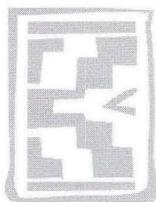

Multiplication of the V4 strain of Newcastle disease virus in Madin Derby bovine kidney cells

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ABSTRACT

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This study describes a reproducible cell culture system that permits the growth and secondary multiplication of the V4 strain of Newcastle disease virus. Allantoic fluid, magnesium chloride and diethylaminoethyl dextran were incorporated in Dulbecco's modified Eagle's medium to encourage secondary viral multiplication without adversely affecting healthy Madin Derby bovine kidney cell growth.

Keywords: Cell culture, MDBK cell, Newcastle disease virus, V4 strain replication

INTRODUCTION

Newcastle disease virus (NDV) contains two surface glycoproteins [fusion (F) and haemagglutinin neuraminidase (HN)]. These are responsible for attachment of the virus to cell surface receptors that enhance fusion of viral and cellular membranes (Scheid & Choppin 1974). It has also been demonstrated that there is a strict correlation between the cleavability of precursor fusion glycoprotein (F_0) in cells in culture and the virulence and the host range of NDV isolates (Samson & Fox 1973 & 1974; Nagai, Ogura, Klenk & Rott 1976; Nagai & Klenk 1977; Scheid & Choppin 1977).

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The content of basic amino acid residues at the cleavage site of the glycoprotein may influence the cleavability of F_0 in avirulent strains of the virus (Glickman, Syddall, Iorio & Sheehan 1988). The precursor fusion protein of virulent strains of NDV is cleaved in a wide variety of cell types. Isolates classified as avirulent pathotypes of NDV grow in very limited cell types such as cells of the chorioallantoic membrane of embryonating chicken eggs or of the embryo. It has been reported that the Australia-Victoria (V4) strain of Newcastle disease virus (NDV-V4), which is a lentogenic strain, readily undergoes productive infection of chicken embryo (CE) cells to produce infectious virions (Kaplan & Bratt 1973). The V4 strain of NDV represents an apathogenic biotype of NDV that has been shown to have extremely low pathogenicity for chickens and chicken embryos (Kendal & Allan 1970). It produces little or no cytopathogenicity in cell culture but some permissive cell types such as chick embryo cells may still show distinct cytopathic changes (Rott 1964; Alexander, Reeve & Poste 1973).

It has also been demonstrated that there is a necessity for the cleavage of both glycoproteins (F

and HN) of apathogenic strains to obtain infectious viral particles (Klenk, Nagai, Rott & Nicolau 1977). Apathogenic strains of NDV grown in Madin Derby bovine kidney cells (MDBK) and chicken embryo cells produce inactive virus with uncleaved glycoproteins whereas virulent strains produce infectious virions with cleaved glycoproteins (Nagai *et al.* 1976). These differences have been exploited to differentiate pathogenic viruses from apathogenic ones. It has been shown that additional proteases, such as trypsin, in the medium are necessary to cleave the glycoproteins (F and HN) for the virion to be infective (Nagai & Klenk 1977) and diethylaminoethyl dextran (DD) and magnesium ions enhance plaque formation (replication of progeny virus) of lentogenic strains of NDV (Barahona & Hanson 1968).

In conventional culture systems the Newcastle disease lentogenic viruses and V4 strain of NDV will infect mammalian cells. However, they fail to produce infectious progeny virus and secondary multiplication of the virus is limited. The development of a reliable and reproducible cell culture system which permits multiplication of V4 and similar lentogenic viruses is of practical significance as it may serve as an additional diagnostic test and method for studying of the virus.

In this article, trials of the growth and secondary multiplication of NDV-V4 strain in MDBK cells are described. Secondary multiplication of the virus was observed by cytopathic effect (CPE) of the virus and by the demonstration of viral antigen in the cell cultures using indirect immunoperoxidase staining. Several panels of monoclonal antibodies, which are directed against specific epitopes of different proteins of various isolates of NDV, were produced (Lana, Marquardt & Snyder 1983; Russel & Alexander 1983; Lamichhane 1988; Meulemans, Gonze, Carlier, Pett, Burny & Le Long 1987). These monoclonal antibodies may be incorporated in the immunoperoxidase stage of the culture systems and will ensure specificity of the staining reaction.

MATERIALS AND METHODS

Cell culture and viruses

A MDBK cell line at passage level 120–125 was used for the growth of the lentogenic and velogenic strains of NDV and stored in liquid nitrogen. Cell growth and storage of MDBK cells were carried out using a procedure adapted from the general methods described by Lamichhane (1988). The growth of MDBK cells was carried out in microtitre plates and contained the following components per well: 50 µℓ MDBK cells [suspended in Dulbecco's modified Eagle's medium (DMEM) and 5 % foetal bovine serum (FBS)], tested viruses (titrated in DMEM plus 5 % FBS) and modified DMEM.

The V4, Indonesian velogenic (V. Ind 2) and Indonesian lentogenic (L. Ind 1) laboratory strains (Graduate School of Tropical Veterinary Science, James Cook University of North Queensland) of NDV were reproduced in 10-day-old embryonating chicken eggs according to the method described by Hanson (1980). The viruses were found to have a titre of 10^8–10^9 egg infective doses 50 (EID_{50})/mℓ of stock virus and these were used to infect MDBK cells. The growth characteristics of these strains were used to compare with the NDV-V4 strain.

Modified medium

DMEM supplemented with 5 % FBS and various concentrations of allantoic fluid (AF), $MgCl_2$ and diethylaminoethyl dextran (DD) was used as growth medium for MDBK cells. Allantoic fluid was produced from 10-day-old embryonating hen's eggs.

The effect of various concentrations of additives (Table 1) to DMEM on the growth of MDBK cells was first investigated after which selected concentrations of additional ingredients were added to the medium for viral growth. Serial ten-fold dilutions of the virus were also used in each variation of the media and viral growth was observed. Approximately 2 x 10^4 to 3 x 10^4 MDBK cells/well were added in 50 mℓ of each selected medium. Additional ingredients DMEM plus 5 % FBS, 5 % AF, 20 mM $MgCl_2$ and 200 mg/mℓ DD, were prepared at three times the final concentration. One part of modified medium was diluted with two parts of the other components in the basal medium.

Experimental design

To determine the effect of additional ingredients in the growth medium of MDBK cells as well as on the secondary multiplication of NDV-V4, a two stage experimental trial was performed.

- Effect of supplemented ingredients (Table 1) and interaction of optimal concentration of supplement on MDBK cell growth.
- Effect of the optimum growth conditions on the secondary multiplication of NDV-V4 strain.

To determine the effect of the additional ingredients in DMEM on the growth of MDBK cells in microtitre

cell culture plates, an approach that accommodated all the different concentrations of the components with eight replicates was used. A replicate of the layout was carried out in another four microtitre cell culture plates to check the repeatability and variability of the results. In each case, controls of MDBK cells in DMEM plus 5 % FBS (basal medium) were incorporated to monitor normal cell growth.

A comparison of the cell growth in each well with that of the controls was used to assess the effect of the additional ingredients. Any retardation in growth of the cells and the presence of CPE were considered to be caused by the additional ingredients. The absence or the presence of the effect was recorded daily until the cell growth in the control wells reached 90 % confluency. The mean percentage of growth retardation was calculated.

Each of the selected ingredients $MgCl_2$, DD, AF and their combinations (Table 2) were investigated for their maximum support of virus multiplication in MDBK cells. Both control MDBK cells (in DMEM plus 5 %) and cells with tested viruses (without additional ingredients) were incorporated to monitor the growth of cells and viruses.

Detection of secondary multiplication of virus

Ten-fold dilutions (10^{-1} to 10^{-5}) of the virus were added to different combinations of supplemented ingredients in DMEM. Eight replicates for each viral dilution were used. This type of design has 320 data points for each of the additive combinations and 64 data points for each of the five virus dilutions. The same design was repeated over time to estimate the repeatability and variability of the result.

With the design mentioned above the multiplication of virus was detected by means of an immunoperoxidase staining technique (Russel & Alexander 1983; Russel, Giffith, & Cannon 1983) in infected MDBK cells. MDBK cells grown in DMEM without any supplements and infected with NDV-V4 were used as virus controls.

Statistical analysis

To evaluate the absence or presence of the effects of additional ingredients on cell growth, data were collected from a balanced design. The frequency of distribution of the mean of the absence and presence of the effects of additional ingredients on cell

TABLE 1 Concentration of additional ingredients tested for the support of maximum growth of MDBK cells in basal medium (DMEM plus 5 % FBS)

Supplemented ingredients		Percentage of growth inhibition	Selected concentration
AF (%)	5	13	5
	10	24	
	15	54	
$MgCl_2$, (mM)	20	30	20
	30	42	
	40	71	
DD (µg/mℓ)	100	15	100
	200	26	
	300	59	

TABLE 2 Percentage of mean of secondary multiplication of NDV-V4 strain in DMEM cells within different combination of additional ingredients in DMEM. Control: growth of NDV-V4 strain in MDBK cells without supplemented DMEM

Dilution	$MgCl_2$	DD	AF	$MgCl_2$ + DD	$MgCl_2$ + AF	DD + AF	$MgCl_2$ + DD + AF	Control V4
10^1	10	11	39	20	5	23	78	1
10^2	8	10	18	18	16	28	95	0
10^3	8	8	16	18	17	38	89	0
10^4	5	8	13	10	12	14	83	0
10^5	1	3	7	8	6	6	38	0

growth was compared. A balanced design was also used to compare the effect of three additional ingredients on the secondary multiplication of NDV-V4 strain and other viruses in MDBK cell cultures. Binomial data [absence (0) or presence (1)] of viral replication were collected from observations under an inverted microscope. The mean percentage of CPE (growth of virus) was calculated.

RESULTS

Cell growth

The frequency of the inhibitory effects of additional ingredients on the growth of MDBK cells in modified DMEM is presented in Table 1. The aim of this trial was to select the maximum concentration of ingredients with acceptable adverse effects on MDBK cell growth. The highest concentration of all ingredients used in the trial significantly altered the growth of the cells with a mean percentage of 55–73. The following concentrations of additional ingredients in the DMEM were selected for their moderate effects (10 %) on cell growth: 5 % allantoic fluid, 20 mM $MgCl_2$, 200 mg/mℓ DD and 5 % FBS.

Virus multiplication

The mean percentage of replication of the NDV-V4 strain in different combinations of the cell media containing additional ingredients is illustrated in Table 2. The combination of allantoic fluid and either DD or $MgCl_2$ and $MgCl_2$ plus DD average did not produce any significant effects. Each supplement on its own did not enhance the growth of the virus. The greatest replication of V4 virus was observed when all additional ingredients were included in DMEM at the 10^{-1} to 10^{-4} dilutions of the virus. When additional ingredients were added separately and in combination to the medium, wells containing high concentrations of virus had extensive CPE and viral antigen was detected in almost all of the cells. Wells containing low concentrations of virus had individual cells in which virus antigen was detected. When progeny virus replication (secondary multiplication) occurred, foci of infected cells could be clearly seen in stained monolayers. To assess the general application of the cell culture, velogenic and lentogenic strains of NDV were tested using the same procedure as those described above. No significant differences regarding virus multiplication were found in comparative tests to determine whether the V4 strain-infected cells produced less progeny virus relative to velogenic and lentogenic strains (Fig. 1).

DISCUSSION

It is believed that the role of the HN glycoprotein is to remove neuraminic acid from the primary recep-

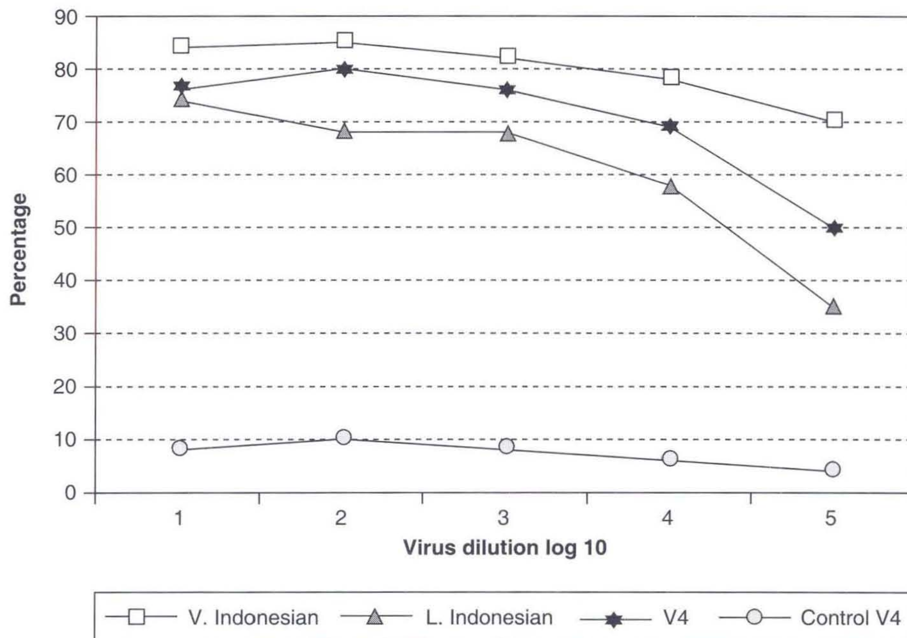

FIG. 1 Percentage of mean of multiplication of velogenic (V) and lentogenic (L) isolates of NDV in supplemented DMEM. Control V4 without supplemented DMEM

tor following adsorption and to facilitate fusion of cleaved F protein to the cell (Huang, Rott, Whan, Klenk & Kohama 1980). This interaction with host cell receptors is the first step in the process of initiating infection. For some avirulent strains of NDV, such as Queensland V4 and Ulster 2C, the HN protein is synthesised as an inactive procurosr HN_0 in a wide range of cells and requires proteolytic activation before the virion can become infective (Nagai *et al.* 1976).

It has been demonstrated that DD facilitates the uptake of complete virions by cells in culture (Kaplan Wiktor, Maes, Campbell & Koprowski 1967; Barahona & Hanson 1968). However, the use of DD alone as one additional ingredient to the medium in this study did not induce a significant increase of multiplication of the progeny virus.

The infectivity of viral particles most likely depends on the proteolytic cleavage of the precursor molecules. It has been reported that the protein responsible for the cleavage of F_0 is a host cell enzyme present in a limited range of cell types and that this cleavage occurs intracellularly (Seto, Garten & Rott 1981).

It is known that serum contains protease inhibitors. It has been reported that allantoic fluid is possibly a suitable source of protease, and that it can remain active in the presence of serum in a cell culture system (Harper 1989). Allantoic fluid also contains protease inhibitors similar to those present in serum. The advantage of the use of allantoic fluid over the use of trypsin is that the protease is less affected by serum that is needed for the viablity of cells in culture.

One of the advantages of using MDBK cell culture systems is that the cells are fast growing and stable. The cell monolayer facilitates its use in microtitre assays when using the cells as the solid-phase in an indirect immunoperoxidase test.

The demonstration of NDV in infected allantoic fluid by HA followed by HI using known reactive serum is widely used in many laboratories. In the assay described in this study the virus is cultured in embryonating chicken eggs, which takes a minimum of 13–15 days to isolate the virus. In some instances, depending on the aim of the study, the use of a reliable cell culture system followed by an appropriate diagnostic technique (e.g. indirect immunoperoxidase) has potential advantages in that it is time saving and economical when compared to the HA assay after passage of virus in embryonating chicken eggs.

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