Cell Culture Studies of a Neonatal Calf Diarrhea Virus

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ABSTRACT

The effects of a neonatal calf diarrhea virus on cell cultures were investigated. Bovine embryonic kidney cell cultures were the most satisfactory for production of virus. Cytoplasmic changes detected after inoculation with a high multiplicity of virus were: 1) cytoplasmic vacuoles; 2) some eosinophilic cytoplasmic inclusions; and 3) some degeneration of cells and detachment from the monolayer. Cultures stained with fluorescein-labeled antibody showed cytoplasmic fluorescence as early as four hr after infection with the maximum fluorescence at five days. No cross reactions were observed between the neonatal calf diarrhea virus and reovirus type 1 or type 3 by the fluorescent antibody technique. Plaques were small and were not produced consistently. The optimal adsorption time was one to two hr. The maximum titer was reached at 18 hr, with the cell-associated titer remaining higher than the cell-free titer until that time. An interferon was produced by cultures infected with either ultraviolet-inactivated or untreated virus.

RÉSUMÉ

Cette étude visait à déterminer les effets d'un virus de la diarrhée néo-natale des veaux sur certaines cultures cellulaires. Celles de rein de foetus de veau s'avérèrent les plus favorables à la multiplication de ce virus. On constata les changements cytoplasmiques suivants, après l'inoculation de ces dernières avec une forte dose du virus: 1) vacuoles cytoplasmiques; 2) quelques inclusions intracytoplasmiques acidophiles et 3) dégénérescence d'un certain nombre de cellules, ainsi que leur détachement de la couche cellulaire. La coloration des cultures cellulaires, à l'aide d'anticorps fluorescents, révéla une fluorescence cytoplasmique, aussi tôt que quatre heures après leur infection; cette fluorescence atteignit sa plus grande intensité, cinq jours après l'infection. L'immunofluorescence ne révéla pas de réaction croisée entre le virus de la diarrhée néo-natale des veaux et le réovirus du type 1 ou 3. Les plages étaient petites et leur production, inconstante. Le temps d'adsorption optimal se situait entre une et deux heures. On obtint la concentration virale maximale en 18 heures, et la concentration du virus attaché aux cellules demeura plus élevée que celle du virus libre, jusqu'à ce moment. Les cultures cellulaires ensemencées avec un virus inactivé aux rayons ultraviolets ou intact produisirent un interféron.

INTRODUCTION

Infectious diarrhea of newborn calves is a major disease problem in both dairy herds and in beef cow-calf herds. Mebus *et al* (7) produced neonatal calf diarrhea in experimental calves by inoculation with feces or bacteria-free filtrates prepared from feces obtained from diarrheic calves. Viral particles with a diameter of approximately 65 nm were observed by electron microscopic examination of partially purified and concentrated material. The authors also described preparation of fluorescein-labeled antibody and staining technique. Virus was

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detected by the immunofluorescent technique in samples collected from herds located in Nebraska, South Dakota, Illinois, and California (13).

Welch (12) reported that the morphology and some chemical characteristics of purified virions appeared to be similar to those of the reovirus group and bluetongue virus. Fernelius *et al* (1) also stated that the virus resembled reoviruses, but since it was not neutralized by antiserum against reovirus-1 or dog reovirus, they concluded that it should be considered as a reovirus-like agent.

Adaptation of this virus to cell culture was described by Mebus *et al* (6) and by Fernelius *et al* (1). Cytopathic effects (CPE), detected by observation of cell cultures with a light microscope, were described. Length of time required for detection of CPE, progressive cytopathic changes, or studies of stained preparations were not given.

The purpose of this investigation was to study cell cultures infected with the neonatal calf diarrhea virus and to compare with those infected with reovirus type 3.

MATERIALS AND METHODS

Viruses

Two isolates of neonatal calf diarrhea virus were used. The Cody isolate was received as the 135th passage in bovine embryonic kidney (BEK) cell cultures and the Lincoln isolate as the 119th passage[1].

Primary BEK cell cultures were prepared according to the method of Madin *et al* (4). Growth medium consisted of 0.5% lactalbumin hydrolysate[2] (LAH) in Hanks' balanced salt solution (HBSS), 10% calf serum, 100 units of penicillin and 100 μg of streptomycin per ml. Monolayers were washed three times with HBSS, inoculated with 0.1 ml of virus suspension, and incubated at 37°C. During the adsorption period the containers were tilted frequently to insure even distribution of virus. The inoculum was removed after one to two hr incubation, the monolayers washed three times with HBSS to remove unadsorbed virus, and maintenance medium (MM) added. MM was composed of 0.5% LAH and 0.1% yeastolate[3] in HBSS plus 100 units of penicillin and 100 μg of streptomycin per ml.

Infected BEK cell cultures were usually harvested five to eight days after inoculation by three cycles of quick freezing in a dry ice-alcohol bath and thawing in a water bath at room temperature. In addition, they were shaken vigorously with glass chips. The fluid was centrifuged at 850 X *g* for 20 min at 4°C. The clear supernatant fluid was stored at -65°C and later used as the virus inoculum.

Virus Assay

Test tube cultures of BEK cells were washed three times with HBSS, inoculated with 0.1 ml of ten-fold serial dilutions of neonatal calf diarrhea virus, allowed to adsorb for one to two hr at 37°C, and 0.9 ml MM added. Five test tubes were used for each dilution and MM was used as the diluent. After five days incubation at 37°C, smears were prepared according to the method of Mebus *et al* (6), stained by the direct fluorescent antibody (FA) technique (7), and examined by fluorescent microscopy. (Preliminary investigations, conducted by checking titers each day, indicated that the maximum titer was obtained at five days and remained stable for at least two additional days). No cross reactions were observed between fluorescein-labeled infectious bovine rhinotracheitis (IBR) virus or bovine virus diarrhea (BVD) virus antisera and cell cultures infected with neonatal calf diarrhea virus, or between fluorescein-labeled neonatal calf diarrhea virus antisera and cultures infected with IBR or BVD viruses. Smears containing fluorescent cells were considered positive. No attempt was made to estimate the percent of fluorescent cells. Titers were calculated according to the method of Reed and Muench (8).

Cytopathogenicity

CPE was studied by direct microscopic examination of BEK monolayers grown in T-60 flasks and inoculated with $10^{5.5}$ or $10^{2.5}$ 50% cell culture infective dose ($CCID_{50}$) of virus. Cells were also grown on cover

[1]From C. A. Mebus, Department of Veterinary Science, University of Nebraska, Lincoln, Nebraska.

[2]Obtained from Nutritional Biochemicals, Cleveland, Ohio, U.S.A.

[3]Obtained from Difco, Detroit, Michigan, U.S.A.

slips and in test tubes, infected, and at intervals stained with hematoxylin and eosin (H&E), acridine orange (AO) (5), and FA. As a comparison, similar observations were made on reovirus type 3 (Abney strain). Uninfected cells were used as controls and were treated in the same manner.

Host Cell Range

In addition to BEK cells, the following cell cultures were inoculated with virus: bovine testicle cells, second and third passages; primary and secondary bovine embryonic thyroid cells; primary and secondary bovine embryonic adrenal cells; BHK-21, Vero, KB, and mouse L cell lines. Cultures were examined daily for CPE and stained with FA after five days incubation.

Plaques

Monolayers grown in 60 mm diameter plastic petri dishes and incubated in a humidified CO_2 incubator were used in attempting to plaque neonatal calf diarrhea virus. Cultures were inoculated with virus as previously described. After unadsorbed virus was removed various media, containing either 0.9% ionagar[4] or 1.5% methylcellulose[5], were used as overlays. Media tested were: LAH in both HBSS and Earle's balanced salt solution (EBSS); Eagle's minimum essential medium (MEM) in both HBSS and EBSS; MEM with a 2X concentration of vitamins and amino acids; medium 199 in both HBSS and EBSS; and 0.1% bovine serum albumin, 0.1% yeastolate, 0.5% LAH in EBSS. Penicillin and streptomycin were included in concentrations previously described. Fetal calf serum was used in concentrations varying from zero to 20%. In certain experiments either DEAE-cellulose or DEAE-dextran was added at the same time as the inoculum, or added with the overlay, or both (9). In certain experiments a second overlay containing 0.01% neutral red was added four days after the first overlay. In other experiments, no neutral red was used, but at intervals after the first overlay, the monolayers were stained with crystal violet (3). Vesicular stomatitis virus (VSV), a bovine enterovirus (ECBO), and pseudorabies (PR) virus were also used in each experiment to determine if conditions were satisfactory for production of plaques.

The immunofluorescent plaque technique of Spendlove *et al* (10) was also tested.

Adsorption

BEK monolayers in T-15 flasks were washed, inoculated with 0.5 ml fluid containing 10^4 $CCID_{50}$ of virus, and the flasks rocked during adsorption periods ranging from five min to three hr. At the end of each adsorption period the inoculum was removed, cells washed five times with HBSS, and 2 ml MM added. After five days incubation at 37°C samples were titered.

Multiplication Cycle

Prior to starting the experiment the average number of cells per T-15 flask was determined by counting the cells from 15 individual flasks with a hemacytometer. For determination of the multiplication cycle, monolayers were washed three times with HBSS and inoculated with 0.5 ml virus suspension. The multiplicity of infection was calculated to be ten. After adsorption for one hr at 37°C flasks were washed three times with HBSS and 2 ml MM added. At intervals, the medium from two flasks was removed, pooled, and saved. This sample was designated as "cell-free virus". Immediately after removing the medium, the monolayers were washed three times with HBSS, two ml MM added, and the cells harvested as previously described. These samples were designated as "cell-associated virus". All samples were stored at -65°C and later titrated.

Interferon

Ultraviolet-treated and untreated virus suspensions were tested as agents for production of interferon. Five ml of undiluted or a ten-fold dilution of virus were placed in an uncovered petri dish 18 inches from a germicidal ultraviolet light and rotated for five min. The virus suspension was then immediately diluted 10^{-1} or 10^{-2} in HBSS and 1 ml amounts added to washed BEK monolayers in T-60 flasks. Ten ml MM were added after a two hr adsorption period. Fluid was decanted from an equal number of flasks at 24 hr intervals for five days and centrifuged at 850 × *g* for

[4]Obtained from Oxoid, Division of Oxo Limited, London, England.

[5]Obtained from Matheson Scientific, Kansas City, Missouri, U.S.A.

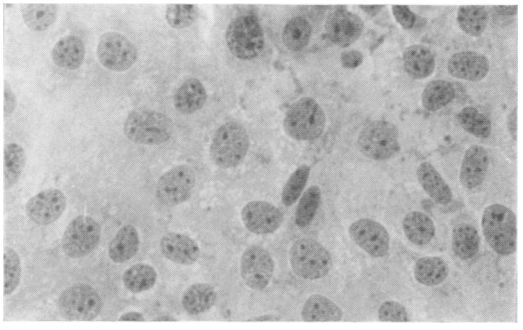

Fig. 1. Uninfected BEK monolayer. H & E stain. X400.

20 min at 4°C. These preparations were designated as "UV". Other preparations, designated as "active", were produced in the same manner, except the virus was not treated with ultraviolet light. The UV preparations were titered in order to check for virus activity.

Interferon preparations were assayed by the 50% plaque reduction test (11) and inhibition of CPE (11), using VSV as the challenge virus. PR virus was also used as a challenge virus to test for lack of antiviral specificity. The interferon titer was calculated as the reciprocal of the dilution at which the plaque number was reduced to 50% of the control count. The end point of the inhibition of CPE test was taken as the reciprocal of the dilution at which half the cultures were protected when CPE was noted in virus-infected control cultures.

To determine if the preparations could be neutralized by specific antiserum, samples were mixed with neonatal calf diarrhea virus antiserum at a ratio of 9:1, placed at 4°C for 18 hr, centrifuged at 850 × *g* for 15 min, and tested for activity. In order to test for possible antiviral action, undiluted interferon was mixed with 100 pfu or 1000 $CCID_{50}$ of VSV, allowed to stand at room temperature for one to two hr, and added to BEK monolayers. Preparations were also tested for activity after centrifugation at 105,000 × *g* for two hr, after heating at 56°C for two hr, and for stability at pH 2.0 and 12.5.

RESULTS

CYTOPATHOGENICITY

When cells were infected with $10^{5.5}$ $CCID_{50}$ of neonatal calf diarrhea virus, CPE was detected in unstained monolayers 24 hr after infection. The affected cells were thin, elongated, almost crescent-shaped, and adhered to the monolayer by a single process. Some small, rounded cells, which had detached from the monolayer, were floating in the fluid medium. At 48 hr the number of detached cells had increased, leaving some areas of the glass devoid of cells. The areas of detached cells never exceeded 50% of the monolayer even when cultures were incubated for as long as 12 days.

Uninfected BEK cells stained with H&E are shown in Fig. 1. When cells infected with $10^{5.5}$ $CCID_{50}$ were stained with H&E 24 hr postinoculation, small cytoplasmic vacuoles were noted, especially in the perinuclear region (Fig. 2, arrow a). As the infection progressed with increasing time, the vacuoles increased in size and number. In some cases they appeared to coalesce to form large vacuoles (Fig. 3) and some of the affected cells contained pyknotic nuclei. Some cells, each containing one eosinophilic, cytoplasmic inclusion body (Fig. 2, arrow b) were seen one to two days after infection. Few cells containing multiple inclusions were observed (Fig. 4, arrow a) four days postinoculation. Detached cells, floating free in the medium, were small, rounded, (Fig. 4, arrow b) or still elongated (Fig. 4, arrow c) and contained pyknotic nuclei with intensely stained, basophilic, nuclear chromatin.

CPE of cells infected with neonatal calf diarrhea virus and stained with AO were similar to that observed with H&E. The cytoplasmic vacuoles appeared as black areas, in sharp contrast to the red staining of the cytoplasmic ribonucleic acid. The cytoplasm of the detached cells was a deep red color and the nuclei were small,

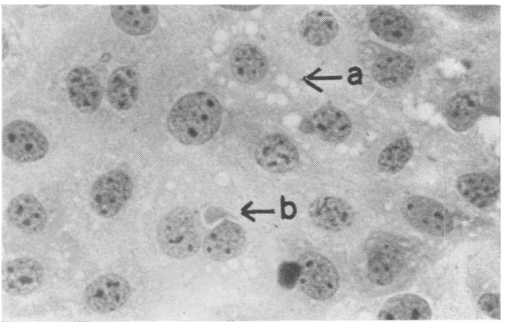

Fig. 2. CPE 24 hr after inoculation with $10^{5.5}$ $CCID_{50}$ of neonatal calf diarrhea virus. Arrows indicate: (a) cell containing numerous cytoplasmic vacuoles; (b) cell containing a cytoplasmic inclusion and vacuoles. H & E stain. X400.

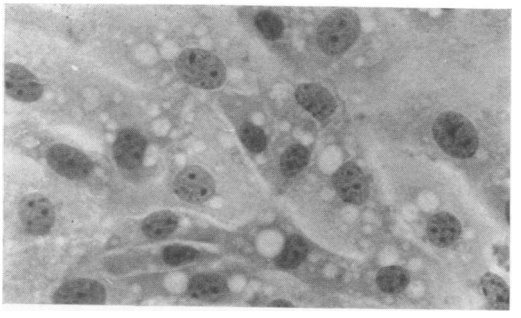

Fig. 3. Large cytoplasmic vacuoles in cells two days after inoculation with $10^{5.5}$ $CCID_{50}$ of neonatal calf diarrhea virus. H & E stain. X400.

rounded, and stained a brilliant yellow color. However, no nuclear or cytoplasmic inclusions were seen.

CPE was very difficult to detect when cells were infected with $10^{2.5}$ $CCID_{50}$ of virus. One or two days after inoculation there was a slightly greater number of cells floating in the medium than in comparable uninfected cultures. Furthermore, as the cells aged, the degenerative process in noninfected cultures and cultures infected with a low multiplicity of infection was so similar that a definite CPE could not be discerned.

Unstained BEK monolayers infected with reovirus type 3 (Abney) did not show CPE until ten to 14 days. Cells became small, round, and detached from the monolayer. Approximately 25% to 50% of the cells were affected. Cytopathic changes were detected as early as two to six days postinoculation when cover slip preparations were stained with H&E (Fig. 5). Some cells contained cytoplasmic vacuoles and cytoplasmic inclusions. Degenerated cells were round and very small, with intensely stained eosinophilic cytoplasm, and pyknotic nuclei.

When cover slip preparations or smears of cells infected with neonatal calf diarrhea virus were stained by the FA technique, fluorescent cells were first detected four hr postinoculation. A few cells, which were usually grouped together in one area, contained discrete perinuclear cytoplasmic fluorescent granules. At six hr the number of fluorescent cells had increased, although they were still few in number. At eight hr they were more numerous and the entire cytoplasm stained diffusely green. By 24 hr approximately 50% of the cells fluoresced (Fig. 6). After 24 hr some cells detached from the monolayer and these cells were filled with fluorescent staining material.

The cytoplasm of some cells inoculated with reovirus type 3 (Abney) and stained by the FA technique with fluorescein-labeled antibody prepared from reovirus type 1 antiserum[6] or from reovirus type 3

[6]From the National Animal Disease Laboratory, Veterinary Science Research Division, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa.

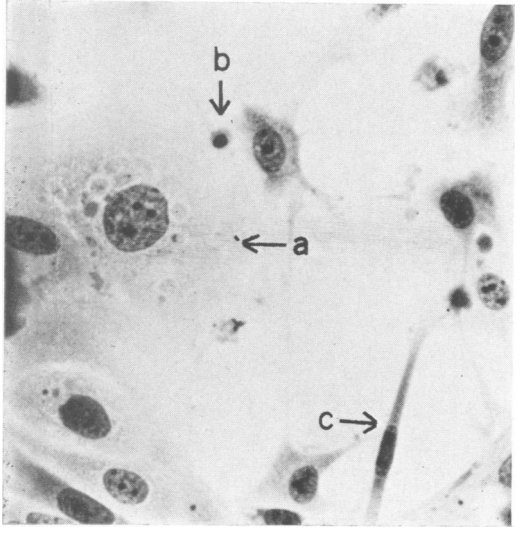

Fig. 4. CPE four days after inoculation with $10^{5.5}$ $CCID_{50}$ of neonatal calf diarrhea virus. Arrows indicate: (a) cell containing multiple inclusions; (b) small, rounded cells, detached from monolayer; (c) elongated cell. H & E stain. X400.

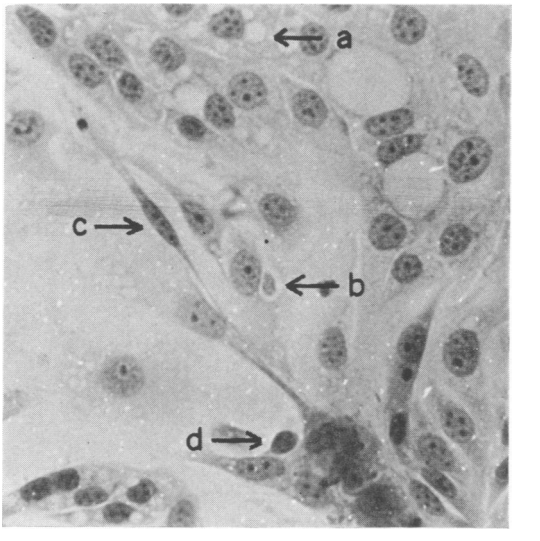

Fig. 5. CPE two days after inoculation with reovirus. Arrows indicate: (a) cell containing cytoplasmic acuoles; (b) cell containing cytoplasmic inclusion; (c) thin, elongated cell; (d) rounded cell with pyknotic nucleus. H & E stain. X400.

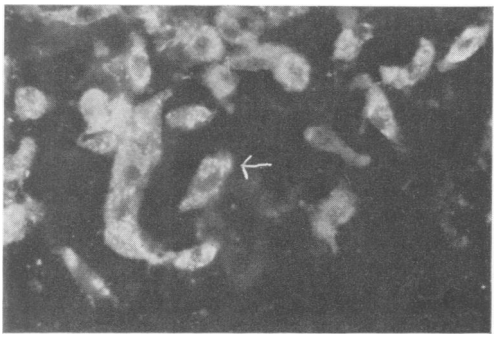

Fig. 6. Smears prepared 24 hr after inoculation with neonatal calf diarrhea virus. Arrow points to a cell containing small fluorescent granules. FA stain. X250.

antiserum[7] contained fluorescent strands which surrounded the nucleus. Other cells contained large cytoplasmic aggregates of fluorescent material and degenerating cells were filled with fluorescent material. No fluorescence was detected in cells infected with neonatal calf diarrhea virus and stained with fluorescein-labeled reovirus type 1 or type 3 antibody. Also, cells infected with reovirus type 3 and stained with fluorescein-labeled neonatal calf diarrhea virus antibody did not fluoresce.

Host cell range

Very minimal cytopathic changes were detected in cell cultures other than BEK. Only a few fluorescent cells were observed at 10^{-1} or 10^{-2} dilutions of virus.

Plaques

Attempts to plaque neonatal calf diarrhea virus were generally unsuccessful. The most satisfactory results were obtained with primary BEK monolayers and a single overlay consisting of 0.5% LAH in HBSS, 5% fetal calf serum, and 0.9% ionager. However, plaques could not be reproduced consistently and none were visible when neutral red was included in the overlay. Seven days incubation prior to staining with crystal violet were required before any areas resembling plaques were visible. The largest areas that appeared to be plaques were 0.5 mm in diameter but were very few in number. The majority could be seen only microscopically.

The fluorescent plaque technique did not produce satisfactory results.

[7]Conjugate prepared by M. B. Rhodes, Department of Veterinary Science, University of Nebraska, Lincoln, Nebraska.

Adsorption

The maximum adsorption of virus to cells occurred at two hr, with approximately 85% adsorbed at one hr.

Multiplication cycle

The multiplication cycles of both isolates were similar (Fig. 7). A steady rise in cell-associated virus titer was noted until a maximum was reached at 18 hr. The cell-associated titer remained higher than the cell-free titer until 18 hr when a second cycle of multiplication began.

Interfron

Cell cultures inoculated with UV-treated preparations did not fluoresce when stained by the FA technique, which indicated that the virus was no longer infectious. Interferon was produced both by cells inoculated with UV-treated virus and with active virus. However, preliminary tests indicated that cells inoculated with UV-treated virus produced the highest concentration of interferon at three days post-inoculation. Therefore, this method was used for subsequent experiments. Titers of various preparations ranged from 300 to 600 units per ml. Titers obtained by using PR virus as the challenge virus were not quite as high as those obtained by using VSV as the challenge virus. The interferon was not neutralized by specific antiserum to neonatal calf diarrhea virus, and the titer was

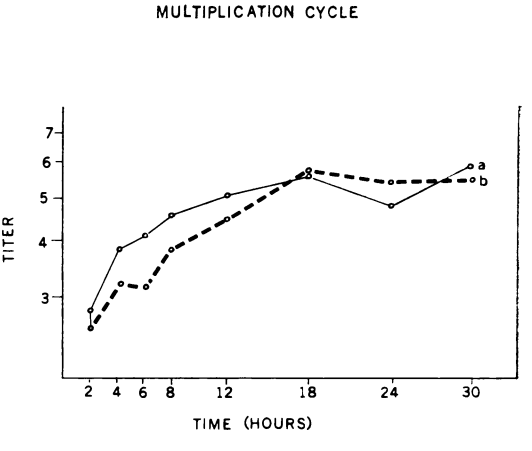

Fig. 7. Multiplication cycle of neonatal calf diarrhea virus. (a) cell-associated virus. (b) cell-free virus. Titers expressed as $\log_{10}$ $CCID_{50}$/0.1 ml.

not appreciably reduced when the preparation was mixed with challenge virus, or after centrifugation at 15,000 × *g* for two hr, or by pH 2.0. There was only a slight reduction in titer after the interferon was heated at 56°C for two hr. However, activity was lost when the preparations were held at pH 12.5 for 48 hr.

DISCUSSION

Previous reports of CPE on cells infected with neonatal calf diarrhea virus were based on direct microscopic examination only. In this study detailed observations were made of both unstained and stained BEK cell cultures. The early changes observed in cells inoculated with a high multiplicity of infection agreed with those reported by Mebus *et al* (6), in that affected cells remained attached to the monolayer by a single process and later detached. Closer examination characterized these cells as being very thin, elongated, often having a crescent shape, and containing pyknotic nuclei. Cytoplasmic vacuoles were very prominent in stained cultures infected with neonatal calf diarrhea virus and with reovirus type 3. This type of CPE has also been reported for cells infected with avian reoviruses (2). The cytoplasmic vacuoles were probably what were referred to as "granulation" by Mebus *et al* (6) and Fernelius *et al* (1) or as "pigmentation" by Fernelius *et al* (1). Cytopathic changes in cultures infected with a low multiplicity of virus were so slight that CPE due to virus could not accurately be distinguished from normal degenerative changes of uninfected cells.

Production of cytoplasmic inclusions in infected cells is characteristic of some viruses, including those of the reovirus group. A few cells containing eosinophilic, cytoplasmic inclusion bodies were seen early in the infectious process when cells infected with neonatal calf diarrhea virus were stained with H&E. However, no inclusions were observed when cells were stained with acridine orange. Cells containing inclusions were relatively few in number so that it is possible they were simply overlooked when the slides were examined.

Fluorescent cells were first detected four hr postinoculation when stained with FA and, even when few in number, could easily be detected. The cytoplasmic fluorescence of cells infected with neonatal calf diarrhea virus was distinctly different from that of cells infected with reovirus type 3. The fluorescent reaction between cells infected with reovirus type 3 and stained with fluorescein-labeled reovirus type 1 antiserum, demonstrated a common antigenicity between these two types. Lack of a cross reaction between cells infected with neonatal calf diarrhea virus and reovirus type 1 or type 3 antiserum, and reovirus type 3 and neonatal calf diarrhea antiserum, indicated that no common antigenicity was detectable by either of these systems.

None of the methods for plaque production tested with neonatal calf diarrhea virus produced consistently satisfactory results. Plaques were generally too small to be visible without a microscope, and no plaques were seen when neutral red was included in the overlay. Since they were visible only when stained with crystal violet, it was not possible to pick virus from the plaque. One of the difficulties encountered may have been due to the necessity of using serum in the overlay medium. Preliminary work indicated that bovine serum, including commercial fetal calf serum, was generally inhibitory to the virus. Primary BEK cells, which appeared to be the most sensitive host cell, degenerated rapidly under a serum-free agar or methylcellulose overlay. Another difficulty may have been due to the type of CPE produced. Infected cells tended to adhere to the monolayer and many of the affected cells showed rather minimal changes. These factors are consistent with poor plaque production.

A substance was produced by cells exposed to ultraviolet-inactivated or untreated virus which, like interferon, reduced the titer of challenge viruses, was not neutralized by specific antiserum, did not have a direct antiviral action, was not sedimented by centrifugation at 103,000 × *g* for two hr, and was stable at pH 2.0.

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Book Review

THE FEEDLOT. *Edited by I. A. Dyer and C. C. O'Mary. Published by The Macmillan Company of Canada Limited. 1972. 224 pages. Price $15.50.*

This book has been written from information provided by 15 people who offered their various expertise regarding the feedlot beef industry in the United States. The editors used this information to prepare their manuscripts.

The fifteen chapters in the book include: location of a feedlot, economics of feedlots and financing role of the feedlot consultant, feedlot design and management, feed mill operations, preweaning conditioning and stocker management, types of cattle to feed, buying feeders and selling fed cattle, feed preparation, cattle feeding, control of feedlot disease and parasites, manure management, measuring beef quality, integration in the feeding industry and the future of the feedlot industry.

In the preface to this book, the editors point out that it is impractical to describe in detail every aspect of cattle finishing, especially since management decisions appropriate to individual feedlots must be made. While this may be true, I believe that more detailed information needed to be supplied in many chapters to make this book particularly useful for an experienced feedlot operator.

However, the book does contain a wealth of basic information for the novice cattle feeder and for all students interested in the feedlot cattle industry. The information is based on conditions in the United States, but in most cases can be related to Canadian circumstances. — *R. A. Curtis.*