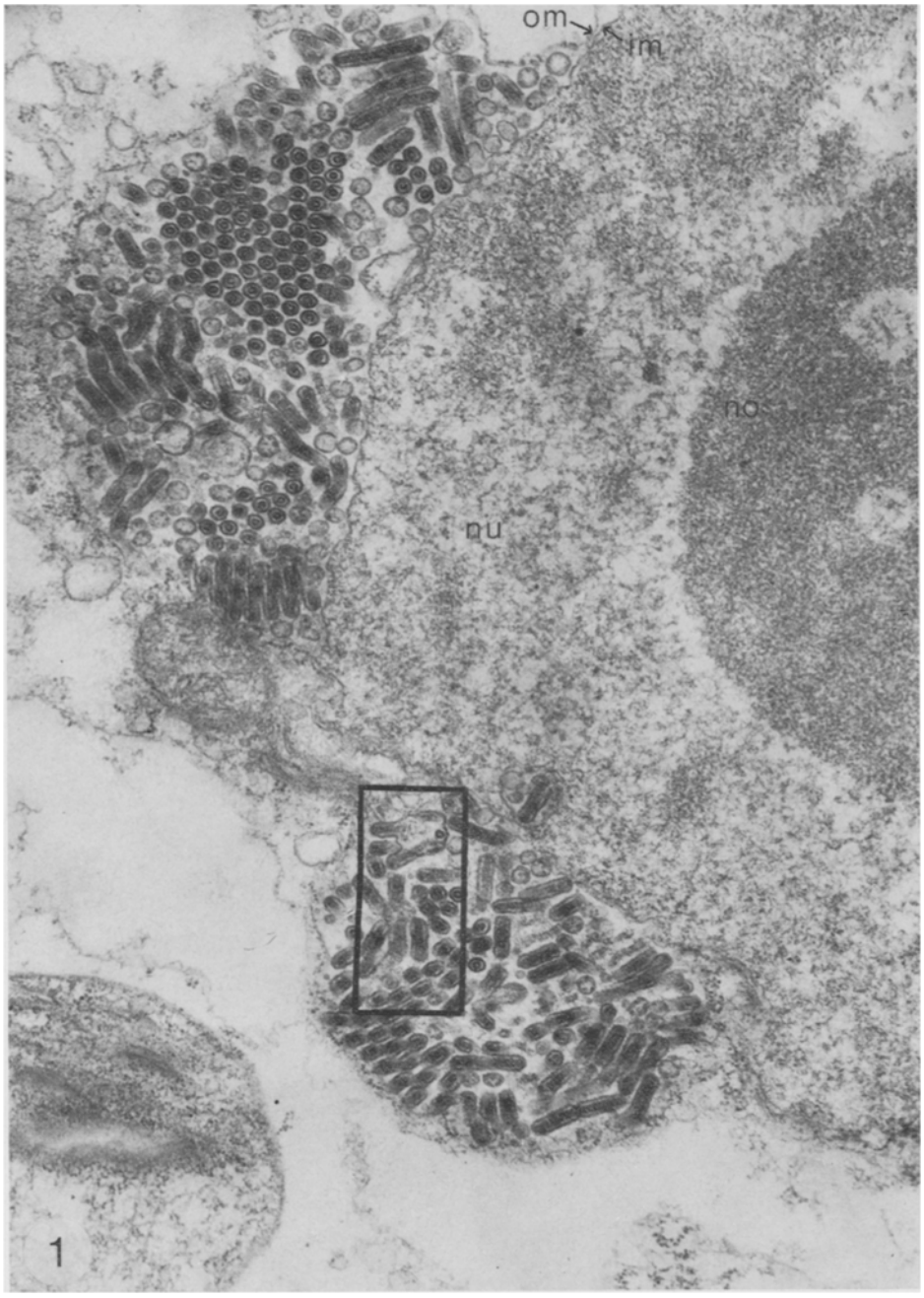

Fig. 1. Deel van een geïnfecteerde cel waarin een deel van de kern (nu) en nucleolus (no) en virusdeeltjes in ruimten tussen de lamellen van de kernmembraan (perinucleaire ruimten); im = binnenste kernmembraanlamel, om = buitenste kernmembraanlamel; vergr. 27.000 ×; het omlijnde deel is vergroot weergegeven in Fig. 6.

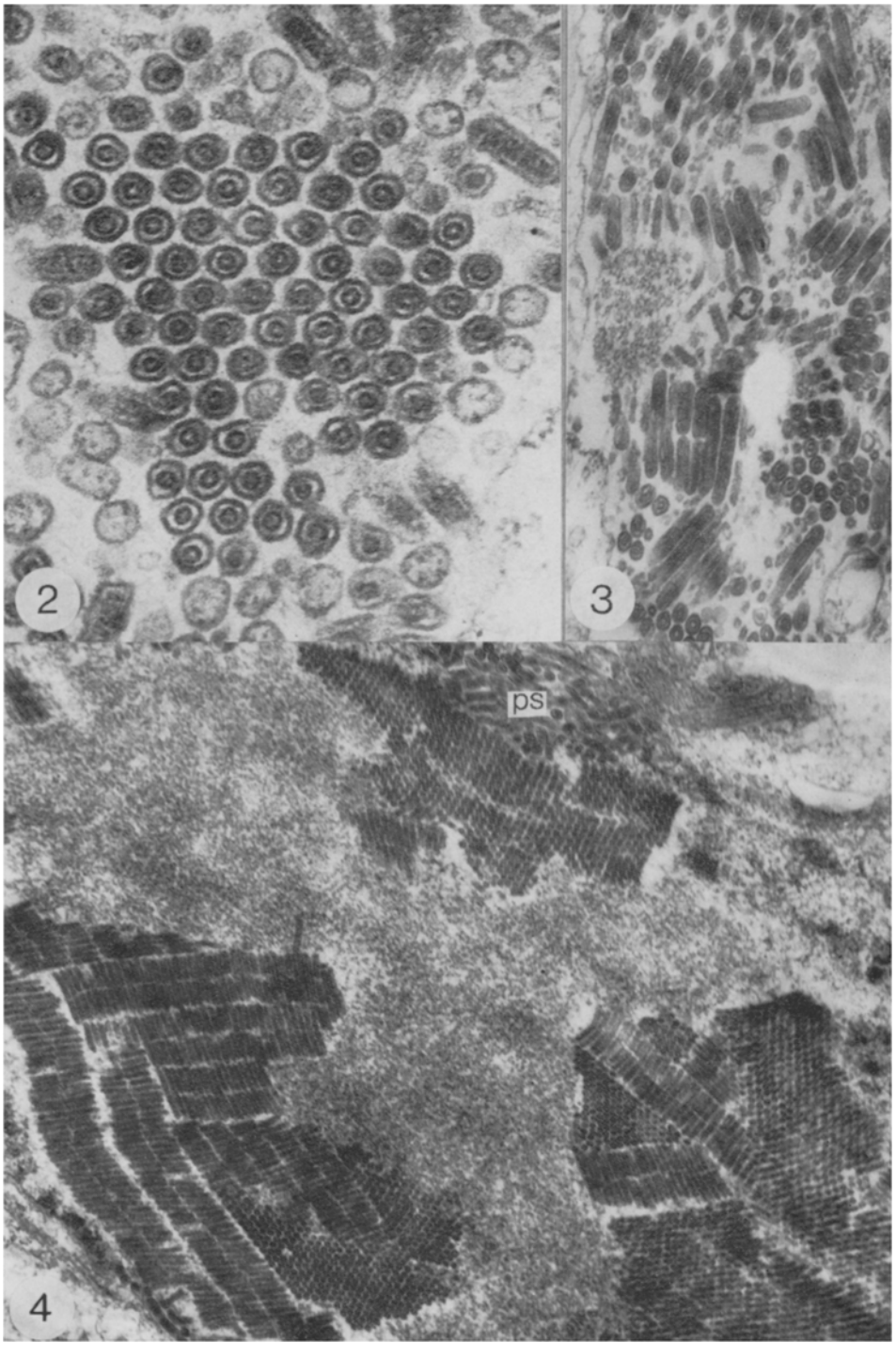
2
3
ps
4

Fig. 2. Cross section of some virus particles at higher magnification (× 80,000).
Fig. 3. Bacillary particles in which a narrowing in the middle of some of the long ones can be observed (× 40,000).
Fig. 4. Part of a nucleus with great numbers of small particles (inner cores) in crystalline array. In the perinuclear space (ps) some coated particles can be observed. The non-coated particles do not enter the nucleolar material. × 25,000.

Fig. 2. Dwarsdoorsnede van enkele virusdeeltjes bij sterker vergroting (80.000 ×).
Fig. 3. Bacilvormige deeltjes waarin bij enkele halverwege een vernauwing kan worden waargenomen (40.000 ×).
Fig. 4. Deel van een kern met grote hoeveelheden kleine deeltjes in kristallijne rangschikking. In de perinucleaire ruimte (ps) kunnen enkele omhulde deeltjes worden waargenomen. De niet-omhulde deeltjes komen niet in de nucleolus voor; vergr. 25.000 ×.

Fig. 5. Part of a nucleus (nu) and perinuclear space (ps) at high magnification (× 80,000).

Fig. 5. Deel van kern (nu) en perinucleaire ruimte (ps) bij sterke vergroting (80.000 ×).

Fig. 6. Enlarged part of Fig. 1 showing the single membrane structure enveloping three inner cores (× 143,000).

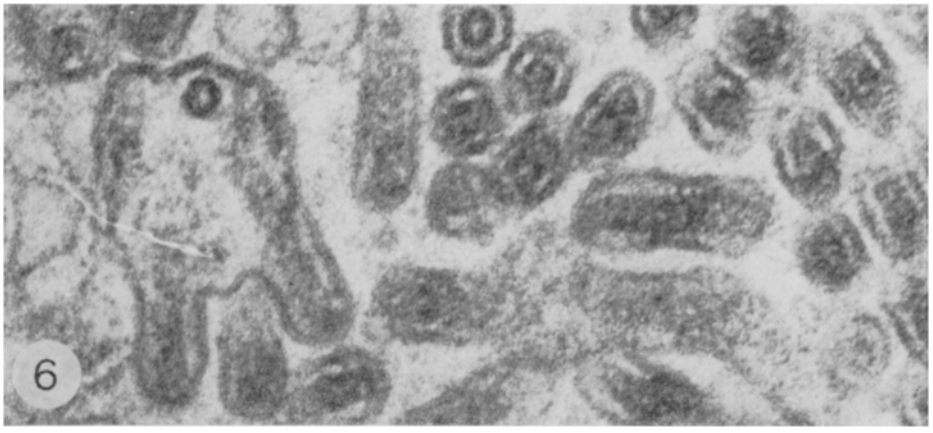

Fig. 6. Vergroot deel van Fig. 1, waarin een enkelwandig membraan drie "inner cores" omgeeft (143.000 ×).

Fig. 7. Accumulation of sac-like envelopes not containing inner bacillary cores in the cisternae of the endoplasmic reticulum (× 40,000).

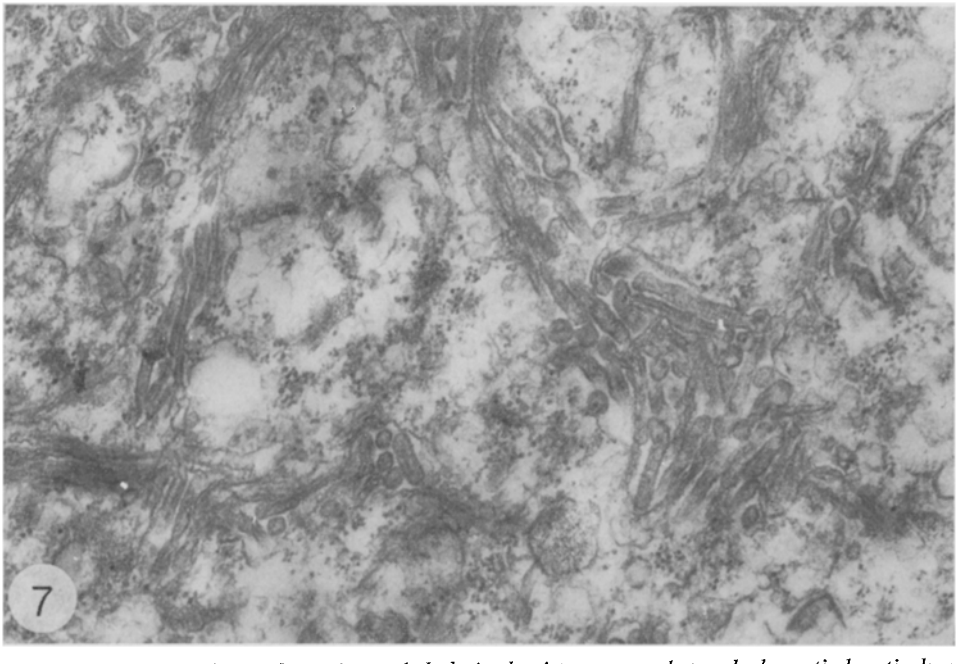

Fig. 7. Ophoping van lege zakvormige omhulsels in de cisternae van het endoplasmatisch reticulum (40.000 ×).

nucleoplasm into the perinuclear space, when becoming enveloped by an outer membrane evidently belonging to the inner nuclear membrane lamella. In a few instances two or more inner cores were found surrounded by a common envelope (Fig. 6). Empty sacs formed by detached nuclear membrane were also found in perinuclear spaces and in cisternae of the endoplasmic reticulum (Fig. 7).

In ultrathin sections of neoplastic tissues as well as of leaf areas without enations elongated virus particles were observed. They often occurred in bundles. Many of such threads were easily detected in negatively stained chop preparations of neoplastic tissues of graft-infected broad bean and of broad bean plants mechanically inoculated from enation-diseased white clover plants. The latter broad bean plants never showed any sign of enation formation. Two types of elongated particles were discerned viz. flexuous threads of around 475 mμ and more rigid rods of about 665 mμ long. By means of sap transmission tests the first virus was identified as white clover mosaic virus and the second one as a representative of the potato virus S group. Bacilliform particles were not observed by negative staining neither in neoplastic tissue nor in mechanically inoculated plants.

Discussion

The elongated viruses cannot be considered responsible for enation formation because such abnormalities were not produced in plants mechanically inoculated and infected with both viruses; thus they seem to be mere contaminants. The exclusive association of the bacilliform particles with tumorous tissues as found in ultrathin sections highly suggests that the particles represent the actual incitant of the clover enation disease. If so then the virus differs basically from those of wound tumour and of maize rough dwarf. Particles of the latter diseases are polyhedral and 60 mμ in diameter for wound tumour (Bils and Hall, 1962) and 50–70 mμ for maize rough dwarf (Gerola and Bassi, 1966). These and some other enation diseases have recently been found associated with dense accumulations of such spherical or polyhedral viruses, e.g. wound tumour (Shikata et al., 1964), maize rough dwarf (Gerola and Bassi, 1966) and sugar-cane Fiji disease (Gianotti et al., 1968). Therefore, the group of virus diseases characterized by histoid tissue outgrowths as brought together by Bos and Grancini (1968) seems heterogeneous in epidemiology (type of vectors) as well as in etiology.

In recent years a number of bacilliform plant viruses have been described. They are mentioned in chronological order of their discovery or of the detection of their bacilliform shape: lettuce necrotic yellows virus (Harrison and Crowley, 1965; Chambers et al., 1965), maize mozaic virus I (Herold et al., 1960; Herold and Munz, 1965, 1967), potato yellow dwarf virus (MacLeod et al., 1966), wheat striate mosaic virus (Lee, 1964, 1967, 1968), *Gomphrena* virus (Kitajima and Costa, 1966), an un-named virus in *Plantago lanceolata* (Hitchborn et al., 1966), sowthistle yellow vein virus (Richardson and Sylvester, 1968), broccoli necrotic yellows virus (Hills and Campbell, 1968) and rice transitory yellows virus (Shikata and Chen, 1969).

Some have particles that are always or partly with one rounded and one flat end (bullet-shaped) and of slightly different sizes, but always of strikingly similar ultrastructure. Epidemiologically, however, this group of viruses is as heterogeneous as is the group of enation viruses. Some are easily transmitted mechanically, others not at all; one is spread by aphids, others by leafhoppers. A close relationship of these plant

viruses to the animal virus vesicular stomatitis (Howatson and Whitmore, 1962) and some others is now generally accepted. The ultrastructure of vesicular stomatitis virus has been studied in much detail by Simpson and Hauser (1966).

The supposed clover enation virus has many morphological features in common with the other bacilliform viruses. Some of these have been found to exclusively occur inside the nucleus and in perinuclear spaces. Kitajima and Costa (1966), for *Gomphrena* virus, and MacLeod et al. (1966), for potato yellow dwarf virus, gave a detailed description of particle formation from pre-existent elements in the nucleoplasm (evidently the free nucleo-capsid) to "mature" particles individually enveloped in material similar to the inner lamella of the nuclear membrane through a process of budding; in this way the complete particles are supposed to land in the perinuclear space.

We have observed stages of a similar process with our clover enation virus. Great amounts of nucleocapsids were frequently found free in the nucleoplasm often occurring in crystalline array. Such particles showed a very regular size, suggesting that 200–220 mμ is the normal length and double lengths a result of linear aggregation of two inner cores during budding. Sometimes it was observed that one envelope contained two or three inner cores.

Other bacilliform viruses were found in the cytoplasm only, such as of maize mosaic I (Herold et al., 1960; Herold and Munz, 1965) and lettuce necrotic yellows (Chambers et al., 1965). Particles of wheat striate mosaic virus were localized in the cytoplasm, between nuclear membranes, and in the nucleus (Lee, 1967).

Since several bacilliform viruses are now being intensively studied in various laboratories for their structure, localization, and nature, undoubtedly in a near future more data will become available on their relationships and identification. Because their outer coat seems to be composed of non-specific plant material, serology of such coated viruses may be a doubtful expedient.

Samenvatting

Morfologie en intracellulaire lokalisatie van bacilvormige virusdeeltjes die samengaan met de klaver-enatieziekte

In ultradunne coupes van tumorachtige weefsels van witte-klaverplanten die door enting kunstmatig waren geïnfecteerd met het klaver-enatievirus waren de kernen en nucleoli sterk vergroot. Zulke kernen bevatten grote hoeveelheden kristallijn gerangschikte buisvormige deeltjes (Fig. 4 en 5). In de ruimten tussen de plaatselijk uiteengedrukte kernmembranen kwamen meestal onregelmatige ophopingen van zulke deeltjes voor (Fig. 1 en 3), hier echter voorzien van extra omhulsel (Fig. 5). Zulke bacilvormige deeltjes waren ongeveer 200 × 80 mμ groot. Soms bevonden zich in één omhulsel twee of meer buisvormige deeltjes (Fig. 6). Buiten de kern in de cisternae van het endoplasmatisch reticulum werden onregelmatig gevormde lege omhulsels aangetroffen (Fig. 7). In normale weefsels werd het virus niet gevonden.

Twee andere virussen met draadvormige deeltjes van ongeveer 475 en 665 mμ lang werden ook in niet-neoplastisch weefsel gevonden. Door middel van negatieve kleuring werden ze gemakkelijk in sap van fijngesneden weefsel aangetoond. Ze gingen snel over met sap en komen waarschijnlijk slechts als verontreiniging voor.

Enatievirusziekten zijn heterogeen in verspreidingswijze en oorzaak. Van een aantal is onlangs gevonden dat ze samengaan met dichte ophopingen van grote polyedrische virusdeeltjes in zieke weefsels.

Het hier ontdekte virus vertoont veel gelijkenis met een nog maar kort bekende, maar gedurende de laatste jaren snel groeiende groep van bacilvormige virussen, die uiteenlopen in veroorzaakte symptomen en verspreidingswijze.

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