The Detection of Foot-and Mouth disease Virus Antigens in Infected Cell Cultures by Immuno-peroxidase Techniques

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SUMMARY

Three immuno-peroxidase techniques (direct, indirect and peroxidase-anti-peroxidase) were compared for their potential in foot-and-mouth disease research. Each technique was shown to offer a simple and efficient means for the detection of the virus of foot-and-mouth disease and of virus-infection-associated (VIA) antigen in infected cells.

Immuno-peroxidase techniques have been used by several investigators for the detection of viruses and a review has been published by Kurstak (1971). The peroxidase technique, like the immunofluorescence technique, can be utilized either directly or indirectly. With the direct technique, anti-virus globulins are labelled with horseradish peroxidase (PO) and reacted directly with virus antigens. The virus-immunoglobulin-PO complexes are detected by the brown reaction product resulting from the enzymic action of PO on H₂O₂ and 3,3'-diamino-benzidine. With the indirect technique, virus antigens are first reacted with unlabelled antibody and then the virus-antibody complexes are exposed to an antiglobulin globulin labelled with PO.

A third technique has been described by Dougherty, Marucci & Distefano (1972) which is a modification of the technique of Sternberger et al. (1970). In this technique, complexes of the virus and hamster-antivirus globulin are exposed to rabbit anti-hamster serum, applied in antibody excess. Under these conditions some rabbit anti-hamster binding sites remain unreacted and subsequently combine with soluble complexes of PO and hamster anti-PO. This is referred to as the peroxidase-anti-peroxidase (PAP) technique. We have explored the application of the three techniques for the detection of foot-and-mouth disease virus (FMDV) antigens in infected cell cultures. The specificity and sensitivity of the different techniques were compared and their potential for antibody measurement investigated.

The FMDV strains used were of bovine origin at 6th passage in primary bovine kidney cells. These viruses were subtypes A24, O1, and C3. The bovine enterovirus (BEV) was isolated in this laboratory from experimental cattle and used at 3rd passage in primary bovine kidney cells.

Antisera were obtained from hyperimmunized guinea pigs, convalescent cattle or guinea pigs immunized with purified inactivated FMDV (to produce anti-140 S sera) (Cowan, 1968). Hyperimmune guinea pig serum previously adsorbed with purified 140 S and 12 S antigens (Cowan, 1968) was also used. This serum was specific for the VIA antigen produced by cells during FMDV replication (anti-VIA antigen serum) (Cowan & Graves, 1966; McVicar & Sutmoller, 1970).

Globulin fractions were precipitated by adding an equal vol. of saturated ammonium sulphate to heat-inactivated sera (56 °C for 30 min) that had been clarified by centrifuging at 15,000 rev/min for 1 h. This precipitate was washed with half-saturated ammonium sulphate, dissolved in phosphate-buffered saline (PBS) and dialysed against 0.1 M-phosphate
buffer solution, pH 6.8. Protein concentrations were determined from $E_{280}^1$ ($E_{1%}^{1cm} = 14.0$) and adjusted to 10 mg/ml.

Globulin fractions of anti-virus sera for the direct method and the rabbit anti-GP globulin globulin fractions for the indirect method were labelled with PO as described by Kurstak (1971). Briefly, 12 mg of PO (type VI, Sigma Chemical Co.) was dissolved in 0.5 ml of 0.1 M-phosphate buffer solution, pH 6.8, and mixed with 0.5 ml (approx. 5 mg) of globulin preparation followed by the addition of 0.05 ml of a freshly prepared 1% aqueous solution of glutaraldehyde (Polyscience Company). After 2 h at room temperature the mixtures were dialysed against PBS and clarified by centrifuging.

For the preparation of peroxidase-anti-peroxidase (PAP), guinea pigs were injected intramuscularly with 0.25 mg of PO in 0.25 ml PBS combined with equal vol. of complete Freund’s adjuvant (Calbiochem). Fourteen days later they were given an intraperitoneal injection of 0.1 mg of PO in an aqueous solution, and bled 7 days later.

The method of preparing the soluble complexes of peroxidase-anti-peroxidase was essentially as described by Sternberger et al. (1970) and Dougherty et al. (1972). Anti-peroxidase globulins were precipitated with PO at equivalence. The thoroughly washed precipitate was dissolved by mixing with a large excess of PO and the complexes made homogeneous by adjusting to pH 2.4 and immediately to pH 7.1. Insoluble material was removed by centrifuging and soluble PAP complexes were separated from unbound PO by precipitation with half-saturated ammonium sulphate. The precipitated complexes were washed twice with half-saturated ammonium sulphate, dissolved in PBS and dialysed extensively against PBS. During dialysis a moderate precipitate formed which was removed by centrifuging. The collected supernatant fluid (PAP) was dispensed in 1.0 ml amounts and stored at -70 °C.

The immuno-peroxidase reactions were made in secondary cultures of bovine kidney cells grown in disposable plastic 35 mm multi-dishes (Linbro Chemical Co.) inoculated with approx. 10 p.f.u. of FMDV. The virus was adsorbed for 30 min. The cell culture was overlaid with 0.6% gum tragacanth in culture medium (Mirchamsy & Rapp, 1968) and incubated at 37 °C in 4% CO₂ for 18 h. As a consequence of this procedure the infection was localized as plaques. After removal of the gum overlay, the cultures were rinsed with PBS and fixed for at least 45 min in absolute ethyl alcohol at 4 °C. After a rinsing with PBS, 0.1 ml of the following reagents were added to the cultures in the order listed for each method.

(a) The direct technique: anti-virus globulin labelled with PO.

(b) The indirect technique: GP anti-virus serum or GP anti-VIA antigen serum and rabbit anti-GP globulin labelled with PO.

(c) The peroxidase-anti-peroxidase technique: GP anti-virus serum or GP anti-VIA antigen serum, rabbit anti-GP serum in antibody excess and GP peroxidase-anti-peroxidase complexes (PAP).

The culture dishes were shaken every 5 min for 20 min to redistribute the reagent over the cell sheet. Between each reaction at room temperature the cultures were rinsed by 4 changes of PBS of 3 min each. In each technique the final step was the addition of a freshly prepared PO indicator consisting of 10 mg of 3,3'-diamino-benzidine tetrahydrochloride in 10 ml of 0.01% H₂O₂ in PBS. When the staining was fully developed, usually within 10 min, the culture dishes were submerged in a 2% formalin solution, the cultures dehydrated with ethyl alcohol and covered with a film of immersion oil. The cell sheets were then observed with a light microscope.

For a comparison of specificity and sensitivity of the different techniques, the antigens were reacted with series of twofold dilutions of the reagent to be tested and depending upon the intensity of staining, the reaction was graded from negative to 4+ (Fig. 1). The reaction
Short communications

Fig. 1. Infected cells in the margin of FMDV, type C, plaques stained by the PAP technique. The reaction was graded 1+ to 4+ according to the intensity of staining. Guinea pig anti-C-140S serum diluted 1/6400, 1/3200, 1/1600, 1/800 (low-power dry objective) gave 1+, 2+, 3+ and 4+ reactions, respectively.

was scored 3+ or 4+ when the cytoplasm of both the rounded and normal cells appearing at the periphery of the plaques were uniformly dark brown or black. With a 2+ score the rounded cells were brown but the peripheral cells stained more lightly and often had a granular cytoplasm. The reaction was scored 1+ when only the rounded cells were stained. Even though this grading system is necessarily subjective, it was found to be dependable because the end-point was based on observations of cultures stained with serial dilutions of the reagent being assayed.

For the direct technique PO-labelled bovine anti-A or anti-O globulin was used with cultures infected with FMDV of types A, O, C or with BEV. In the homologous systems, bovine PO-labelled globulin could be diluted 1/20 or 1/40 to obtain a 2+ reaction. A strong cross-reactivity was observed between the FMDV of types A and O, and a lesser cross-reactivity between type C and A or O. Bovine anti-FMDV PO-labelled globulin gave staining of BEV-infected cells. This was expected since most of our cattle sera contain antibodies against BEV. Guinea pig PO-labelled globulin against type O did not cross-react with BEV.

Some preliminary experiments were performed to examine the reasons for the cross-reactions using as blocking agents specific antisera against the FMDV particle (140 S) or VIA antigens. The homologous staining reaction was blocked by undiluted homologous
Table I. Virus antigen detection by the indirect technique using peroxidase labelled antibody and the peroxidase-anti-peroxidase (PAP) technique

<table>
<thead>
<tr>
<th>Guinea pig serum reacted with antigen</th>
<th>Indirect technique: cell cultures infected with</th>
<th>PAP technique: cell cultures infected with</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>O</td>
</tr>
<tr>
<td>Hyperimmune anti-A</td>
<td>200*</td>
<td>80</td>
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<tr>
<td>Anti-A-140S</td>
<td>200</td>
<td>10</td>
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<tr>
<td>Hyperimmune anti-O</td>
<td>40</td>
<td>400</td>
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<tr>
<td>Anti-O-140S</td>
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<td>200</td>
</tr>
<tr>
<td>Hyperimmune anti-C</td>
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<tr>
<td>Anti-C-140S</td>
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<td>20</td>
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<td>Anti-VIA antigen</td>
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<tr>
<td>Normal</td>
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<td>Anti-BEV</td>
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A, O, C; foot-and-mouth disease virus of type A, O, or C. BEV; bovine enterovirus.

* Reciprocal of serum dilution giving a 2+ staining.

GP hyperimmune serum or bovine convalescent serum and partially inhibited by homologous anti-140 S serum. Some inhibition occurred with the heterologous sera. However, cross-reactions were blocked completely by anti-VIA antigen sera, indicating that cross-reactivity was due largely to the production of VIA antigen by infected cells.

The results of the indirect and the peroxidase-anti-peroxidase (PAP) technique are summarized in Table I. The results of the indirect technique show that with the anti-140 S sera, the dilutions producing 2+ staining were at least tenfold higher for the homologous than for the heterologous reactions. A higher degree of heterologous reaction was observed with the GP hyperimmune sera. The results obtained with the anti-VIA antigen serum indicate, as with the direct method, that this cross-reactivity was probably due to reactions with the VIA antigen. Antigens in infected cells did not react with normal serum. Cells infected with FMDV did not stain when reacted with GP anti-BEV serum and cells infected with BEV reacted only with GP anti-BEV sera.

The results of the PAP technique show stronger reactions than those obtained by the indirect method. This enhancement was particularly pronounced with the anti-140 S sera. A high degree of cross-reactivity was noted not only with hyperimmune sera but also with anti-140 S sera which indicated that antigens other than VIA antigen may have been involved. In particular FMDV type C proved to be a strongly reacting antigen. In further experiments the cross-reactivity was removed or partially inhibited from the anti-140 S sera by adsorption with a heterologous 12 S antigen. For instance, the 12 S antigen of type C, when added to anti-type O 140 S serum, inhibited most of the staining of cells infected with virus of type C as well as some of the staining of cells infected with virus of type O. Conversely, adsorption of the anti-type A-140 S serum with the 12 S antigen of type O removed most of the type O and some of the type C cross-reactivity. Antigenic relationships between the different types of FMDV, as indicated by the PAP technique, are not unexpected in view of the extensive cross neutralization which occurs between the 7 types of FMDV (Cottral, 1972). The results obtained with normal GP serum, GP anti-BEV serum and BEV-infected cultures were similar to those obtained with the indirect method.

Each of the techniques tested appears to have certain advantages. The PO-labelled globulins for the direct and indirect technique are easiest to prepare, but are relatively costly in terms of the amount of peroxidase used and there is usually some background staining.
The least amount of background staining occurred with the PAP technique. The initial preparation of PAP is more complicated than the preparation of PO-labelled globulin but the technique is then as simple as the other two. Moreover, PAP is used in a 1/50 dilution and can be aspirated from the cell sheet and reused, as suggested by Dougherty et al. (1972).

Attempts were made to block with bovine convalescent sera the reactions of GP antisera to virus antigen in the indirect or PAP techniques. However, considerable staining of infected cells occurred as a consequence of a strong cross-reaction of the rabbit anti-GP globulin serum with bovine globulin. Adsorption of the rabbit anti-GP globulin serum with bovine immunoglobulin resulted also in the loss of most of the anti-GP activity. Thus, adsorption was not feasible and attachment of rabbit anti-GP globulins to bovine immunoglobulins may pose some problems when using the indirect or the PAP technique for the staining of bovine tissue sections for virus antigen if GP anti-virus sera are used as a reagent.

We also tested PO techniques for the detection of FMDV and VIA antigen in infected BHK-21 and PK-15 cell cultures. The results obtained with these cell lines were very similar to those with secondary bovine kidney cells.

Staining by PO techniques gave results very similar to the fluorescent antibody techniques with regard to antigen distribution in cells during the various stages of virus replication. However, immuno-peroxidase techniques have the advantage that the staining is permanent and that specimens can be examined with an ordinary light microscope. Their specificity and technical simplicity make these a useful tool for the detection of FMDV and of VIA antigen in FMDV-infected cells and tissues. Such investigations on pathogenesis are in progress.

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REFERENCES


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