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RAPID IDENTIFICATION OF INFECTIOUS PANCREATIC NECROSIS VIRUS IN INFECTED CELL CULTURES BY IMMUNOPEROXIDASE TECHNIQUES

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Abstract: Direct and indirect immunoperoxidase (IP) techniques were evaluated for their potential in identifying infectious pancreatic necrosis (IPN) virus. Both techniques were shown to offer a relatively simple, rapid and efficient means for the specific identification of IPN virus in infected cells. The direct IP method resulted in less nonspecific staining; however, the indirect method was clearly specific and utilized commercially available reagents.

INTRODUCTION

The identification of infectious pancreatic necrosis (IPN) virus depends on the development of characteristic cytopathic effects in susceptible cell cultures followed by confirmation using specific neutralization tests.[1,7] The development of more rapid serological tests for the identification of IPN virus is needed. Previously we have shown that the direct immunofluorescent technique was a sensitive and rapid method for detecting IPN virus in infected cell cultures[5] as have Tu *et al.*[6] Despite its rapidity, however, immunofluorescence has failed to gain widespread acceptance as a routine diagnostic tool for identifying IPN virus, perhaps because of a number of inherent difficulties.

In recent years, the immunoperoxidase (IP) method has been utilized to identify a variety of viral antigens in cell culture. This technique provides several advantages over fluorescent antibody methods including the elimination of background staining and the fading of fluorescence as well as providing a permanent slide record. The IP method, like immunofluorescence, can be utilized either directly or indirectly. With the direct technique, antivirus globulins are labeled with horseradish peroxidase (PO) and reacted directly with virus antigens. The virus-immunoglobulin - PO complexes are recognized by the brown reaction product produced by the enzymatic action of PO on H_2O_2 and 3,3′-diamino benzidene. In the indirect technique, viral antigens first are reacted with unlabeled antibody and then the virus-antibody complexes are treated with antiglobulin globulin labelled with PO.

This study reports the adaptation of the IP technique for the rapid identification of IPN virus in infected cell cultures.

MATERIALS AND METHODS

Cell cultures. The permanent cell line (AS) established from Atlantic salmon by Nicholson and Byrne[3] was propagated at 20 C in growth medium (GM) consisting of Eagle's minimum essential medium with Earle's balanced salts solution and supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml).

Viruses. The Dry Mills strain of IPN virus was originally provided by D. Locke of the Main Department of Inland

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Fisheries and Game and was propagated in RTG-2 cells in this laboratory as described previously.[2,4] This strain of IPN virus was isolated from an outbreak of IPN at the Dry Mills Hatchery, Gray, Maine, in 1967. The ATCC VR299 and Berlin, New Hampshire, strains of IPN virus were kindly supplied by Roger Dexter, Craig Brook National Fish Hatchery, East Orland, Maine. Stock cultures of the viruses were prepared by infecting monolayer cultures of RTG-2 cells propagated at 20 C in 60-cm^2 culture flasks. After adsorption of the virus for 1 h at 20 C, GM was added. When cytopathic effects were extensive (usually 2 to 4 days), the remaining cells and supernatant fractions were sonically treated for 1 min at 20 kc/s with a Sonic Dismembrator.[2] This suspension was then clarified by centrifugation at 2,000 x g for 15 min. The virus suspensions were then filter sterilized and stored in ampules at -80 C.

Antiserum. Antiserum to the Dry Mills strain of IPN virus was prepared in rabbits by injecting 1.0 ml of stock virus preparation intravenously at weekly intervals for 4 to 6 weeks. Sera with very high virus neutralization titers were obtained.

Enzyme-glubulin conjugates. Specific IPN antisera were conjugated with horseradish peroxidase (PO) according to the following procedure. Globulin fractions of IPN antisera were obtained by precipitation in half-saturated ammonium sulfate, followed by suspension in 0.1M phosphate buffered saline (PBS), pH 6.9. The globulin fractions were then adsorbed onto ethanol-fixed RTG-2 and/or AS cell monolayers. Following dialysis for 24 h against 2 l. of 0.1M PBS, pH 6.9, 10 mg of (PO) (specific activity 290 pu/mg) were added to 1.0 ml of dialyzed globulin (10 mg protein/ml) plus 0.3 ml of 0.1M PBS, pH 6.9. The mixture was stirred and 0.15 ml of 0.5% aqueous solution of glutaraldehyde was added in 25 μl aliquots. After 4 h of incubation at room temperature without stirring, the preparation was dialyzed against two l of 0.1M PBS, pH 6.9. The opalescent preparation was precipitated by ammonium sulfate and resuspended in PBS and applied to a Sephadex G-100 column. Fractions were eluted with 0.01M PBS, pH 7.5 and assayed spectrophotometrically at 280 nm and 403 nm. The fractions which showed coincident peaks at 280 and 403 nm were combined and stored at -20 C until further use.

Direct IP Method. Coverslip cultures of infected and uninfected AS cells were washed with PBS, fixed in 95% ethanol and incubated with the PO-antibody conjugate (.21ml/cm^2) for 1 h at room temperature. After incubation, the cell monolayers were washed three times with PBS to remove non-specifically adsorbed antibody. The coverslips were then incubated for 4 min in a saturated solution of 3,3′ diaminobenzidine tetrahydrochloride in 0.05M-tris-HCl buffer (pH 7.6) with 0.01% H_2O_2. The antigen bound peroxidase catalyses the reduction of H_2O_2 to H_2O and the parallel oxidation of the 3,3′diaminobenzidine tetrahydrochloride to a dark brown percipitate.

Indirect IP method. Coverslip cultures of infected and uninfected AS cells were washed and fixed as described for the direct IP method and incubated with anti-IPN (Dry Mills) antisera for 1 h at room temperature. The slides were then washed twice with PBS, air-dried, and incubated with a 1:20 dilution of goat anti-rabbit IgG-PO conjugate[3] at room temperature for 1 h. These slides were then processed as described for the direct IP method.

[2] Quigley-Rochester, Inc., Rochester, New York.

[3] Cappel Laboratories, Inc. Downington, Pennsylvania.

RESULTS

Direct IP Method. When viewed by light microscopy, uninfected AS cells stained by the direct IP method appeared unaffected except for a negligible level of pale tan background staining (Fig. 1). The cells exhibited little contrast, with only slightly outlined nuclei surrounded by the faint tan staining cytoplasm. Infected cells, however, showed sharp contrast, defined nuclei and extensive dark brown cytoplasmic granulation. Degenerate cells with extensive CPE generally appeared dark brown with darker staining globules or aggregates in the cytoplasm (Fig. 2). The appearance of IPN infected cells following IP staining, including the distinctive dark staining globules, was reminiscent of IPN infected cells stained by immunofluorescence.[5] Viral antigen could be detected in AS cells 6-8 h after infection.

The Dry Mills IPN PO-antibody conjugate gave similar results with AS cells infected with the ATCC VR-299 and Berlin, N.H. strains of IPN virus. We have previously shown that these three strains of IPN virus appear to be antigenically distinct in virus neutralization tests.[5]

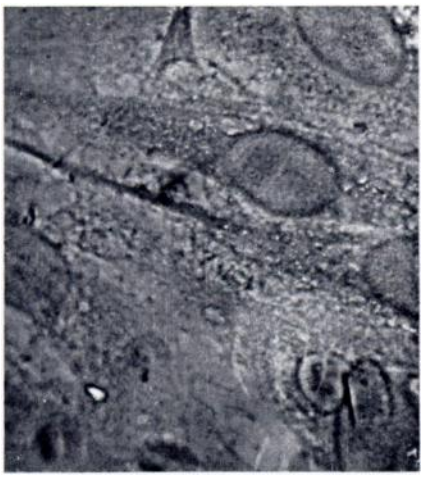

FIGURE 1. Uninfected AS cells stained by the direct immunoperoxidase (IP) technique. (×970)

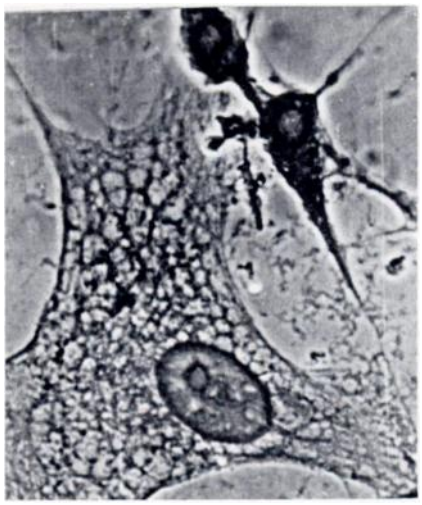

FIGURE 2. AS cells infected with the Dry Mills Strain of IPN virus at an input multiplicity of 100 $TCID_{50}$ per cell and stained by the direct immunoperoxidase (IP) technique (8 hours post infection). (×970)

Indirect IP method. Uninfected AS cells stained by the indirect IP method and examined using light microscopy appeared generally similar to those prepared by the direct method (Fig. 3) although slight non-specific staining was encountered, with slightly darker tan staining cytoplasm. However, infected AS cells stained by the indirect IP method were easily distinguished with darker brown staining similar to that observed with the direct method (Fig. 4). Again, specific staining was detected in AS cells infected with the ATCC VR299 and the Berlin, New Hampshire strains treated with Dry Mills IPN virus antisera.

The specificity of the indirect IP method was demonstrated by the lack of staining in infected cells treated with goat anti-rabbit IgG-PO conjugate but omitting reaction with IPN antisera.

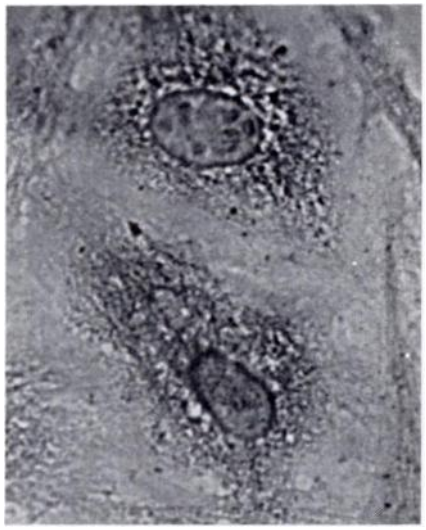

FIGURE 3. Uninfected AS cells stained by the indirect immunoperoxidase (IP) technique. (×970)

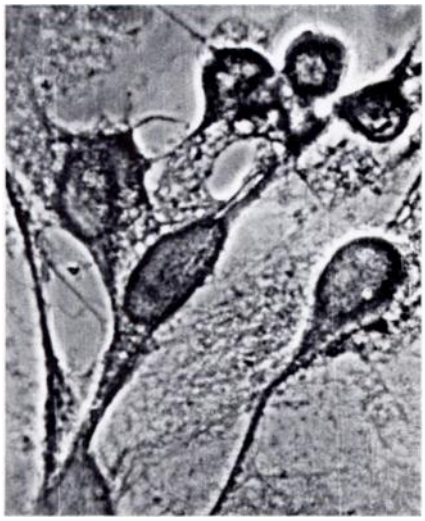

FIGURE 4. AS cells infected with the Dry Mills Strain of IPN virus at an input multiplicity of 100$TCID_{50}$ per cell and stained by the indirect immunoperoxidase (IP) technique (8 hours post infection). (×970)

Also, AS cells infected with infectious hematopoietic necrosis virus were not stained by the IP method using IPN viral antiserum.

DISCUSSION

The results clearly demonstrate that the immunoperoxidase (IP) method can be used for the rapid detection and identification of IPN virus in infected cell cultures. The technique is simple, rapid, specific and reproducible. The rapidity of the method offers an advantage over complicated and time consuming neutralization tests. Furthermore, our preliminary studies using three different strains of virus indicate that, although not allowing specific typing, the technique may identify an isolate as IPN virus using antisera prepared against any isolate. This is similar to results obtained using immunofluorescence.[5] The necessity for a polyvalent antisera for use in the more strain specific neutralization test is thus obviated. The IP method also offers major advantages over immunofluorescence including simpler methodology, easier interpretation of results and the availability of a permanent slide record.

The direct IP method gave somewhat better results than the indirect procedure. In general, the level of background, nonspecific staining was less with the direct method. Alternatively, the indirect IP method has the distinct advantage of utilizing commercially available antisera PO conjugates, thus obviating the need to prepare specific IPN antisera-PO conjugates. Therefore, investigators presently using IPN antisera can employ the indirect IP method with a minimum investment of time and money.

Therefore, this study shows that the IP method is a relatively simple, sensitive serological technique for the identification of IPN virus in infected cell cultures and provides numerous advantages over neutralization and immunofluorescent methods.

Acknowledgements

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