Immunofluorescent Cell Assay of Neonatal Calf Diarrhea Virus

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ABSTRACT

A reliable plaque assay procedure has not yet been described for the neonatal calf diarrhea virus. Therefore, a previously developed immunofluorescent cell counting procedure was adapted to assay this virus.

Adsorption of the virus to bovine kidney cells plateaued at 60 minutes. The optimal staining time was between 20 and 24 hours postinfection. Infected cells began releasing from the coverslips if the cultures were incubated longer than 24 hours.

This procedure has proven successful with virus grown in cell culture as well as virus present in fecal samples.

RÉSUMÉ

Personne n'a encore décrit une méthode fiable d'essai par les plages, relative au virus de la diarrhée néo-natale du veau. Par conséquent, les auteurs ont adapté à ce virus une méthode de comptage cellulaire immunofluorescente déjà mise au point.

L'adsorption du virus aux cellules rénales bovines atteignit un sommet en 60 minutes. Le temps idéal pour procéder à la coloration se situait entre 20 et 24 heures après l'ensemencement. Les cellules infectées commencèrent à se détacher des lamelles, lorsqu'on prolongeait l'incubation au delà de 24 heures.

Cette méthode s'est avérée efficace tant avec le virus de culture cellulaire qu'avec celui de fèces virulentes.

INTRODUCTION

In initiating a study on neonatal calf diarrhea virus (NCDV)[1] a previously developed immunofluorescent cell (IFC) counting procedure (4) was adapted and used to assay the virus. The results obtained using the IFC method are reported in this communication. The technique described should greatly facilitate studies of NCDV since a reliable plaque assay procedure is lacking. The IFC counting procedure permits a quantitative assay of infectious NCDV in less than 24 hr.

MATERIALS AND METHODS

Media and Cells

The growth medium consisted of autoclavable Eagle's minimum essential medium[2] plus 5% fetal bovine serum and antibiotics. The serum was left out of the

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[1]NCDV has also been referred to as Nebraska calf diarrhea virus and as a reo-like virus.

[2]Difco Laboratories, Detroit, Michigan.

maintenance medium used on infected cells.

The Madin Darby bovine kidney (MDBK) cells were obtained from the American Type Culture Collection.

Virus

Vaccine (Scourvax-Reo) NCDV prepared by Norden Laboratories, Lincoln, Nebraska was used in this study. The wild-type NCDV was a gift from Dr. C. A. Mebus, University of Nebraska.

Immunofluorescent Cell Staining

Our basic fluorescent antibody methods and IFC counting have been described (4, 5). The conjugates used were provided by Norden Laboratories and by the Department of Veterinary Science, University of Nebraska (2). The cells used in the immunofluorescence studies were grown on circular coverslips 15 mm in diameter contained in 60 mm Petri dishes.

Effect of Washing Cell Sheets on NCDV Adsorption Time

Cells in the first of two sets of coverslip cultures were washed with 4 ml of maintenance medium that was allowed to remain on the cells for at least five minutes at room temperature. After washing, each coverslip culture was inoculated with 0.02 ml of a virus dilution and incubated at 37°C in a CO_2 incubator. At designated intervals the adsorption period for the cultures was terminated by washing the cell sheets with maintenance medium. The infected cell sheets were then incubated at 37°C in maintenance medium. Eighteen hours after infection all cultures were fixed in cold acetone, stained with antiviral fluorescent antibody and the number of infected cells in each culture was determined.

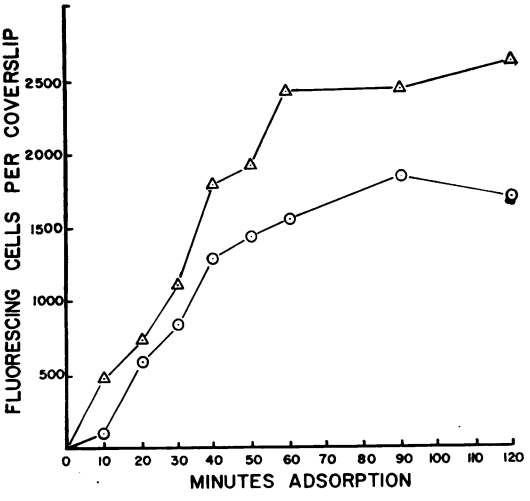

Fig. 1. Effect of adsorption time on the number of immunofluorescent cells. MDBK cells in coverslip cultures were inoculated with 0.02 ml of NCDV. Adsorption at 37°C was allowed to proceed for indicated intervals, then terminated by washing away the unadsorbed virus. The cells were stained 18 hr after infection. Cells washed prior to inoculation △ and cells not washed prior to inoculation (⊙).

Optimal Postinfection Time for IFC Staining

Coverslip cultures of MDBK cells infected with NCDV were incubated at 37°C. At prescribed intervals a set of infected cultures contained in a Petri dish was removed and stained with fluorescent antibody.

Reproducibility of the IFC Assay Technique

Thirty coverslip cultures of day old cells were divided into three sets and inoculated with 1:20, 1:40 and 1:80 dilutions respectively, of an NCDV stock. After an 18 hr incubation at 37°C the cultures were stained and the fluorescent cells on each coverslip were counted.

Counts were made in two ways. The entire culture was scanned or a single scan was made from an edge through the center to the other edge using a Zeiss GFL fluorescence microscope with a 25X objective and a 12.5X eye piece. One strip counting has been discussed in an earlier communication (5).

RESULTS

NCDV Adsorption Time

Under the conditions described, adsorption of the virus plateaued at 60 min (Fig. 1). The washed cells consistently produced higher titers. During the first ten min of

TABLE I. Reproducibility of the IFC Assay

Viral dilutions	1:20	1:40	1:80
No. of coverslips examined	10	10	10
Mean No. FC[a] per coverslip[b]	320 (± 16%)	150 (± 13%)	79 (± 18%)
Mean No. FC[a] per strip[b]	28 (± 21%)	11 (± 27%)	7 (± 29%)

[a]FC, fluorescent cells
[b]Coefficient of variation

adsorption titers were almost ten times greater with washed than with unwashed cells. Thereafter, titers using washed cultures averaged 20% to 40% higher.

Postinfection Time for Staining

Fluorescent cells were first observed in cultures that had been stained eight hr after infection (Fig. 2). This number increased until 20 hr postinfection, remained constant for four additional hours and then decreased slowly.

Reproducibility of the IFC Assay

When more than 28 fluorescent cells were counted on a whole coverslip or a single strip our greatest variability was ± 21% (Table I).

There was a direct relationship between the concentration of virus in the inoculum and the number of fluorescent cells observed in the stained cultures. Consequently, only a single virus particle was required to produce infection in a cell.

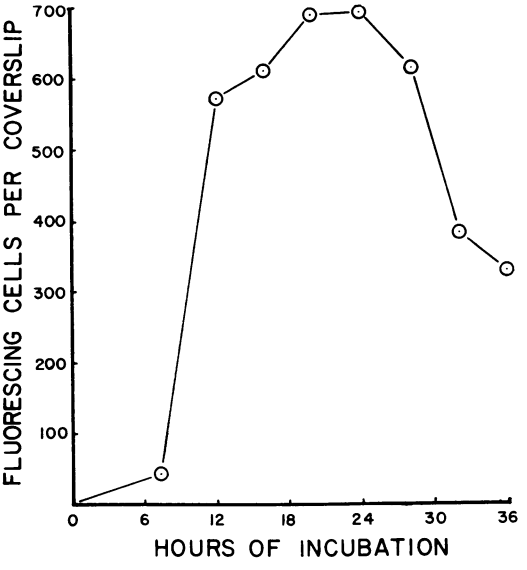

Fig. 2. Effect of incubation time at 37°C on the number of immunofluorescent cells per coverslip. MDBK cells were inoculated with NCDV and assayed at the indicated times postinfection.

DISCUSSION

Attempts to assay NCDV by the plaque method have only been partially successful (6). As a consequence, previous investigators have used a fluorescent antibody procedure (3, 6) in which groups containing five test tube cultures of bovine kidney cells were infected respectively with different viral dilutions. After five days of incubation smears of cells made from the tube cultures were stained with fluorescent antibody. The number of positive and negative smears obtained with each viral dilution would then be determined and the viral titer calculated using the Reed-Muench formula.

While that procedure has permitted very successful work to be done with NCDV it has some limitations, viz. as an end point dilution method it lacks the accuracy of direct counts, five days are required and five smears must be prepared and stained for each viral dilution. The assay method we describe in this communication requires less effort and is more rapid and accurate. It should expedite additional studies with NCDV.

Preliminary studies (LaSalle and Spendlove, unpublished results) have shown that by 24 hr postinfection many infected cells have enlarged and spread over adjacent cells. In so doing the peripheral areas of cytoplasm often lose contact with the attachment surface. To prevent sloughing in the IFC counting procedure, movement of infected cell cultures must be minimized prior to staining. Even with this precau-

tion cell losses increase after 24 hr of infection. Growing cells on collagen coated surfaces did not prevent such loss of infected cells.

It is our experience that 2500 infected cells on a 15 mm coverslip can be accurately counted. In attempting higher counts, even with a grid in the ocular, some cells may be counted twice and others may be missed. A time saving simplification of the counting procedure is the use of one strip counts (5). The area examined by a single scan across a coverslip can easily be determined and the factor required to convert one strip counts to whole coverslip counts then calculated. The multiplication factor calculated for the one strip counts in this study was 16.7. When whole coverslip and one strip counts were made on the same cultures the factor for one strip counts was found to be 12.3. This discrepancy between calculated and observed multiplication factors can be explained on the basis of the cells not growing to the edge of the coverslip.

We have used this IFC procedure to detect NCDV in diarrheic fecal specimens. The wild-type NCDV produced easily detectable quantities of antigen in MDBK cells. We are currently using the assay described in this paper to determine antibody titers against NCDV.

NCDV is antigenically related to the reoviruslike agent associated with diarrhea in infants and young children (1). The assay described in this communication may be useful for epidemiological investigations of the reoviruslike agent that affects infants. The readily obtainable NCDV vaccine and fluorescent antibody could be used in a virus-neutralization survey of human sera.

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BOOK REVIEW

RUMINANTS: CATTLE, SHEEP AND GOATS — GUIDELINES FOR THE BREEDING, CARE AND MANAGEMENT OF LABORATORY ANIMALS. *Published by the National Academy of Sciences, Washington, D.C. 1974. 72 pages. Price $5.50.*

The leading word in the title of this publication is Guidelines. So often the title means little to the contents described. In this case the guidelines are just that. From the history which provides the background through facilities, procurement, management and laboratory procedures, the total concept of animal care is dealt with in an orderly and informative manner. Detailed examples are given where necessary but mainly principles are emphasized.

The section on anesthsia may be oversimplified by inferring that you can learn how to anesthetize by reading a book.

This is an excellent book of guidelines. — *J. D. Schroder.*