Neth. J. Pl. Path. 88 (1982) 33-37

The effect of pretreatments of carbon-coated formvar films on the trapping of potato leafroll virus particles using immunosorbent electron microscopy

EMMY VAN BALEN

Research Institute for Plant Protection (IPO), Wageningen

Accepted 21 September 1981

Abstract

The application of electrical charges on carbon-coated formvar films had a marked effect on the trapping of virus particles by immunosorbent electron microscopical techniques. On grids, positively charged with ethidium bromide, numbers of virus particles were high, and almost equal to those trapped with the aid of protein A in combination with a negative charge.

Additional keywords: ethidium bromide, glow discharge, electrically charged grids, protein A.

Introduction

Several authors (e.g. Brlanski and Derrick, 1979; Kojima et al., 1978; Lesemann et al., 1980; Milne and Luisoni, 1975, 1977; Ohki et al., 1980; Roberts and Harrison, 1979) have described the effect of coating of electron microscope grids with antisera, or their γ-globulins, on the trapping of particles of the viruses concerned. This method, commonly known as the Derrick method, enables the detection of virus particles that otherwise might escape observation, e.g. because they occur at a low concentration in some of their hosts or vectors or in a limited number of cells (Brlanski and Derrick, 1979; Kojima et al., 1978; Ohki et al., 1980; Roberts and Harrison, 1979).

Since the numbers of virus particles trapped may vary greatly between grids and even between squares of a grid, efforts were made to improve the trapping (Lesemann et al., 1980). On grids, treated with protein A, prior to coating with antiserum to sugar cane mosaic virus and tobacco mosaic virus (TMV), Shukla and Gough (1979) observed larger numbers of virus particles than on films coated only with crude antiserum.

Protein A is known to bind to the Fc portion of lgG molecules in such a way that one molecule of protein A binds two lgG molecules.

Applying these techniques I found rather big differences in spreading and quantity of the viruses on the grids. My efforts were aimed to get a higher yield of virus particles and a more even spreading of the viruses.

Materials and methods

Potato plants (*Solanum tuberosum* cv. Bintje) were used, infected with potato leaf-roll virus (PLRV) and kept in a glasshouse at c. 15°C.

Veins were removed from full-grown leaves and homogenized with an Ultra-Turrax blender, in phosphate buffer (0.06 M, pH 6.5) at 0°C, 5 ml of buffer solution being applied to 1 g of vein tissue. The homogenate was centrifuged at 3090 *g* for 10 min. The supernatant was used in our experiments for detection of PLRV particles.

The γ-globulin fraction of PLRV antiserum was kindly supplied by Ing. D.Z. Maat and had a concentration of 1 $mg.ml^{-1}$. Protein A, a cell wall protein from *Staphylococcus aureus,* had a concentration of 0.1 $mg.ml^{-1}$ in an aqueous solution of 7.7 mM NaN_3 (Shukla and Gough, 1979).

A number of experiments were performed to study the effect of the different treatments. Three of them will be described in the following. In the first experiment the formvar-coated grids were coated with carbon, either a thick layer (evaporation 10 sec), or a thin layer (evaporation 3 sec).

Prior to γ-globulin coating and application of virus these two batches of grids were given pretreatments, which consisted of either a) the application of a negative electrical charge, by exposure to HV glow discharge in argon for 5 min at a pressure varying from 10^{-1} till 6×10^{-1} torr in an Edwards vacuum coater, model 306, or b) the application of a positive electrical charge by floating on ethidium bromide (30 $\mu g.ml^{-1}$ in distilled water) for 15 min (Sogo et al., 1979). Untreated carbon-coated formvar grids, stored at room temperature for two weeks or more were considered to be neutral.

In the second and third experiment γ-globulin and virus were applied to the three batches of pretreated grids provided with a thick carbon layer (negatively charged, positively charged and neutral), either directly or after coating with protein A.

In all experiments the grids were rinsed between treatments with two drops of distilled water and drained briefly with filter paper. After coating with γ-globulin for 15 min the grids were incubated for 1 h with the virus preparation. Rinsing was then done by floating them on a phosphate buffer solution (0.06 M, pH 6.5) for 5 min. Negative staining was done with 2% aqueous uranyl acetate, followed by rinsing with a drop of distilled water, draining with filter paper, and drying in a cool air current for approximately 1 min. The grids were not allowed to dry during the process, except after staining. They were examined in a Philips EM 300, at an accelerating voltage of 60 KV.

Results

Results of experiment 1 are summarized in Table 1. A thick carbon layer and positive electrical charge gave the highest yield of trapped PLRV particles, and the best contrast.

The effect of protein A coating was tested on neutral, positively and negatively charged grids, provided with a thick carbon layer. The results, summarized in Table 2 show, that negatively charged, protein A-coated grids trapped the largest number of particles and had very good staining quality, they were followed by the positively charged non-protein A-coated grids, which also showed good staining quality.

Table 1. Effect of carbon layer thickness and surface charge on the staining quality and on the numbers of PLRV particles trapped on grids.

Carbon layer	Surface charge	Estimation of virus particles[1]	Staining quality[2]
thin (evaporation 3 sec)	positive	+ +	o
	neutral	+ +	oo
	negative	+ +	oo
thick (evaporation 10 sec)	positive	+ + +	ooo
	neutral	+	oo
	negative	+	o

[1] + = few particles: (0-1 per selected area (SA) = 25 cm² at a magnification of 27000); + + = regularly found (0-5 per SA); + + + = many particles (1-25 per SA).

[2] o = poor contrast, particles hard to distinguish amongst other debris; oo = moderate contrast, particles readily distinguishable against clear background; ooo = good contrast, particles readily distinguishable against clear background.

Tabel 1. Het effect van de dikte van de koolstoflaag en de elektrische oppervlaktelading van de vliezen op de kwaliteit van de kleuring en het aantal gehechte deeltjes van het aardappelbladrolvirus.

Table 2. Effect of surface charge and protein A application on the staining quality and on the numbers of PLRV particles trapped on grids.

Surface charge	Protein A	Estimation of virus particles[1]		Staining quality	
		2nd experiment	3rd experiment	2nd experiment	3rd experiment
positive	applied	+	+ +	oo	ooo
positive	not applied	+ + +	+ + +	ooo	oooo
neutral	applied	+ +	+	ooo	o
neutral	not applied	+	[3]	oo	[3]
negative	applied	+ + + +	+ + +	oooo	ooo
negative	not applied	+ +	[3]	o	[3]

[1] + = a few particles (0-1 per SA = 25 cm² at a magnification of 27000); + + = regularly found (0-5 per SA); + + + = many particles (1-25 per SA); + + + + = very many particles (1-40 per SA).

[2] o = poor contrast, particles hard to distinguish amongst other debris; oo = moderate contrast, particles readily distinguishable against clear background; ooo = good contrast, particles immediately distinguishable against clear background; oooo = excellent contrast, particles immediately distinguishable against clear background.

[3] Not included in the experiment.

Tabel 2. Het effect van de elektrische oppervlaktelading en de behandeling met proteïne A op het aantal aan de vliezen gehechte deeltjes van het aardappelbladrolvirus.

Discussion

The technique of immunosorbent electron microscopy (ISEM) is generally considered to be a valuable tool in the diagnosis of plant viruses. The results of my experiments are in agreement with this opinion. Although the experiments were largely limited to PLRV, they indicate that trapping virus particles on the grid can be improved by different pretreatments of the grid. Using carbon-coated formvar films it was found that a fairly thick carbon layer is needed, possibly to exclude a less favourable influence of the formvar film on the trapping of γ-globulins. Protein A is known to increase the number of particles (Shukla and Gough, 1979) trapped by γ-globulins. I found that a protein A coating was particularly effective in combination with a negative charge of the grid. A similarly high particle yield was obtained on positively charged grids, coated with γ-globulins only. The latter method has two advantages as compared with the former. Grids can more readily be charged positively, by floating them on ethidium bromide solution, than charged negatively by glow discharge. Moreover, protein A is rather expensive. Using ethidium bromide, however, one should be aware of its carcinogenic properties.

Glow discharge, to obtain a negative surface charge, is generally used to make the carbon-coated formvar films hydrophilic. Against our expectation we observed a decreasing effect on the number of PLRV particles trapped. The staining quality was low and it was often hard to distinguish between virus particles and debris.

In my experiments stored untreated carbon-coated grids were considered to be neutral. They may well have had slightly negatively charged binding sites. Similarly it is not possible to estimate the amount of charge applied to the grids by glow discharge and ethidium bromide. Ethidium bromide may just neutralize the negatively charged binding sites and thus improve the trapping of negatively charged proteins. Immunosorbent electron microscopy would greatly gain in value as a diagnostic method, if the virus particles trapped would be spread evenly on the grid. Till now this is definitely not the case. None of the treatments I applied, whether electrical charge or protein A, led to improvement in this respect.

Acknowledgements

Thanks are due to M.A.G. Sengers and H.G.J. Welten for their skilful technical assistance.

Samenvatting

Het effect van voorbehandelingen van koolstof-formvar vliezen op de adsorptie van aardappelbladrolvirusdeeltjes in de immuno-elektronenmicroscopie

Het aanbrengen van elektrische lading op koolstof-formvar vliezen beïnvloedde de adsorptie van virusdeeltjes aan het vlies, bedekt met homoloog antiserum (γ-globulinen). Op vliezen met een positieve lading werd een veel groter aantal virusdeeltjes waargenomen dan op de neutrale of negatief geladen vliezen. Positief geladen vliezen werden verkregen door behandeling met ethidium-bromide. Negatief geladen vliezen werden verkregen door behandeling met geïoniseerd argongas. Op de nega-

tief geladen vliezen bleken grote hoeveelheden verontreinigingen van allerlei aard te precipiteren. Bovendien was het beeld van de virusdeeltjes vaag. Negatief geladen vliezen gaven wel goede resultaten ten aanzien van het vangen van virusdeeltjes in combinatie met proteine A en γ-globulinen.

References

Brlanski, R.H. & Derrick, K.S., 1979. Detection of seedborne plant viruses using serologically specific electron microscopy. Phytopathology 69: 96-100.

Kojima, M., Chou, Tg. & Shikata, E., 1978. Rapid diagnosis of potato leafroll virus by immune electron microscopy. Ann. phytopath. Soc. Japan 44: 585-590.

Lesemann, D.E., Bozarth, R.F. & Koenig, R., 1980. The trapping of tymovirus particles on electron microscope grids by adsorption and serological binding. J. gen. Virol. 48: 257-264.

Milne, R.G. & Luisoni, E., 1975. Rapid high-resolution immune electron microscopy of plant viruses. Virology 68: 270-274.

Milne, R.G. & Luisoni, E., 1977. Rapid immune electron microscopy of virus preparations. In: Karl Maramorosch & Hillary Koprowski (Eds), Methods in virology, VI: 265-281.

Ohki, S.T., Shohara, K. & Inouye, T., 1980. A simple sero-electron microscopical technique (SDN method) for detection and diagnosis of cucumber mosaic virus. Ann. phytopath. Soc. Japan 46: 51-53.

Roberts, I.M. & Harrison, B.D., 1979. Detection of potato leafroll and potato mop-top viruses by immunosorbent electron microscopy. Ann. appl. Biol. 93: 289-297.

Shukla, D.D. & Gough, K.H., 1979. The use of protein A, from *Staphylococcus aureus,* in immune electron microscopy for detection plant virus particles. J. gen. Virol. 45: 533-536.

Sogo, J.M., Rodeno, P., Koller, Th., Venuela, E. & Salas, M., 1979. Comparison of the A-T rich regions and the *Bacillus subtilis* RNA polymerase binding sites in phage ϕ 29 DNA. Nucleic Acids Res. 7: 107-120.

Address

Instituut voor Plantenziektenkundig Onderzoek (IPO), Postbus 42, 6700 AA Wageningen, the Netherlands.