Investigation of Immunoperoxidase-labelled Rotavirus in Tissue Culture by Light and Electron Microscopy

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SUMMARY

A tissue culture-adapted strain of bovine rotavirus, grown in calf kidney monolayers, has been examined by light and electron microscopy after immunoperoxidase labelling. Some of the characteristic problems associated with pre-embedding methods have been demonstrated. Preparative techniques involving pretreatment of infected cells with a detergent followed by a detergent/fixative combination have enabled virus antigen to be labelled while maintaining satisfactory ultrastructural preservation.

Immunoenzyme techniques are becoming more widespread for use in diagnostic and research procedures in microbiological studies, particularly for the investigation of virus antigens in cell systems. The morphogenesis of a variety of viruses has been examined by electron microscopy using horseradish peroxidase-labelled specific antibody (e.g. Leduc et al. 1969; Dmochowski et al. 1974; Kurstak & Kurstak, 1974; Bohn, 1980) but such studies have as yet been relatively few in number largely because of the difficulties, in pre-embedding techniques, of exposing intracellular antigens to conjugate molecules while maintaining adequate ultrastructural preservation. Alternative methods, generally involving greater difficulty, have been explored to some extent and the subject of technique has been reviewed and discussed by Williams (1977). In the present study, a pre-embedding method has been employed in an examination of peroxidase-labelled bovine rotavirus in tissue culture, and light and electron microscopic observations have been compared.

Primary calf kidney (CK) cells infected with a bovine rotavirus were used throughout this work; the virus was a CK-adapted tissue culture strain obtained originally from Dr G. Woode, IRAD, Compton, Newbury, Berks. and had a titre of $10^{5.5}$ TCID$_{50}$/ml after further passaging in tissue culture. Cells were grown according to previously described methods (Chasey, 1977), on glass coverslips in Leighton tubes for light microscopy, and on the end surfaces of 2 oz plastic bottles (Nunc A/S Denmark) for electron microscopy. Confluent monolayers were infected with 0.1 to 0.5 ml of stock virus and these, together with uninoculated control cultures, were prepared for immuno-labelling and microscopy 24 or 48 h p.i.

Anti-rotavirus immunoglobulin was precipitated by sodium sulphate from a rabbit antisera to the bovine rotavirus (neutralization titre 320) and conjugated to horseradish peroxidase (Type VI, Sigma, Poole, Dorset) by the sodium periodate method of Nakane & Kawaoi (1974). The conjugate, without purification, was diluted 1:1 in PBS and stored in aliquots at $-20^\circ$C. Working dilutions were made before use. 3,3'-Diaminobenzidine was used as enzyme substrate and 10 mg were shaken in 10 ml 0.05 M-sodium phosphate buffer, pH 7.3 (SPB), and activated with one or two drops of 20 vol. hydrogen peroxide.

For light microscopy, monolayers were immuno-labelled by one of three basic protocols, all steps being performed at room temperature. (i) Coverslips were rinsed in 0.05 M-SPB and fixed in acetone for 5 min. After air drying the cells were covered with one drop of conjugate, diluted between 1:5 and 1:20 from the stock, and incubated in a moist chamber.
for 30 min. The coverslips were washed in buffer and incubated subsequently for between 5 and 15 min with the enzyme substrate solution, before rinsing in distilled water and air drying. (ii) Coverslips were rinsed and incubated with a 0.1% solution of saponin (Merck Erg. B.6) in SPB for 5 min. They were subsequently covered with SPB containing 0.05% saponin, 0.05% glutaraldehyde and 1% paraformaldehyde for 10 or 20 min. After rinsing, the cells were incubated with the conjugate for 30 min and then treated as in (i). (iii) Coverslips were treated as in (ii) but the primary incubation with saponin was omitted. Non-infected control coverslips were prepared according to method (i). Infected control coverslips for antigen 'blocking' tests were treated similarly but were incubated additionally with undiluted anti-rotavirus immunoglobulin before incubation with the conjugate. Control cultures, both infected and non-infected, were also prepared by incubation with a peroxidase solution in place of the conjugate. All coverslips were mounted in DPX mountant on glass slides and examined in bright field in a Leitz Ortholux microscope.

Monolayer cultures grown in plastic bottles were labelled and prepared for electron microscopy in situ. Cells were either incubated with the conjugate after only a brief pre-fixation step or were subjected initially to combinations of fixatives and detergents before addition of the conjugate. The conjugate was applied in all cases at dilutions of the stock ranging between 1:5 and 1:20 and incubation times initially of 3 to 5 h were later extended to 17 to 18 h. SPB (0.05 M), pH 7.3, was used throughout and washing times between steps varied between 30 min and 18 h. Four basic protocols, sometimes with minor changes, were used as follows: (i) monolayers, after brief rinsing, were pre-fixed for 10 min in a solution containing 2% paraformaldehyde and 0.1% glutaraldehyde. After a wash in several changes of buffer, the cells were incubated with the conjugate, washed and fixed in 3% glutaraldehyde for 10 min. After washing again, the cells were covered with the enzyme substrate solution for 30 min and rinsed finally in buffer. (ii) Monolayers were rinsed briefly and incubated with a 1 x 10^-4 M-digitonin solution for 1 min before washing and pre-fixation in 2% paraformaldehyde for 10 min. The procedure was subsequently as in (i). (iii) Monolayers were rinsed and incubated with a 0.1% saponin solution for 5 min followed by an incubation with a solution containing 0.05% saponin, 0.05% glutaraldehyde and 1% paraformaldehyde for 10 min. After washing, the conjugate was added and the cells processed as above. (iv) As for (iii) but the primary incubation step with 0.1% saponin was omitted. Controls, in conjunction with the experiments on the coverslip cultures, consisted of non-infected monolayers incubated either with the anti-rotavirus conjugate or with free peroxidase in solution. During processing, monolayers were monitored in an inverted microscope.

After labelling, all monolayers were post-fixed in 1% osmium tetroxide for 1 h, followed by rapid dehydration in ethanol and embedded with slight modifications according to a previously described method (Chasey & Alexander, 1976; Perre & Foncin, 1977). Thin sections, usually unstained, were examined in either a Philips EM 300 electron microscope or a Joel 100 B instrument, both operating at 80 kV.

Examination of infected monolayers fixed in acetone and incubated with the anti-rotavirus−peroxidase conjugate showed distinctive 'islands' of reaction product (Fig. 1 a), each consisting of deeply coloured rounded cells surrounded by less densely stained cells. Reaction product was localized in the cytoplasm and was often granular in appearance. The surrounding regions of uninfected monolayer usually appeared virtually colourless when examined in bright field, and non-specific background staining was minimal. Essentially similar results were obtained with infected coverslips pre-treated with saponin followed by the saponin/glutaraldehyde/paraformaldehyde mixture (Fig. 1 b). If the pre-treatment with saponin alone was omitted, however, the distinctive staining pattern was characteristically altered. Only the central, heavily infected rounded cells at each focus of infection
Fig. 1. Light micrographs of rotavirus-infected CK monolayers after immunoperoxidase labelling. (a) Cells were grown on glass coverslip and fixed in acetone. The focus of densely labelled cells is surrounded by cells of weaker contrast. Non-infected areas are unlabelled. (b) Cells as in (a) but pre-treated for 5 min in 0.1% saponin followed by 10 min fixation in the saponin/glutaraldehyde/paraformaldehyde mixture. Labelling pattern is essentially similar to acetone-fixed preparations. (c) Cells as in (b) but without saponin pre-treatment. Only densely labelled cells are visible. (d) Monolayer of cells grown in plastic culture bottle for electron microscopy and treated as in (c); photographed through the plastic on which cells were grown, after curing of the Araldite. Labelling pattern is comparable with that shown in (c). (e) Cells as in (c) but fixed for 20 min. Intensity of labelling is severely reduced.
Fig. 2. Electron micrographs of rotavirus-infected CK monolayers after immunoperoxidase labelling. All sections are unstained. (a) Heavily labelled extracellular virus particles are present after primary fixation of cells in 2% paraformaldehyde/0.1% glutaraldehyde mixture for 10 min. Intracellular virus particles (V) are unlabelled. (b) Heavily labelled cell, dead at the time of fixation, prepared as in (a). There is a general distribution of reaction product throughout the disorganized cytoplasm but none in the nucleus. (c) Infected cell in a monolayer pre-treated with 0.1% saponin for 5 min followed by 10 min fixation in the saponin/glutaraldehyde/paraformaldehyde mixture. Large numbers of virus particles are present and some areas show strong labelling. (d) Part of an infected cell prepared as in (c). Budding particles, rough endoplasmic reticulum and precursor material are labelled while 'complete' virus within the cisternae remains unlabelled. (e) Part of an infected cell pre-treated with $1 \times 10^{-4}$ M-digitonin for 1 min followed by a 10 min fixation in 2% paraformaldehyde. Virus particles budding through the rough endoplasmic reticulum are intensely labelled but those within the cisternae are unlabelled. Diffuse reaction product is also present in the vicinity of the budding particles.
were labelled and all others tended to remain uncoloured (Fig. 1c, d). Labelled cells were also detectable in infected monolayers pre-treated with the saponin/glutaraldehyde/paraformaldehyde mixture for 20 min instead of 10 min, but such cells were fewer in number and the intensity of colour was severely reduced (Fig. 1e).

Non-infected control monolayers were completely unlabelled, with little or no background staining, and infected monolayers incubated with unconjugated peroxidase were similarly unlabelled. Infected cells pre-treated with unconjugated antiserum before incubation with the conjugate showed a similar labelling pattern to those cells incubated with conjugate alone, but the intensity of colour was considerably reduced. The monolayers in this type of control tended to exhibit a slightly higher overall background colouration.

Infected cultures pre-fixed with paraformaldehyde and not treated with specific membrane-disrupting procedures before incubation with the conjugate appeared, in general, totally unlabelled when examined by electron microscopy. Extracellular virions adjacent to the plasma membranes were, however, heavily labelled and each particle appeared to be completely enveloped in electron-dense reaction product. By comparison, unlabelled virions within the cytoplasm of infected cells exhibited very low contrast (Fig. 2a). Disintegrating cells, presumed to have been dead at the time of fixation, could be identified as infected and were full of reaction product (Fig. 2b). This was usually distributed generally throughout the disorganized cytoplasm and heavy deposits often obscured remaining ultrastructural features.

Examination of infected tissue culture monolayers exposed to appropriate fixation and membrane-disrupting conditions revealed intracellular reaction product. Ultrastructural preservation was variable and most cells had suffered some degree of disruption and organelle disorganization. The best preparations were obtained when cells were pre-treated with a detergent alone, followed by a detergent/fixative mixture, but otherwise minor changes in the protocol were found to be relatively unimportant. In any one preparation the degree and quality of immunocytochemical labelling was variable and the density and distribution of reaction product could vary not only from one cell to another but also within a particular cell. Digitonin and saponin tended to give comparable results in terms of characteristic labelling patterns although somewhat better ultrastructural preservation was obtained by the use of saponin. Infected cells were easily identified by the presence of virus and many of these contained electron-dense reaction product which distinguished them from adjacent non-infected ones by virtue of their markedly increased contrast (Fig. 2c). Reaction product was clearly associated with the virus particles and the rough endoplasmic reticulum which was distinctly labelled, and adjacent areas of amorphous reaction product, corresponding to precursor material, were usually present. Virions within intact cisternae of the endoplasmic reticulum generally remained completely unlabelled but particles in the process of budding through the membrane, ‘open’ to the cytoplasmic space, were labelled and strongly contrasted (Fig. 2d, e). Non-infected cells in both infected and control cultures were always devoid of reaction product.

The observations of this study have shown that rotavirus antigen can be effectively labelled with peroxidase-coupled specific antibody using the direct method and the techniques employed have enabled comparisons to be made between light and electron microscopic images. The preparation for electron microscopy of monolayers in situ within the culture bottles was a convenient technique to use as it allowed greater control over short incubation times and continuous monitoring of the cells throughout the processing; labelled cultures for electron microscopy (Fig. 1d) could be compared directly with those prepared for the light microscope (Fig. 1c).

Despite the relative success of the pre-embedding method used, some of the difficulties concerning penetration of conjugate molecules, ultrastructural preservation and main-
tenance of antigenicity were well-illustrated. Dead or dying cells with already disrupted plasma membranes could be easily labelled, while intact cells required pre-treatment with detergent. Nevertheless, conjugate molecules were unable in general to penetrate intact regions of rough endoplasmic reticulum and this resulted in uneven labelling.

Ultrastructural integrity although not optimal was generally adequate after treatment of cells with saponin. This detergent has been employed previously by Bohn (1978, 1980) and Hall et al. (1978) in similar studies.

Rotavirus, in common with many viruses, appears to lose much of its antigenicity after excessive glutaraldehyde fixation. Ciampor et al. (1974), in an ultrastructural investigation of influenza virus using peroxidase-conjugated antibody, fixed cells in 2.5% glutaraldehyde for 1 h before labelling and it is perhaps significant that the electron density of the developed reaction product in their micrographs is extremely low.

An important feature of the present study, illustrated particularly clearly in the light micrographs, was the distinctive labelling of cultures with a good relative contrasting between infected and non-infected cells. There was little or no background colouring and the use of unpurified conjugate, containing both free peroxidase and unconjugated immunoglobulin molecules, was not detrimental to the overall quality of the preparations.

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REFERENCES


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