Localization by Immunogold Cytochemistry of Viral Antigen in Sections of Plant Cells Infected with Red Clover Mottle Virus

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SUMMARY

Red clover mottle virus (RCMV) was detected by light and electron microscopy in sections of systemically infected pea leaf cells at successive stages of early infection using the combination of a sensitive immunocytochemical method with colloidal gold as a marker of antigen and a low temperature embedding procedure. Gold label was present in the cytoplasm at approximately the same time as the virus-induced membranous inclusions were established in the infected cells as judged by conventional electron microscopy of adjacent tissue. Labelling occurred simultaneously in the cytoplasm and among the membranes of the inclusion but not over the vesicles contained inside the inclusion. Viral antigen was localized by light microscopy with colloidal gold, nominally 8 nm, coupled to protein A and by electron microscopy with colloidal gold, nominally 5 nm, coupled to swine anti-rabbit immunoglobulins. Background staining was low and healthy tissue exposed to specific RCMV antibodies also had a very low level of gold particles.

INTRODUCTION

Red clover mottle virus (RCMV) is a member of the comovirus group (Bruening, 1978). In the cell, RCMV induces cytoplasmic membranous inclusions containing vesicles with fine fibrils presumed to be RNA (Tomenius & Oxelfelt, 1982). The membranous inclusions are characteristic of comoviruses (Martelli & Russo, 1977), and it has been demonstrated for two comoviruses, cowpea mosaic virus and Echtes Ackerbohnenmosaik virus, that replication of the viral genome is likely to be associated with the vesicles in the inclusions (de Zoeten et al., 1974; Hatta & Francki, 1978).

The present immunocytochemical investigation was undertaken to detect the distribution of viral antigen in RCMV-infected cells and to ascertain the relationship of antigen with the membranous inclusions and other cellular components. The membranous inclusions may have a role in the synthesis of coat proteins or in the assembly of virus particles, but this has not yet been demonstrated. In infected cells, the virus particles, unless they form aggregates or crystals, are not readily distinguished from ribosomes in the cytoplasm, which complicates the study of the early stages of infection. Hatta & Francki (1981) have had limited success in distinguishing small polyhedral viruses from ribosomes by digesting sections with RNase.

Immunocytochemistry is a possible method for the recognition of complete virus particles or viral antigens in sectioned material. Resins (methacrylates–acrylates) polymerized at low temperature are superior to the standard epoxy resins in preserving native molecular structure and antigenicity of proteins (Carlemalm et al., 1982; Craig & Goodchild, 1982; Bendayan & Shore, 1982). Colloidal gold is an excellent marker for immunocytochemistry both for the electron microscope (Roth et al., 1978; De Mey et al., 1981; for virus antigen, see Garzon et al., 1982; for pea seed storage proteins, see Craig & Millerd, 1981; Craig & Goodchild, 1982) and the light microscope (De Mey et al., 1981; Roth, 1982). In this paper we describe immunogold methods that detect RCMV antigen in ultrathin and thick pea leaf sections after low temperature embedding. We have used these methods to follow the early stages of infection.
Virus and host plant. Six-day-old plants of pea, Pisum sativum L. cv. English Sabre, were mechanically inoculated with RCMV (strain S) (Oxelfelt, 1976). The plants were kept in a greenhouse at 20 to 24 °C and a 16 h light period. Systemic vein clearing symptoms appeared on the third leaf pair at day 7 post-infection. Tissue pieces were excised from the middle region close to the midvein of the third leaf at days 4, 5, 6, 7, 8 and 9 post-infection. In some experiments, mainly for light microscopy, pieces were excised from leaves with mosaic symptoms at days 13 and 14 post-infection.

Chemicals. Butyl methacrylate (Fluka), glycol methacrylate embedding kit JB-4 (Polysciences), Lowicryl K4M (Juniper Ultra Micro, Stockholm), protein A (Pharmacia), swine immunoglobulins to rabbit immunoglobulins and rabbit immunoglobulins to human serum albumin (Dako, Copenhagen), and tetrachlorauric acid (Merck), were used in this study.

Immunogold reagents. Colloidal gold particles of nominal size 20 nm, 8 nm or 5 nm were prepared by reducing 0-01 % solutions of AuCl₃, HCl with trisodium citrate, ascorbic acid or white phosphorus respectively, essentially as described by Frens (1973) and Slot & Geuze (1981). Swine anti-rabbit immunoglobulin was coupled to 20 nm or to 5 nm colloidal gold by the method of De Mey et al. (1981). Protein A was coupled to 8 nm colloidal gold after the method of Slot & Geuze (1981), except that the final solution was made up in 0.5 % ovalbumin in 0.05 M-Tris-saline, pH 7.6.

Antibodies to RCMV. The virus was purified as described by Abdelmoeti & Oxelfelt (1982). Infected leaves were homogenized in phosphate buffer containing EDTA and diethyldithiocarbamate; the extract was filtered through gauze, centrifuged and the supernatant treated with polyethylene glycol 6000; after centrifugation the precipitate was washed and resuspended in phosphate buffer containing EDTA and NaCl, and the virus was further purified by chromatography on Sepharose CL-4B. The method gives a preparation of RCMV free of extraneous protein as judged by analytical ultracentrifugation and by electrophoresis in polyacrylamide gels containing SDS (Oxelfelt, 1976). Rabbits were injected intramuscularly with virus mixed with Freund’s incomplete adjuvant, followed by booster injections at 2-weekly intervals. The animals were bled and the immunoglobulins purified from the antiserum by ammonium sulphate precipitation and Sephadex G-200 chromatography. The final preparation of immunoglobulins to RCMV does not react with extracts of uninfected pea leaves in enzyme-linked immunosorbent assays (ELISA); it is highly reactive with the virus particles and extracts of infected leaves.

Recently, we have obtained antibodies to the larger coat protein subunit. The procedure was briefly as follows. A preparation of pure RCMV was boiled for 5 to 10 min in the presence of 1 % SDS, 5 mM-2-mercaptoethanol and 0-1 M-diethanol disulphide, to disrupt the virus and depolymerize the coat protein (Oxelfelt, 1976). The coat proteins were separated by electrophoresis on SDS-polyacrylamide gels; and pieces of gel containing the larger subunit (about 40000 mol. wt.) were cut out, washed, homogenized in phosphate-buffered saline (PBS) containing 0.1 % SDS, mixed with an equal volume of Freund’s adjuvant, followed by booster injections at 2-weekly intervals. The animals were bled and the immunoglobulins purified from the antiserum by ammonium sulphate precipitation and Sephadex G-200 chromatography. The final preparation of immunoglobulins to RCMV does not react with extracts of uninfected pea leaves in enzyme-linked immunosorbent assays (ELISA); it is highly reactive with the virus particles and extracts of infected leaves.

Antiserum from the rabbit after two booster injections was treated with ammonium sulphate to obtain a partially purified immunoglobulin fraction, which was dissolved in PBS and used in double-immunodiffusion studies as a control for similar studies with the antibodies to the intact virus. For immunodiffusion tests, 5 × 5 cm glass plates were covered with a layer of 1 % agarose in barbital–barbitone buffer, pH 8-6.4 ml per plate. A central well (4 mm diam.) containing 20 μl antibody solution was surrounded by 2 mm wells containing 10 μl antigen solution lying on radii at 1 cm distance from the central well. The antigen solution used was one of the following: (i) pure intact virus at 125 μg/ml in 0-025 M-phosphate buffer containing 0-05 M-NaCl and 0-025 M-EDTA, pH 6; (ii) the same preparation but with the protein coat completely depolymerized by boiling for 10 min in the presence of 1 % SDS, 2-mercaptoethanol and diethanol disulphide as described above; (iii) the same preparation as (i) but containing 1 % SDS, unheated.

Tissue preparation. Leaf pieces, about 1 mm across, from infected or healthy plants were fixed in 3 or 4 % glutaraldehyde or 4 % paraformaldehyde in 0-1 M-phosphate buffer, pH 7-1, for 2.5 to 3 h at 0 °C. They were washed overnight at 4 °C in PBS, which sometimes included 7 % sucrose. The pieces were dehydrated in acetone at 0 °C (either gradually through a series beginning with 30 % acetone or abruptly by direct transfer from buffer to absolute acetone, with three changes) and then left in acetone at 20 °C for 1 to 2 h. The pieces were then passed to a mixture of equal parts of acetone and Lowicryl K4M, a polar cross-linked methacrylic–acrylic polymer (for electron microscopy) or butyl methacrylate containing the Lowicryl initiator, benzoin methylether at 5 mg/ml (for light microscopy). Inhibitors had been removed from the methacrylate by shaking it with 2 % Na₂CO₃ and with distilled water, after which it was dried with Na₂SO₄. After 2 to 3 h, leaf pieces were transferred to fresh initiated Lowicryl or methacrylate and left to infiltrate at −20 °C for a week or more with two changes of medium. Leaf pieces were then put in 8 mm Taab polythene capsules containing fresh medium, and placed over dry ice in a thermos and left to polymerize about 15 cm from a CAMAG fluorescent tube emitting u.v. light at 350 nm; the temperature in the neighbourhood of the capsules was about −30 °C. In one experiment for light microscopy, JB-4 embedding medium was used instead of butyl methacrylate, otherwise following the same procedure.

For ultrastructural studies in parallel with the immunocytochemistry adjacent leaf pieces from the third leaf
infected for 4, 5, 6, 7 and 8 days were fixed conventionally in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7, for 2 h, and in 2% OsO4 for 6 h in the same buffer. After dehydration in ethanol including overnight staining in 70% ethanol saturated with uranyl acetate, the tissue was embedded in Spurr's medium. Sections were post-stained with uranyl acetate and lead citrate.

**Immunocytochemical staining for electron microscopy.** Thin sections of tissue, 50 to 70 nm, were mounted on Formvar-coated Ni-grids, and floated on drops of 1% bovine serum albumin (BSA) in Tris-saline, pH 7.6, for 5 min at room temperature to reduce non-specific adsorption of immunoglobulins. The grids were then transferred to drops of antiviral immunoglobulins, at a concentration of 20 µg/ml in Tris-saline containing 1% BSA, and incubated overnight (about 18 h) at 4°C. After washing with Tris-saline buffer (a mild jet spray from a plastic bottle for 20 s, grids placed on drops of buffer for 5 min, and repeated jet spray wash for 20 s), the grids were incubated on drops of the immunogold reagent (swine immunoglobulins coupled with 5 nm colloidal gold) diluted 1:10 or 1:5 in 1% BSA in Tris-saline, for 4 h at room temperature. After washing as above followed by an additional jet spray wash and placing for 5 min on drops of buffer, the grids were finally washed with 30 drops of double-distilled water, dried, and stained for 10 min with 5% uranyl acetate in water. Some of the grids were also stained with lead citrate for 1 to 2 min. As control procedures antiviral immunoglobulins were replaced by immunoglobulins to human serum albumin at the same concentration to check for unspecific binding of immunoglobulins to infected tissue, primary immunoglobulins were omitted or the standard procedure was applied to sections of healthy tissue.

**Immunocytochemical staining for light microscopy.** Butyl methacrylate sections, cut at 5 µm, were floated on distilled water and attached to slides at room temperature. Slides were passed through xylene (two passages of 1 min each) to remove the methacrylate, and transferred quickly through acetone and water to Tris-saline. Aldehyde groups on the sections were blocked with 1% BSA in Tris-saline for 5 min at room temperature when gold coupled to swine immunoglobulins was subsequently applied (or 0.5% ovalbumin in Tris-saline if protein A-gold was used). Antiviral immunoglobulins were diluted in Tris-saline containing 1% BSA or 0.5% ovalbumin. The standard concentration was 20 µg/ml, applied overnight at 4°C. After washing in Tris-saline (three passages of 2 min each) slides were treated with the gold reagent (20 nm gold coupled to swine Ig or 8 nm gold coupled to protein A, diluted 1:1 with Tris-saline containing 1% BSA or 0.5% ovalbumin), for 2 to 6 h at room temperature. After washing in Tris-saline, slides were counter-stained in 0.01% toluidine blue for 15 to 30 s, dehydrated through graded alcohol dilutions (a few seconds in each), passed through xylene and mounted in synthetic Canada balsam.

As control procedures the concentration of antiviral immunoglobulin was varied from 5 to 40 µg/ml, antiviral immunoglobulin was replaced by immunoglobulin to human serum albumin at the standard concentration to check for non-specific binding of immunoglobulins to infected tissue, or the standard procedure was applied to sections of healthy tissue.

JB-4 sections were treated as in the standard procedure, except that they were first etched in xylene for 15 min and digested with 250 µg/ml Sigma protease V for 2 h at 37 °C before washing in Tris-saline and applying antibodies (after Mozdzen & Keren, 1982).

**RESULTS**

**Immunodiffusion tests**

When the intact virus was allowed to diffuse against antibodies to whole virus, one immunoprecipitate band appeared within 24 h; when it diffused against antibodies to the coat protein subunit, no precipitate appeared even after 3 days. When completely depolymerized virus (after boiling in SDS and reducing agents) diffused against antibodies to whole virus, no immunoprecipitate formed, whereas when it diffused against antibodies to coat protein subunit, one immunoprecipitate band appeared within 24 h. When virus treated with 1% SDS at room temperature diffused against either antibody, one precipitate band formed in each case, although this did not occur until the second day with the antibodies to intact virus. The position of the immunoprecipitate band was characteristic, being close to the antigen well for the intact virus, whether previously treated with SDS or not, and about half way between the antigen and antibody wells for the coat protein subunits; this is as expected owing to the great difference in molecular sizes between intact virus and protein subunits.

In principle, the antibodies to whole virus might still react with the native protein subunits, even though they do not react with subunits prepared by SDS treatment. This is very unlikely, however, since the treatment with SDS does not affect the reaction between antibody and intact virus, and, in general, antibodies prepared from rabbits injected with a protein that has been denatured with SDS react specifically both with the native protein and with the treated protein.
Fig. 1. Pea plant cell infected with red clover mottle virus 6 days post-infection. (a) Proliferation of the outer part of the nuclear membrane (arrows) and a young membranous inclusion in the cytoplasm (MI). Ribosomes and/or virus particles are relatively few among the vesicles in the inclusion compared to the adjacent cytoplasm. N, Nucleus. Bar marker represents 250 nm. (b) Virus-like particles in a double row in a plasmodesma. Bar marker represents 250 nm.
when it is allowed to renature in the absence of SDS (see Burridge & Jordan, 1980). We have not found a suitable method of preparing subunits from virus without the use of SDS.

Electron microscopy

Preservation of cell organization, organelles and antigenicity in tissue fixed with 3 or 4% glutaraldehyde and embedded in the low denaturing embedding medium was sufficient to localize the antigen. Membranes were not preserved, but were seen in negative contrast. Nuclei and chloroplasts were in excellent condition, comparable with that expected with conventional epon embedding. In tissue fixed with 4% formaldehyde, however, chloroplasts were disorganized and ruptured, and also most of the antigenicity was lost.

The presence and degree of development of membranous inclusions indicated the stage of infection in mesophyll cells determined from the investigation of tissue embedded in Spurr's epoxy resin. At day 4 post-infection no virus-induced membranous inclusions were seen in the third leaf, which was still expanding. Vesicles began to accumulate in mesophyll cells to form the inclusion on days 5 and 6 post-infection and were visible always together with dictyosomes in the cytoplasm close to the nuclear membrane, which seemed to be involved in the synthesis of membranes (Fig. 1a).

Accumulations of vesicles were occasionally seen as early as day 4 or 5 post-infection in immature xylem vessels and sieve tubes; thus, virus particles could have been present in these cells before they were detected in the surrounding mesophyll tissue. The number of inclusions increased rapidly in mesophyll cells between days 5 and 6 post-infection (from 14 to 56%), and on day 8 membranous inclusions were visible in 68% of sectioned cells. Virus-like particles were first observed on day 6 in the plasmodesmata, where they often occurred in double rows (Fig. 1b). Thus, intracellular alterations in mesophyll cells began 5 days after infection, 2 days before vein clearing was visible on the leaves.

Small aggregates of virus-like particles were seen from day 7 post-infection in the cytoplasm often in the vicinity of the membranous inclusion and also in the vacuole (Fig. 2). Label was not detected in mesophyll cells infected for 4 or 5 days; on the 6th day label was localized in the cytoplasm and the membranous inclusions. From day 7 to day 9 post-infection the label was intense in the inclusions as well as in the cytoplasm (Fig. 3). Gold labelling was observed among the membranes of the inclusion but not over the vesicles (Fig. 3). Nuclei, chloroplasts and mitochondria remained unlabelled. Gold was also present in the mature xylem vessels adhering to the cell walls. A diffuse staining was occasionally present in vacuoles and in the lumen of sieve tubes.

Sections of healthy tissue treated by the standard procedure and sections of infected tissue exposed to immunoglobulins to human serum albumin usually showed no label (Fig. 4). When primary immunoglobulins were omitted there was no labelling.

Light microscopy

Immunocytochemical staining was equally strong after formaldehyde and glutaraldehyde fixation. There was no advantage in including sucrose in the wash buffer after fixation, nor in abrupt rather than gradual dehydration in acetone, procedures found necessary by Takamiya et al. (1980).

When the standard staining procedure was applied to butyl methacrylate sections of infected tissue with mosaic symptoms, gold label could be seen as a red stain generally distributed in the ground cytoplasm of mesophyll and epidermal cells, but not over nuclei or plastids, nor did it obviously accumulate in vacuoles. It could also be seen in conducting tissue. Staining was brilliant with the smaller probe (protein A–8 nm gold), but much less so with the immunoglobulin–20 nm gold reagent, presumably because of poorer penetration. Toluidine blue stained nuclei and cuticles deep blue while leaving the rest of the cell very lightly stained. Unfortunately, the membranous inclusions were not always recognized.

Healthy tissue was essentially unlabelled with gold, as was infected tissue treated with immunoglobulins to human serum albumin. The absence of non-specific red gold staining even from conducting tissue in control slides was striking. When antiviral immunoglobulins were
Fig. 2. Pea plant cell infected with RCMV 8 days post-infection. Aggregates of virus-like particles (arrows) in the vacuole (V) and in the cytoplasm with associated amorphous dark-staining material (arrowhead) are shown. C, Chloroplast; MI, membranous inclusion present in a young phloem cell above. Bar marker represents 250 nm.
Fig. 3. Lowicryl embedding. Membranous inclusion (MI) in a mesophyll cell 9 days after infection with RCMV. Gold label (black dots, arrows) is present inside the inclusion and in the adjacent cytoplasm, but not over the vesicles (VS, see inset), chloroplast (C) and mitochondrion (M). Virus-like particles were generally not seen after 18 h exposure to antiserum. Bar markers represent 250 nm.

applied at higher dilutions to infected tissue, the subsequent gold staining was less intense without any obvious change of position within the cells.

Gold staining was not observed in 4 μm sections of JB-4 material. Thinner sections might have given better results (the embedding material cannot be removed as can butyl methacrylate), or more vigorous etching, but butyl methacrylate sections seemed to be of comparable quality as regards preservation of structure.
Fig. 4. Lowicryl-embedded healthy tissue exposed to antiviral immunoglobulins. Non-specific staining (arrows) is present in low concentration over the cytoplasm, nucleus (N) and chloroplast (C). Bar marker represents 250 nm.

DISCUSSION

The results of this study demonstrate that RCMV antigen can be effectively localized by immunogold methods in ultrathin and thick leaf sections after fixation in 3 to 4% glutaraldehyde and low temperature methacrylate embedding.

Viral antigen was localized by electron microscopy in the cytoplasm, including the virus-induced membranous inclusions, 6 days after infection in mesophyll cells, 1 day before vein clearing symptoms were visible on the leaves. Thus, viral antigen did not accumulate in detectable amounts until the inclusion was established and replication of the viral genome had presumably started. The low background labelling over nuclei, chloroplasts and mitochondria was comparable to that in healthy controls exposed to RCMV-specific antibodies, indicating that virus particles do not accumulate in any of these organelles.

Localization of small polyhedral viruses in the cytoplasm and their site of assembly may be complicated by several factors. Virions can be identified only if they form large aggregates or crystals (Milne, 1967; Hatta & Francki, 1981). RCMV does not develop large aggregates in or close to the membranous inclusion (Tomenius & Oxelfelt, 1982) unlike three other comoviruses (Stefanac & Ljubesic, 1971; Kitajima et al., 1974; Bowyer et al., 1980). However, small virus aggregates are sometimes seen in the cytoplasm next to membranous inclusions. If coat protein and newly synthesized viral RNA are assembled in association with or within the membranous inclusion, the particles are rapidly translocated, as label occurs all over the cytoplasm early in infection. Label is intense among the membranes of the inclusion and is sometimes associated with the membranes of the vesicles, but is not present over the vesicles. This indicates that virus structural protein possibly synthesized on cytoplasmic ribosomes as with cowpea mosaic virus (Owens & Bruening, 1975), may combine with newly made viral RNA outside the vesicles into complete RCMV particles.

In this study we used as primary antibodies immunoglobulins developed in rabbits against whole virus particles and specific to them according to our tests. The immunodiffusion tests strongly support the view that the antibodies to whole virus do not react with unpolymerized
protein subunits. This is entirely in line with experience with other viruses (see van Regenmortel, 1982; McMillen & Consigli, 1977). To follow the course of RCMV infection in more detail it will be necessary to develop antisera specific to coat protein subunits, to enzymes induced by the virus or to virus double-stranded RNA (for discussion of antisera against similar components of other viruses, see van Regenmortel, 1982).

Our work with thick leaf sections was intended mainly to develop methods that could later be applied to ultrathin sections of Lowicryl K4M. Butyl methacrylate was chosen as low temperature embedding medium for thick sections because it can easily be removed from the sections, thus avoiding the need for etching to allow penetration of antibodies. The work is of wider interest, however, since we found that staining with the protein A–8 nm gold reagent, in particular, gave good results that were much easier to interpret owing to the virtual absence of non-specific staining than corresponding sections of freeze-substituted material embedded in wax and stained by fluorescent antibody methods.

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REFERENCES


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