DEVELOPMENT OF INDIRECT IMMUNOPEROXIDASE ASSAYS FOR THE DETECTION OF AVIAN ADENOVIRUSES IN INFECTED CELL CULTURE

M.D. Ahmad[1] and G.W. Burgess
Department of Biomedical and Tropical Veterinary Sciences, James Cook, University of N.Qld., AUSTRALIA
[1]Present Address: Animal Sciences Institute, National Agricultural Research Centre, Islamabad, PAKISTAN

ABSTRACT

Monolayer indirect immunoperoxidase (IIP) assays were developed for the improvement of routine viral isolation and titration. The instant identification of virus with increased sensitivity (10-100 fold) was the characteristic of IIP. Polyclonal chicken antiserum reacted with all the avian adenoviruses (FAV) strains studied. Viral isolation and titration was made possible without the need of blind passages. However, the isolates were also characterized or typed by studying their reaction pattern with a panel of monoclonal antibodies (MAbs) produced to FAV strain 398A.

INTRODUCTION

The role of FAVs (Group 1) as an etiologic agent is a controversial issue (McFerran, 1991; Reece *et al.*, 1987; McCracken and Adair, 1993). Viral isolation either in cell culture or in embryonating eggs is thought to be an important diagnostic aid (McFerran, 1989). Avian adenoviruses produce basophilic intranuclear inclusion bodies and round type cytopathic effect (CPE) in cell culture (Mustaffa-Babjee and Spradbrow, 1975).

Viral isolation in cell culture has several limitations. Frequently several blind passages are needed before the CPE are observed (Cook, 1983; McCracken and Adair, 1993; Saifuddin and Wilks, 1990a). It is, therefore, not possible in most of the cases to titrate the infectious virus from tissues of infected birds. The development of CPE is slow and visual appraisal of end point titers is often equivocal.

Modern techniques aimed at direct detection of viral antigens have been applied to a number of pathogens. These techniques are sensitive and rapid and with appropriate reagents they can be very specific. The detection to FAV antigen in infected tissues has also been reported using immunoperoxidase (avidin biotin complex) staining (Saifuddin *et al.*, 1991). Reports are also available for the detection of non-cytopathogenic strains of viruses by immunostaining (Janmat, 1990; Kung, 1989). With the aid of infected cell cultures immune responses in birds have been detected by immunofluorescence staining following adenovirus infection in SPF birds (Adair *et al.*, 1980).

This paper describes the development of an IIP technique for detection and quantification of FAVs in cell culture with an aim to detect definite endpoints, the detection of FAV contaminants in cell culture, the determination of viral neutralization endpoints and FAV strain identification using MAbs (Ahmad, 1996).

MATERIALS AND METHODS

Cell culture

Primary chicken embryo liver cell culture (PEL) were prepared from 14 days old SPF and commercial chicken embryos. The test strains of FAVs were titrated (tenfold dilution's) in microtitre plates (Villegas and Purchase, 1980). Aliquots of 50μL of PEL cells resuspended in medium 199 at a concentration of 0.9% v/v (supplemented with 10% fetal bovine serum, 10mM tricine and 10 mM $NaHCO_3$) were added to each well. Plates were sealed and incubated in an air tight humid box at 37°C.

Fixative

Microtitre plates were fixed with parafomaldehyde fixative after 24, 48, 96 and 120 hours of PEL incubation. Plates were fixed 15, 20 and 30 minutes and were washed with TEN-T wash buffer and dried over a paper towel.

Primary antibody

Hyperimmune SPF chicken antiserum or MAbs produced to FAV 398A strain (Ahmad, 1996) were used as primary antibodies for detecting the viral antigen in infected PEL.

Chicken antiserum was standardized for the assay from 1/100, 1/200 and 1/400 dilution's in TEN-TC

dilution buffer while hybridoma supernatants were used undiluted and ascitic fluids were standardized from 1/100, 1/200, 1/400 and 1/800 dilution's. Aliquots of 50μL were added to each well. Plates were dried by gentle tapping onto a paper towel.

Monoclonal antibodies were used either in the form of undiluted hybridoma supernatants or ascitic fluids standardized from 1/100, 1/200, 1/400 and 1/800 dilutions.

Anti-species conjugate

Horseradish peroxidase (HRPO) conjugated with rabbit antichicken IgG was standardized for hyperimmune chicken serum by diluting 1/500, 1/1000, 1/2000 and 1/4000, in TEN-TC and aliquots of 50μL were added to each well. Horseradish peroxidase goat anti-mouse conjugate was standardized for MAbs at dilution of 1/500 and 1/1000 for the assay.

Plates were allowed to react for one hour at room temperature with conjugate and then washed three times with TEN-T wash buffer.

Immunoperoxidase substrate solution

Substrate solution was prepared in acetate buffer (pH 5.0) by the addition of 30% H_2O_2 at the rate of 5μL per 10 mL of buffer and 0.5 mL of 3-amino-9-ethylcarbazole (AEC 0.5M) stock solution. Aliquots of 100μL per well were used. Plates were incubated for 30 minutes at room temperature in the dark and washed.

Counter stain

One percent fast green solution in distilled water was used as a counter stain to give contrast with the red coloured infected cells. Aliquots of 100 μL were added to each well and the plates were incubated for 20 minutes. Plates were washed and examined under an inverted microscope.

RESULTS AND DISCUSSION

Cell cultures

The uninfected PEL cultures in flasks were observed as islands of epithelial cells which later formed a uniform monolayer. However, when cells were infected in suspension with FAVs the attachment of PEL onto the surface of the plate was inhibited or delayed. In wells with high concentration of virus a few round cells were found alongwith some fibroblasts. An increase in the number of cells fixed was observed alongwith the decrease in concentration of virus. Within 36 hours, round cells which stained strongly (infected with the virus) could be observed on the margins of the islands of healthy epithelial cells. In uninfected cell cultures the islands of epithelial cell formed a confluent monolayer after 72 hours incubation.

Our observations are in agreement with Adair *et al.*, (1980) who found PEL to be superior to chicken kidney cells and the fluorescence in PEL was confined almost exclusively to the epithelial cells. Insensitivity of fibroblasts of FAV infection noticed in this studied has already been reported (Miller *et al.*, 1972; Adair *et al.*, 1980).

Incubation time

The plates fixed after 24 hours of incubation had a substantial loss of cells during the washing steps. The number of cells fixed gradually increases with the incubation time. Although the virus infected cells can be fixed and detected by the IIP test as early as 18 hours post infection it is recommended for determination of the endpoint of viral titre or serum neutralization (SN) that the minimum incubation period required is 48 hours post infection.

Fixative

Plates were fixed successfully with paraformaldehyde fixative as early as 15 minutes and upto 30 minutes. The optimum fixation time was considered to be 20 minutes. The paraformaldehyde fixative did not mask the antigen. Previously methanol has been used to fix cell cultures in microtitre plates for IF (Adair *et al.*, 1980). The paraformaldehyde fixative used in this study was found to be a fixative of choice to be used for polystyrene microtitre plates where the organic solvent based fixatives can interfere.

Primary antibody

Infected PEL culture in microtitre plates were readily stained with hyperimmune chicken serum. A 1/200 dilution of chicken antiserum proved to be the optimum for the test. A higher concentration of antiserum (1/100) produced an intense red staining reaction with infected cells and a light pink non-specific background. Although the background reaction was reduced with a 1/400 dilution of chicken antiserum. However, intensity of colour of the infected cells was also reduced.

Hybridoma supernatant (undiluted) proved to be a better source of primary antibody for IIP. The infected cells stained dark red to light pink in colour depending on the MAbs used while uninfected control did not show any background colouration. The optimum dilution of ascitic fluid (MAbs) was determined to be 1/400 to 1/800. No background reaction was seen with

most of the ascitic fluids even at a 1/200 dilution. However, the MAbs produced a variety of reactions depending on the characteristics of the MAb used. The MAbs (Ahmad, 1996) made it possible to use IIP for confirming the endpoint neutralization with chicken serum. Some of the MAbs (ascitic fluids of 1F2, 6H2, 9H12 and 7G11) also stained extracellular proteins in the intracellular spaces in infected wells. However, this kind of background staining was absent in uninfected control wells. Infected wells stained with these MAbs could be readily recognized without the aid of a microscope. This is an attractive feature for laboratories carrying out large number of titration's or serum neutralization.

Conjugate

Every batch of conjugate was standardized prior to use. Rabbit anti-chicken conjugate at 1/1000 dilution stained viral infected cells an intense red colour with a light pink coloured background. The background staining of uninfected cells was more intense with a 1/500 dilution of the conjugate. The conjugate dilution of 1/2000 and 1/4000 stained poorly. Both 1/1000 and 1/2000 dilution's of goat anti-mouse conjugate stained equally well. The infected cells had an intense red colour and uninfected cells stained green with the counter stain.

Counter stain

The viral infection was predominant in hepatocytes (epithelial cells) which stained reddish pink. The 1% fast green counterstain produced a brilliant green background and resulted in better contrast with the red coloured infected cells.

Reaction with other serotypes

The chicken antiserum to FAV 398A stained 11 reference serotypes of FAV as well as the 17 Australian isolates of FAVs acquired from different sources. The cross reactivity of polyclonal chicken antiserum with all the references viruses collected from different sources regardless of their serotypes shows the detection of group specific reactions by IIP. The group specific epitopes were detected by IF as cells infected with all the 11 serotypes studied were stained with FAV-1 antiserum. The reaction of polyclonal chicken antiserum (FAV 398A) in the present study with all the FAV strains studied by IIP is in agreement with Adair *et al.* (1980). The FAV infection in PEL results in the production of group specific as well as type specific antigens thus the reaction of polyclonal serum with all the FAVs observed is not surprising.

Viral titration

The IIP assay was found to be very useful for determining the endpoints in viral titration where most of the healthy and infected cells fixed onto the plates. Loss of infected cells with higher concentrations of virus was not unexpected and did not interfere with the interpretation of endpoints. Red staining of small number of infected cells with more than 95% healthy cells stained green with counter stain was the striking feature of endpoints. Thus the endpoint titer's recorded by IIP were 10 to 100 fold and occasionally 1000 fold more than by the visual appraisal of CPE.

Viral titration by IIP made it possible to calculate the viral tires which obviate the individual variations for pathogenicity or pathogenesis studies. The method is also superior to IF which requires special equipment such as dark field ultraviolet microscope and fails to produce permanent records with cytology of infected cells. The plastics used for microtitre plates and fixatives used for cell cultures may interfere with IF or may produce non-specific auto-fluorescence.

These observations supports the hypothesis made that the assay has the ability to detect viruses isolated directly from infected tissues without the need of blind passages where the CPE are not visible by light microscope. In contrast to this the non-specific kind of CPE were observed occasionally in control PEL culture which did not stain red.

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