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Specific gold-labelling of antibodies bound to plant viruses in mixed suspensions

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Abstract

Protein A-gold complexes with gold particle diameters of 7 and 16 nm were prepared and could be stored at 4 °C for at least 5 months without loosing activity. The complexes were used to detect antibodies bound to two plant viruses in mixed suspensions. Depending on the antibodies used, each virus could be labelled specifically with protein A-gold complexes with a gold particle diameter of either 7 nm or 16 nm.

A double-labelling technique was developed by which the viruses in suspension could be labelled specifically with protein A-gold complexes with gold particle diameters of either of the two sizes mentioned. Using this technique it was possible to distinguish and identify two viruses with a similar spherical appearance in the electron microscope in mixed preparations.

Additional keywoords: protein A-gold, immuno-labelling, virus identification.

Introduction

Gold labelling of virus particles in purified suspensions and extracts of infected plant tissue has been shown to be a useful technique in addition to other immuno-electron microscopical techniques as described by Derrick (1973) and Milne and Luisoni (1975). Labelling virus particles with protein A-gold (pAg) (Pares and Whitecross, 1982) and gammaglobulin (IgG)-gold (Lin, 1984) complexes for positive identification appeared to have advantages over the decoration technique described by Milne and Luisoni (1975). The pAg technique makes use of the two binding properties of IgG. Each IgG molecule consists of one region (F_c-fragment), which binds to protein A and two other identical regions ($F(ab')_2$-fragments), which bind to the antigen. Pares and Whitecross (1982) described the 'gold-labelled antibody decoration' (GLAD) with protein A-gold complexes using different strains of tobacco mosaic virus as antigens. With this serological technique distantly related viruses could be distinguished by quantitative analysis of the number of adsorbed gold particles, but such an analysis failed to distinguish between closely related viruses. Lin (1984) showed the merit of IgG-gold complexes for the rapid detection and identification of viruses in leaf-dip preparations of infected plant tissue. A combination of the serologically specific electron microscopical (SSEM) technique, by which viruses in suspension are trapped on grids precoated with antibodies (Derrick, 1973) and subsequent gold labelling resulted in

an increased background labelling. Louro and Lesemann (1984) showed that this aspecific adsorption of protein A-gold complexes to precoated grids could be eliminated almost entirely by using specific antibodies from which the F_c fragment had been removed, leaving the $F(ab')_2$fragments intact. This technique combines the sensitivity of the SSEM technique and the discrimination of the GLAD technique. In the present study we investigated the possibility of labelling two different antigens in mixed preparations with protein A-gold complexes consisting of gold particles with different diameters.

Materials and methods

Viruses. Cowpea chlorotic mottle virus (CCMV) and southern bean mosaic virus (SBMV), cowpea strain, were maintained in cowpea *(Vigna unguiculata* cv. Blackeye Early Ramshorn). Tobacco mosaic virus (Wageningen strain) was maintained in *Nicotiana tabacum* var. White Burley.

CCMV was purified as described previously (Verduin, 1978). Essentially the same purification protocol was used for SBMV but the buffers differed. As homogenization buffer 0.25 M citrate buffer (pH 4.8) containing 0.02 M $MgCl_2$ and 5 mM disodium EDTA was used. Pellets were resuspended in 0.2 M citrate buffer (pH 5.5) containing 0.02 M $MgCl_2$, 0.001 M disodium EDTA and 0.001 M sodium azide. TMV was purified according to procedure 5 as described in Noordam (1973). Mixed suspensions of CCMV and TMV or CCMV and SBMV were prepared in 0.1 sodium acetate buffer (pH 5.0). Each virus was used in a final concentration of 0.01 mg ml^{-1}.

Antisera. Antibodies against CCMV (anti-CCMV), SBMV (anti-SBMV) and TMV (anti-TMV) were elecited in rabbits by one intravenal injection of 1 mg of purified virus followed by two intramuscular injections of 2 mg purified virus in Freund's complete adjuvant with a two-week interval. In double diffusion tests titres of 1/32, 1/512 and 1/64 for CCMV, SBMV and TMV respectively were reached against virus at 1 mg ml^{-1} 8 weeks after the initial injection. Antisera of the three viruses were purified by passing the whole serum through a column of Affigel Blue (1 ml serum per 4.7 ml Affigel Blue). Gammaglobulin was then eluted with 0.02 M Tris/HCl buffer (pH 8.0) containing 0.028 M NaCl and 3 mM NaN_3. The obtained purified gammaglobulin (IgG) suspensions were diluted in 0.1 M sodium acetate buffer (pH 5.0) containing 1% (w/v) bovine serum albumin (BSA).

Preparation of 7 nm and 16 nm gold particles. Colloidal gold was prepared by reduction of hydrochloroauric acid ($HAuCl_4$) with sodium citrate (Slot and Geuze, 1981) or a mixture of tannic acid and sodium citrate (Mühlpfordt, 1982), resulting in suspensions of colloidal gold particles with respective average diameters of 16 and 7 nm. For the production of the colloidal gold suspensions, glassware was cleaned extremely well by boiling in distilled water. Solutions were made in freshly prepared distilled water and filtered through 0.2 μm nitrocellulose filters prior to use. Colloidal gold particles with a diameter of 16 nm were prepared as follows: 247.5 ml of distilled water was boiled in a clean flask under reflux and 2.5 ml of a 25.4 mM $HAuCl_4$ solution was added. Then 7.5 ml of 34 mM tri-sodium citrate was quickly added to the boiling $HAuCl_4$ solution while stirring. The mixture was boiled for 15-30 min under reflux.

Colloidal gold particles with a diameter of 7 nm were prepared by adding a mixture of 2 ml 34 mM tri-sodium citrate and 0.54 ml 1% (w/v) tannic acid quickly to 100 ml of a boiling 25.4 mM $HAuCl_4$ solution. The mixture was then boiled for another 15 min under reflux. The colloidal gold suspensions were allowed to cool, divided in 30 ml portions and stored at 4 °C until use. To determine the diameters of the gold particles in the suspensions thus prepared, the gold particles were allowed to adsorb to grids covered with formvar/carbon and previously incubated for 10 min on a solution of 0.01% (w/v) poly-L-lysin (M_r 70 000). Diameters of gold particles were measured from micrographs taken of each suspension.

Preparation of the protein A-gold complexes. Lyophilized protein A (pA) was dissolved in distilled water to a concentration of 2 mg ml^{-1}. The colloidal gold suspensions were, if necessary, adjusted to a pH between 5 and 6 by adding drops of 0.1 M K_2CO_3 in double distilled water. The optimal amount of pA necessary to stabilize the colloidal gold suspensions was determined by adding various amounts of pA to samples of the gold suspensions as described by Horrisberger and Rosset (1977). If colloidal gold was completely stabilized by protein A, the color of the suspension remained red after addition of NaCl. The minimum amounts of pA necessary to stabilize the suspensions of 7 nm and 16 nm particles were 10 and 5 $\mu g\ ml^{-1}$ respectively.

The appropriate amount of pA was added to 30 ml of gold suspension and the mixture was stirred for 5 minutes. Then 0.3 ml of 5% (w/v) polyethylene-glycol (M_r 20 000) was added. The suspension was centrifuged (16 nm gold: 1 h at 15 000 *g*; 7 nm gold: 45 min at 104 000 *g*; all *g* values are given at R_{av} and all centrifugations were done at 4 °C) and the supernatant removed. The loose pellet containing the pAg complexes was resuspended in the remaining supernatant and left for 2 h at 4 °C. The concentrated suspension was diluted in phosphate buffered saline (PBS: 0.137 M NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 3.1 mM NaN_3) (pH 7.4) and centrifuged for 15 min 250 *g*. The supernatant was then again centrifuged at high speed. After centrifugation the supernatant was removed carefully and the loose pellet of pAg particles was resuspended in the remaining supernatant. The absorption at 520 nm was determined and the pAg suspension was diluted to an $A_{520} = 5$ in PBS containing 1% (w/v) BSA and 3 mM sodium azide. Protein A-gold suspensions were stored at 4 °C.

Single and double gold labelling of virus. Preparations were made on 150 mesh nickel grids covered with formvar and coated with a ca. 3 nm carbon layer. Prior to use the grids were exposed to a glow discharge in air for 10 s. For single gold labelling, grids were incubated on drops of a mixed virus suspension for 10 min, followed by an incubation for 10 min on 1% (w/v) BSA in 0.1 M sodium acetate (pH 5.0) and 10 min on 0.01 mg IgG ml^{-1}. The grids were then washed with 30 drops of 0.1 M sodium acetate buffer (pH 5.0). Grids were further incubated for 10 min on a suspension of 7 nm pAg (pAg-7) particles diluted to $A_{520} = 0.5$ in 1% (w/v) BSA in 0.1 M sodium acetate buffer (pH 5.0). Grids were washed with 30 drops of buffer, 10 drops of distilled water and stained with 0.05 M uranyl acetate in water.

For double gold labelling, grids were treated as described above. After incubation on pAg-7 the grids were washed with 30 drops of buffer and incubated on the second antibody suspension for 10 min, washed with 30 drops of buffer and incubated for 10 min on pAg-16 ($A_{520} = 0.5$). Finally the grids were washed with 30 drops of buffer,

10 drops of water and stained with uranyl acetate. All incubations were at room temperature. Preparations were observed with a Siemens Elmiskop 101 or a Zeiss EM 109 electron microscope.

Results

Colloidal gold suspensions prepared by reduction of chloroauric acid with sodium citrate or tannic acid/citrate using the described methods, repeatedly yielded suspensions containing gold particles with average diameters of 16 nm and 7 nm, respectively. The distribution of particle diameters in suspensions is shown in Fig. 1A (7 nm particles) and Fig. 1B (16 nm particles). As there was no overlap of particle diameters between the 7 nm and 16 nm suspensions, no attempt was made to isolate fractions of more homodisperse particles by centrifugation of pAg complexes on glycerol gradients (as described by Slot and Geuze, 1981). Seven nm and 16 nm gold suspensions stabilized with pA were used in single- and double-labelling experiments.

The pAg complexes remained sufficiently active over a period of at least 3-5 months when kept at 4 °C. Both single- and double-labelling were tested on a mixture of TMV and CCMV. Preparations of this mixture were adsorbed to grids, incubated on specific antibody solutions against TMV or CCMV followed by incubation with pAg-7. The results of these experiments are shown in Figs 2A and 2B. In Fig. 2A the virus preparation was incubated with specific antibodies against TMV. TMV particles were covered with 7 nm gold particles, whereas the CCMV particles were not labelled at all. Fig. 2B demonstrates the reverse situation where the virus preparation was incubated with specific antibodies against CCMV and only CCMV particles were labelled with 7 nm gold particles. Identical results were obtained when labelling was done with pAg-16 instead of pAg-7.

When virus preparations labelled for CCMV with pAg-7 were incubated further with specific antibodies to TMV followed by pAg-16, double-labelling was achieved. CCMV particles were surrounded with 7 nm gold particles and TMV with 16 nm particles (Fig. 3). Cross-adsorption of 16 nm particles to CCMV particles rarely occurred.

These experiments were repeated with a mixture of two spherical viruses, CCMV and SBMV. Due to their similar morphological appearance individual particles of

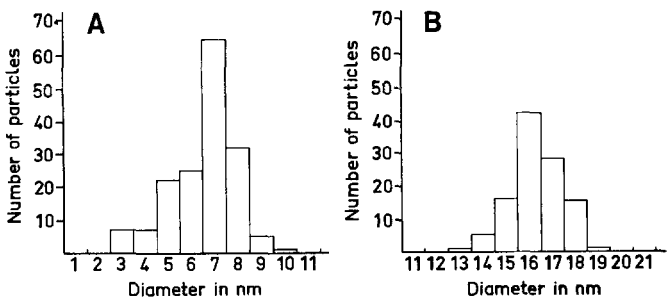

Fig. 1. Histograms illustrating the diameters of colloidal gold particles prepared by the reduction of $HAuCl_4$ with sodium citrate (A) and with a mixture of tannic acid and sodium citrate (B).

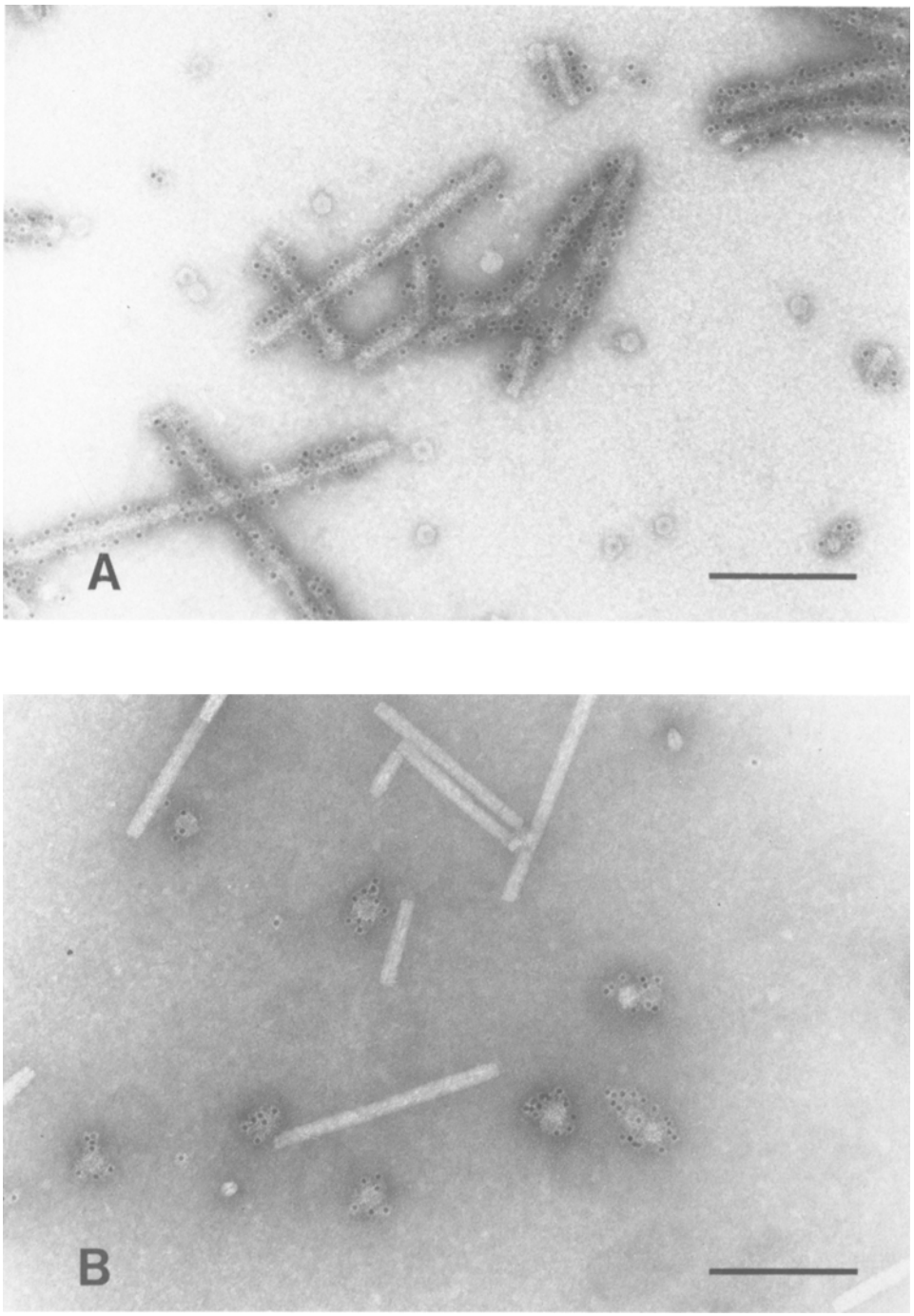

Fig. 2. Specific labelling of TMV (A) and CCMV (B) with protein A-gold complexes with a gold particle diameter of 7 nm (bar represents 200 nm).

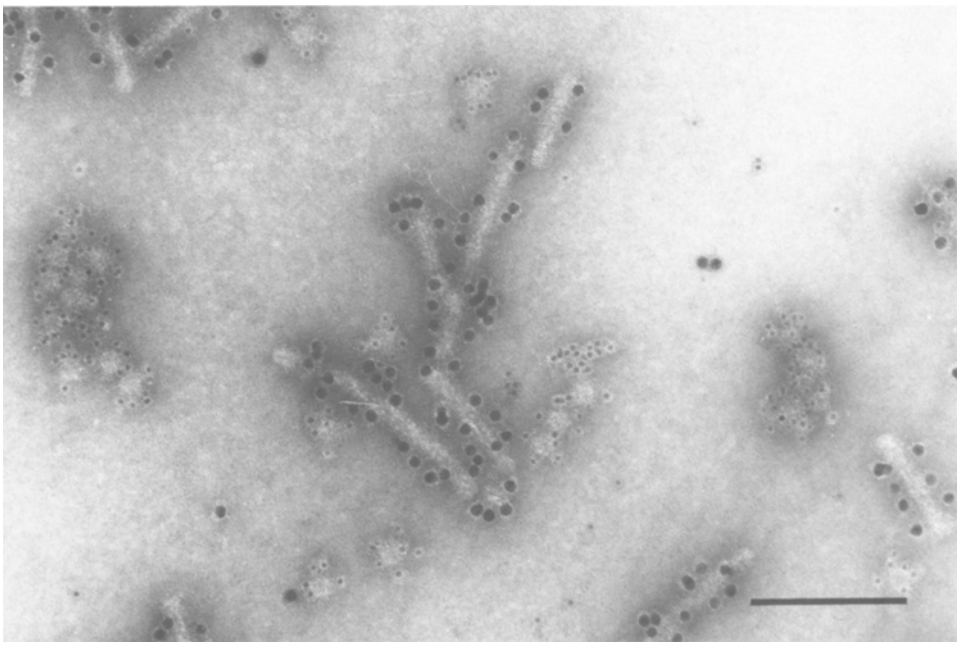

Fig. 3. Double-labelling of two viruses in suspension. TMV is specifically labelled with protein A-gold complexes with a gold particle diameter of 16 nm and CCMV with particles of 7 nm in diameter (bar represents 200 nm).

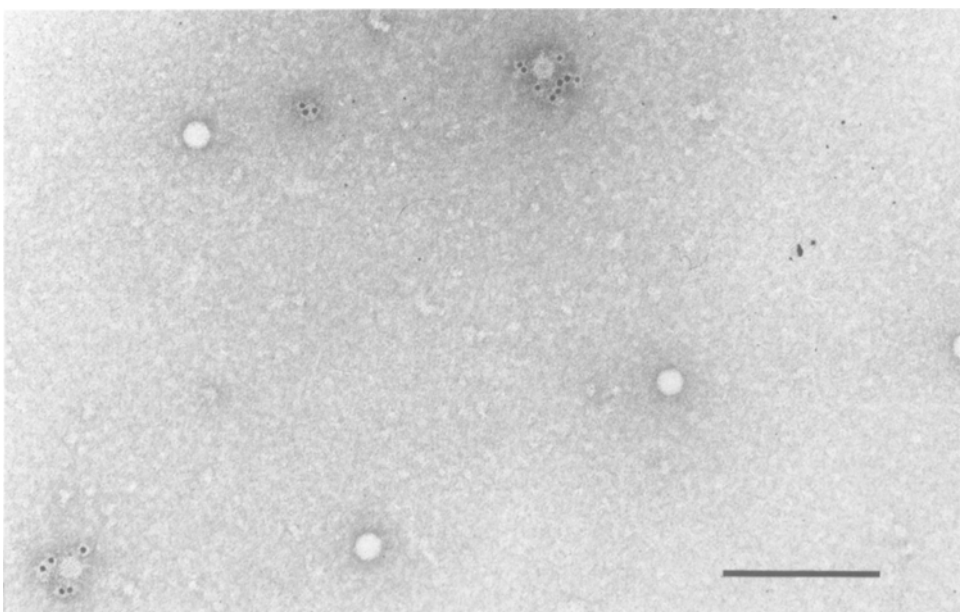

Fig. 4. Specific labelling of CCMV with protein A-gold complexes with a gold diameter of 7 nm, in a mixed suspension of CCMV and SBMV (bar represents 200 nm).

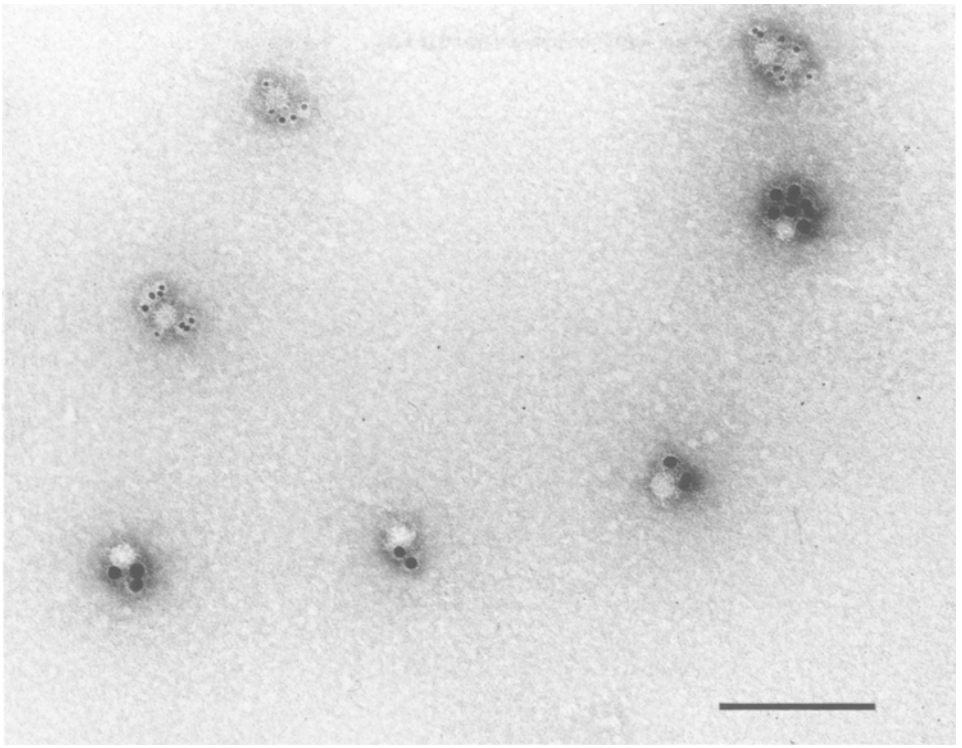

Fig. 5. Double-labelling of CCMV and SBMV. CCMV is specifically labelled wit protein A-gold complexes with a gold particle diameter of 7 nm and SBMV with particles of 16 nm in diameter (bar represents 200 nm).

CCMV and SBMV cannot be identified with the electron microscope when occurring in a mixture. However, Fig. 4 demonstrates specific labelling of CCMV with pAg-7 in a CCMV/SBMV mixture incubated with specific antibodies to CCMV. Some spherical particles are labelled (CCMV) and some have no label (SBMV). In Fig. 5 double-labelling is shown with pAg-7 bound to CCMV particles and pAg-16 to SBMV particles. Cross-adsorption of pAg-16 to the first specific antibodies present on CCMV occurred only rarely. Apparently the pAg-7 blocked almost all available binding sites for pA. Incubation of the preparations with pA (0.01 mg ml^{-1} in 0.1 M sodium acetate buffer (pH 5.0) with 1% (w/v) BSA) in between the first labelling with pAg-7 and subsequent incubation with the second antibody reduced cross-adsorption, but was not used routinely.

Discussion

Preparation of suspensions of gold particles with defined diameters according to the described methods appeared to be easy and reliable. As our studies involve several antigenically different plant viruses we have chosen to work with pAg complexes instead

of immunoglobulin-gold complexes. Absorption of pA to these gold particles was easy and resulted in active and stable electron-dense markers which could be used for the detection of antigen-antibody complexes in the electron microscopy.

Slot and Geuze (1981) and Geuze et al. (1981) demonstrated the use of pA complexes of two different sizes for double-labelling of two antigens in sections of frozen tissue. We have demonstrated here the use of double-labelling for two antigens in mixed plant virus suspensions. Labelling one antigen with pAg-7 and the other with pAg-16 appeared to be possible with minimal cross-adsorption. Not only could CCMV be distinguished from TMV by labelling the first with 7 nm gold particles and the latter with 16 nm particles, but also could CCMV and SBMV particles be identified in mixed preparations of the two viruses. Both viruses could not be clearly identified in mixed preparations due to a similar morphological appearance in the electron microscope. Double-labelling of antigens could be a powerful technique in the detection and identification of viruses in suspensions and in dip-preparations of plant tissue from mixed infections. Continuing the idea of Pares and Whitecross (1982) to distinguish serologically more closely-related viruses, it might be possible to identify and quantify this relation by double-labelling of a single virus. This virus, incubated with mixtures of heterologous and homologous antibodies respectively adsorbed to 7 and 16 nm gold particles will be labelled with 7 and 16 nm gold particles. The ratio of 7 nm to 16 nm gold particles should be a measure to the relationship between these two viruses. The limitations of this method will depend on the maximum number of gold particles which can bind to one virus particle. Further investigations will be needed to demonstrate the usefulness of this technique.

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Samenvatting

Specifieke goudmerking van antilichamen gebonden aan plantevirussen in gemengde suspensies

Proteïne A werd geadsorbeerd aan colloïdale gouddeeltjes met een diameter van 7 en 16 nm. Deze proteïne A-goudcomplexen werden gebruikt voor de specifieke merking van antilichamen gebonden aan virusdeeltjes in mengsuspensies van twee virussen.

Afhankelijk van het antiserum dat werd gebruikt was het mogelijk om, in suspensies van tabaksmozaïekvirus (TMV) en 'cowpea chlorotic mottle' virus (CCMV) of van CCMV en 'southern bean mosaic' virus (SBMV), elk virus afzonderlijk te merken met gouddeeltjes van 7 en 16 nm.

Een techniek voor dubbele merking werd ontwikkeld waarbij de virussen in mengsuspensies specifiek gemerkt konden worden met proteïne A-goudcomplexen die

gouddeeltjes bevatten van de twee genoemde afmetingen. Zo kon in een gemengde virussuspensie van CCMV en TMV, TMV specifiek gemerkt worden met gouddeeltjes van 16 nm en CCMV met deeltjes van 7 nm. In mengsuspensies van CCMV en SBMV werd CCMV specifiek gemerkt met gouddeeltjes van 7 nm en SBMV met deeltjes van 16 nm. Met deze dubbele merking is het mogelijk met een elektronenmicroscoop onderscheid te maken tussen twee gelijkvormige virussen die in één preparaat voorkomen. Tevens is met deze techniek een positieve identificatie van beide virussen mogelijk.

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