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SEQUENTIAL DEVELOPMENT OF SOME BOVINE VIRUS ANTIGENS IN CELL CULTURES STUDIED BY THE FLUORESCENT ANTIBODY TECHNIQUE

by

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TABLE OF CONTENTS

	Page
List of Tables and Figures	iv
General Introduction	1
Paper 1: SEQUENTIAL DEVELOPMENT OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS IN BOVINE TURBINATE CELL CULTURES	3
Summary	3
Introduction	4
Literature Review	5
Materials and Methods	11
Results	13
Discussion	15
Conclusion	15
Table I	17
Figures 1-12	19
References	33
Paper II: SEQUENTIAL DEVELOPMENT OF BOVINE PARAINFLUENZA TYPE 3 VIRAL ANTIGENS IN BOVINE TURBINATE CELL CULTURES STUDIED BY FLUORESCENT ANTIBODY TECHNIQUE	37
Summary	37
Introduction	38
Literature Review	39
Materials and Methods	43
Results	45
Discussion	45
Table II	47
Photomicrographs	48
References	62
Paper III. SEQUENTIAL DEVELOPMENT OF BOVINE VIRUS DIARRHEA (BVD) VIRAL ANTIGENS IN CELL CULTURE	65
Summary	65
Introduction and Review of Literature	66
Materials and Methods	69
Results	71
Discussion	71
Table III	73
Figures	74
References	84
ACKNOWLEDGEMENTS	87

Page

APPENDIX A: METHODS 88

1. VIRUS TITRATIONS 88

A. Microtiter Technique 88
B. Plaque Assay Technique 88

2. PREPARATION OF CONJUGATE 90

A. Fractionation of Serum 90
B. Conjugation of Globulin 91
C. Preparation of Bovine Kidney Powder 92
D. Adsorption of Conjugate 93

APPENDIX B: SOLUTIONS 94

1. Growth Media 94
2. Maintenance Media 94
3. Tris-buffered Saline (TBS) 94
4. PBS (Phosphate Buffered Saline) 94
5. Antibiotics--Stock Solutions 94
6. Conjugation Regents 95

APPENDIX C: RESULTS 96

APPENDIX: REFERENCES 100

LIST OF TABLES AND FIGURES

Page

Paper I:

Table I: Sequential Staining of IBRV Antigens in BT Cells Infected with IBR Virus 17

Fig. 1-12: Photomicrographs Showing Appearance of Viral Antigens in Bovine Turbinate Cells Infected with IBR Virus, and Stained by the Fluorescent Antibody Technique. (All Photographs were taken at 250 x Magnification, unless otherwise indicated). 19

Fig. 13-I: Time course of IBR virus infectivity Titer (PFu/.2ml) 31

Paper II:

Table II: Sequential Development of PI_3-V Antigens in BT Cells Infected with PI_3-V. 47

Fig. 1-11: Immunofluorescent Photomicrographs of Bovine Turbinate Cells Infected with Bovine Parainfluenza Type 3 Virus and Harvested at Various Times 48

Fig. 12-II: Time course of PI_3 virus infectivity titer ($TCID_{50}$/ml) 60

Paper III:

Table III: Sequential Development of BVD Antigens in Cell Culture 73

Fig. 1-8: Photomicrographs Showing Sequence of Appearance of Fluorescent Viral Antigens in Bovine Embryonic Kidney Cells Infected with Bovine Virus Diarrhea Virus 74

Fig. 9-III: Time course of BVD virus infectivity titer ($TCID_{50}$/ml) 82

Appendix:

Table A-a: Initial Titration of IBR, BVD by Plaque Assay 96

Page

Table A-1: Initial Microtiter Titration of the Three Viruses 97

Table A-2: Final Titration of PI_3-V 98

Table A-3: Final Titration of BVD (virus) 98

Table A-4: IBRV Titration by Plaque Assay 99

General Introduction:

The viruses of Infectious Bovine Rhinotracheitis (IBR), Bovine Virus Diarrhea (BVD) and Bovine Parainfluenza type 3 (PI_3) are of considerable importance to the livestock producer and the veterinarian because of the wide range of disease entities they produce, either individually or in combination, in cattle all over the world. A herpes virus, the IBR virus has been incriminated as the cause of upper respiratory disease, conjunctivitis, balanoposthitis, pustular vulvovaginitis, infertility, and in conjunction with PI_3 virus, the shipping fever complex. Some other members of the Herpes group have generated current interest as they have been associated with different types of cancer. The PI_3 virus has been isolated from man, cattle, water buffalo, horses, monkeys, rodents, sheep, swine, etc. and is associated with respiratory disease in cattle. Other members of the Paramyxovirus family, notably rinderpest, canine distemper and Newcastle disease viruses have caused widespread and often devastating diseases in livestock. Human strain of PI_3 causes respiratory diseases in children and some members of the parainfluenza group have recently been indicted as possible etiologic agents in multiple sclerosis. BVD virus is widespread in cattle populations often as subclinical infections, as well as clinical enteritis, abortion and cerebellar hypoplasia.

Understanding of the morphogenesis of these viruses in cells in vitro is a useful step in the understanding of their pathogenesis in living animal. This study was designed to investigate the sequence of development in cell cultures, of the antigens of these viruses. The antigens will be visualized by fluorescent antibody (FA) staining. IBR and PI_3 viruses infect the bovine respiratory tract, hence bovine turbinate cell cultures have been chosen to study the morphogenesis of these viruses. The development of

BVD virus will be studied in Bovine Embryonic Kidney (BEK) cell cultures.

Paper 1:

SEQUENTIAL DEVELOPMENT OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS IN BOVINE TURBINATE CELL CULTURES

Summary

The sequence of appearance and localization of IBR-V antigens in infected bovine turbinate cells were studied by Fluorescent Antibody (FA) technique. Both intranuclear and cytoplasmic fluorescence were observed. Specific fluorescence was first seen at 2 hours post infection, as bright line of fluorescence surrounding the nuclear membrane. Cytoplasmic fluorescence was most intense between 16 and 24 hours post infection, and diminished slightly after that. Nuclear fluorescence was observed between 6 and 12 hours post infection. The production of infective virus was correlated with appearance of fluorescent viral antigens.

SEQUENTIAL DEVELOPMENT OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS ANTIGENS IN CELL CULTURES

Introduction:

Infectious bovine rhinotracheitis virus (IBRV), a herpes-virus, has been incriminated as the cause of upper respiratory disease, conjunctivitis, abortion, balanoposthitis, a fatal calfhood disease, infectious pustular vulvovagivitis, infertility, artificial mastitis, and endometritis (House) (13). Like some human herpes viruses, IBRV has been shown to induce malignant transformation of infected hamster cells (24) (Michalski and Hsiung, 1975). This fact is of particular importance because IBRV could be a contaminant of bovine sera commonly used in tissue culture media. Hence a great deal of interest has been generated in the study of the pathogenesis of IBRV infection, its development in infected cells and the effect on infected cells. Fluorescent Antibody technique was born in 1941 when Coons (6) utilizing the knowledge that the antibody molecule could be covalently linked with other molecules without disturbing its specific activity, successfully coupled fluorescein isocyanate to antiserum. Later Riggs et al. (30) introduced the more stable fluorescein isothiocyanate.

Fluorescent antibody (FA) techniques have been widely used in diagnosis and study of multiplication of viruses (28, 23, 41, 29, 1, 11, 20, 12, 26, 33, 31, 42, 35). This method is particularly useful in visualizing the reproductive cycle of viruses in tissue culture, and in following sequential development of viral antigens (33, 31, 12). FA offers great promise in diagnoses of disease because of the rapidity with

which diagnosis can be made (12). One of the greatest problems with FA is the preparation of specific antisera. Nonspecific staining reduces the sensitivity of FA tests. Fundamental to FA techniques is the production of highly specific antiserums for each virus or viral antigen. Several methods for producing monospecific antibodies, have been described (39, 31, 33, etc.). Christofinia, et al. (4) described a method for producing of monospecific antisera in gnotobiotic animal, against viral antigens.

Literature Review

The development of viral antigens in cell cultures as observed by FA techniques has been studied in several virus-cell culture systems. Reports concerning the location of immunofluorescent antigens have varied widely, even in the same virus. The antigens of New Castle disease have been located in the cytoplasm whereas influenza viral antigens localize in the nucleus (29). Hog cholera antigens have been localized first in nucleus, in cytoplasm and in both (23). Such situations have been encountered for innumerable virus-cell culture systems, and raises the questions why and how viral antigens segregate in different cell compartments. This also raises even more fundamental questions, such as what factors determine whether a virus will multiply in the nucleus or cytoplasm of infected cells, and how does the virus move its component parts from sites of synthesis to sites of assembly (33).

In studies of IBRV by FA technique, Bindrich (2) reported that immunofluorescent antigen were present within the nuclei of infected bovine embryonic kidney (BEK) cells at 5 hours after infection. This nuclear fluorescence increased as infection progressed. Cytoplasmic fluorescence

was not observed.

Gratzek, et al. (11) studied the development of IBRV in bovine testicle cells by fluorescent microscopy and electron microscopy. They observed initial fluorescence at the nuclear membrane 90 minutes post infection. Fluorescence was vesicular, and gradually spread through the cytoplasm. Nuclear fluorescence was absent. Electron microscopic observations revealed a pattern characteristic of the herpesvirus group. Virus capsids were observed in the nuclei of infected cells, and enveloped intracytoplasmic viral particles were always within vesicles.

In similar studies with bovine kidney (BK) cells, Jasty et al. (20) determined the location of viral DNA by acridine orange staining and by Feulgen test reaction; location of viral proteins was by fluorescent antibody (FA) staining, and cellular morphology was studied by hematoxylin-eosin (HE) staining. Fluorescence was first observed at the region of the nuclear envelope at 4 hours post infection. Fluorescence spread rapidly into the cytoplasm, and by 12 hours, the entire cytoplasm emitted bright specific fluorescence. During this period they observed fluorescence at the nuclear envelope, with occasional specks of fluorescence in the nucleoplasm. By 20 hours, they noted that the entire nucleus, in addition to the cytoplasm, emitted fluorescence. Theil, et al. (39) studied IBRV development in MDBK cells by immunofluorescence staining. Fluorescent granules first appeared at the nucleocytoplasmic junction 4 hours after infection, intensified around the nuclear membrane and spread throughout the cytoplasm by the 12th hour after infection. Nuclear fluorescence was not observed, in spite of the fact that they tested six strains of IBRV, (some known to produce intranuclear inclusions), and used conjugates prepared by different methods. Heating the coverslips reduced cytoplasmic

fluorescence, but nuclear fluorescence was not observed in heated coverslips. Schipper and Chow (35), studied IBRV development on bovine embryonic kidney (BEK) cells, and noticed fluorescence first in the cytoplasm adjacent to the nuclear membrane of infected cells at 12 to 24 hours post incubation. Fluorescence spread throughout the cytoplasm. In general, nuclear fluorescence appears to be uncommon (Theil, et al. 5) in IBRV infected cells.

Electron microscopy studies of single cycle infection of tissue culture cells by IBRV (18, 19, 37) have yielded somewhat different results. In all cases, viral particles were first noticed in the nucleus between the 5th (37) and 8th (18, 19) post infection hour. These viral particles were considered to be immature viral nucleocapsids (19), and generally acquired an envelope from the internal lamella of the nuclear envelopes. Envelopment of virus occurred mainly in the nucleus though it was observed occasionally in the cytoplasm (19). In either case the source of the envelope was the internal lamella of the nuclear envelope. Following similar studies by Schwartz and Roizman on Herpes simplex virus, (34) Jasty and Chang (18) showed that bovine kidney cells infected with IBRV develop branching membranous channels in their cytoplasm. The presence of virus particles in these channels suggest that such membranous structures might be concerned with the passage of virus from the perinuclear cisterna to the intercellular spaces. Using acridine orange and Feulgen test, Jasty and Chang (20) has shown that the site of synthesis of IBRV DNA was the nucleus (nuclear envelope and its internal boundary). These and similar evidence (34) suggest that the IBR viral DNA is synthesized in the nucleus; the viral nucleocapsid is assembled in the nucleus (34); and the viral particle is enveloped in the nucleus before being transported

into the cytoplasm.

Electron microscopy and other studies on other members of the herpes group have also shown similar tendencies (10, 13, 22, 25, 31, 32, 33). Lebrun (22) studied the localization of herpes simplex antigens in chorio-allantoic membranes, mouse brain and human epidermoid carcinoma cells grown in tissue culture. Localization of antigens was by means of specific human serum and fluorescein-labelled anti-human gamma globulin. She observed that antigen made its first appearance as a small spherical spot in the nucleus, increased slowly in amount there, and then appeared in the cytoplasm in large amount. The nucleus lost antigen until at the end stages, showing typical type A inclusions, it contained little or no antigen. Taking other similar studies in consideration, Lebrun concluded that the herpes simplex virus contains characteristic antigenic material during its intranuclear stage, that is, before it has acquired its second coat. Roizman, et al. (33) also studied cellular compartmentalization of herpes virus antigens during viral replication. Using human epidermoid carcinoma (HEP-2) cells, labelled pool of human gamma globulin, and rabbit serum prepared against boiled cell debris they demonstrated five distinct immunofluorescent elements, antigenic products specified by herpes virus in HEP-2 cells. These were (a) an amorphous mass filling the nucleus, (b) large and (c) small nuclear granules, (d) cytoplasmic granules, (e) and a powdery material dispersed throughout the cytoplasm. The viral antigens were segregated in the nucleus and cytoplasm; each antigen accumulated in one compartment only. All five immunofluorescent elements were demonstrable with the rabbit serum (Ra-1BC), but the human gamma globulin stained only cytoplasmic antigens. They observed that this restricted reactivity of the antibody contained in the human globulin is not due to its inability

to penetrate the nucleus of the fixed cells--since the same globulin pool was successfully used to demonstrate intranuclear antigen in cells infected with measles virus. These workers offered two hypotheses to explain the compartmentalization of viral antigens (34): the antigenic products specified by virus are synthesized either in the nucleus or in cytoplasm, and that the antigens accumulate where they are made; (b) all antigens are made in one compartment but are selectively transported or become immunologically altered as they cross into other compartments. Sufficient evidence was not yet available to clearly discriminate between these two. However, they tend to favour the second hypothesis. Roizman et al. (33) pointed out that studies in their laboratory indicates that whereas the bulk of viral proteins are synthesized on cytoplasmic polysomes, the nucleus is the site of synthesis of viral DNA, and the assembly of the virion. It could be that viral protein is selectively transported to the nucleus, or that protein acquires a new immunological reactivity in the process of transport from cytoplasm to nucleus (33). In an analysis of polypeptides made in cells infected with herpes simplex virus, Honess and Roizman (13) observed that of the 49 polypeptides specified by the virus, at least two polypeptides showed evidence of posttranslational changes in their mobility. Such changes may be determined by intracellular translocation or may occur during assembly of the virion, and could impart new immunologic reactivity to the protein.

By being able to demonstrate intranuclear viral antigens with antiserum made in rabbit against a 72-hour infected cell stroma (rendered free of infectious, undenatured viruses), Roane and Roizman (31) concluded that the immunologic specificity of a particular antibody may determine whether intranuclear or cytoplasmic antigens are stained by FA. Nii, et al.

(25) in electron-microscopy studies with ferritin-conjugated antibodies (using human and rabbit antisera against herpes virus) found that nuclear and cytoplasmic membranes, viral envelope and aggregates of viral antigen encountered in the nuclear and cytoplasmic matrix were all tagged with ferritin-labelled antibodies. Viral capsids were not tagged. They surmised that the capsid protein was so weak an antigen that sera employed were not sufficiently strong to cause a recognizable tag.

Many other explanations have been given for the failure to locate intranuclear antigens by FA. Theil et al. (39) suggested that the intranuclear inclusions are not rich in virus or viral antigens. They further suggested that because of rapid multiplication of IBR in MDBK cells, viral antigens do not accumulate in the nucleus in sufficient amount to be detected by FA.

One interesting observation made by Theil, et al. was that the development of IBR viral antigens were not affected by 3 pyrimidine analogues--FUdR, IUdR, and ARA-C. They interpreted this as an indication that IBRV immunofluorescent antigens develop independent of DNA synthesis and infectious virus replication. Secondly, that the production of CPE by IBR virus is not a valid indication of infectious virus replication. In their studies of proteins specified by herpes simplex virus, Honess and Roizman (13) reported that of the 49 polypeptides specified by the virus 23 were classified as structural, 16 nonstructural and 10 unassigned. They concluded that there is no simple differentiation into early nonstructural and late structural proteins. Structural proteins are members of both early and late groups. Extrapolated to IBRV, this could explain why the pyrimidine analogues did not block the production of immunofluorescent antigens. Like the herpes virus, some structural proteins

(antigens) could have been part of early proteins. It may also be that DNA synthesis was not completely blocked. Consigli, et al. (5) showed that the presence of 5 methyl deoxycytidine (5MdC) in Parker's 1066 media resulted in failure of FUdR to inhibit specific protein synthesis in polyoma virus. They showed that 5MdC, a compound which on deamination would yield thymidine which is capable of reversing the FUdR effect. It is possible that the inhibiton by the pyrimidine analogues was not complete or was sufficiently reversed to result in synthesis of viral structural proteins.

MATERIALS AND METHOD

Virus: Los Angeles strain IBRV was obtained from Dr. H. Minocha (Department of Infectious Diseases, Kansas State University) and propagated in Madin-Darby Bovine Kidney cells in 2 liter roller bottles. On the fifth post inoculation day, the cells were scraped off the bottle, and clarified. The virus was concentrated by ultracentrifugation at 25,000 rpm for 5 hours. The pellet was resuspended in 3 mls of PBS sonicated, and titrated by plaque assay method. This virus was used for the study.

Growth Media: [a]Eagles Minimum Essential Media (MEM) with 5% Lactoalbumin[b] hydrolysate (LAH), 1% sodium pyruvate, antibiotics and 10% inactivated Fetal Calf Serum.[c]

Maintenance Media: MEM with 5% LAH, 1% Sodium pyruvate, antibiotics (see appendix B) and 10% inactivated horse serum.[c]

[a,b]Grand Island Biological Company, Grand Island, New York 14072.

[c]Kansas City Biological Inc., Lenexa, Kansas 66215.

Cultures: Primary Bovine Turbinate (BT) cells, passage 16 was grown to monolayer in 75 cm plastic cell culture flask.

Coverslip culture: Twenty two mm square coverslips (Corning) were washed in hypochromic acid, and autoclaved. Coverslips were placed in 35 x 10 mm plastic petri dishes, and seeded with 300,000 BT cells in 2 mls of growth media. After 24 hours, the growth media was removed, the coverslips were washed twice with PBS, and each coverslip was infected with 0.2 mls of a 10^{-1} dilution (10^{6} PFU/ml) of the virus described above. The virus was allowed to adsorb for 1 hour at 37°C in a carbon-dioxide incubator. The coverslips were then washed twice with PBS, and 2 mls of maintenance media added. This was regarded as post infection time 0, hour. Samples were collected at the following hours post infection: 0, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, 20, 24, 30, 36 and 48 (hours). At time of collection, each coverslip was washed twice in Tris-buffered Saline (TBS), allowed to dry in a 37° incubator for 10 minutes and fixed in cold acetone for 10 minutes (at -70°C). The coverslip was thereafter air-dried and stored at -70°C for future use. Controls included uninfected coverslips, and coverslips infected with BVD virus. Fluorescent Antibody (FA) staining. In a second experiment, the fixative was a 2:1 mixture of Acetone and Methanol, at -20°C.

The coverslips were taken out of cold storage and allowed some minutes to reach room temperature. They were placed in petri-dishes lined with absorbent paper and flooded with TBS for 5 to 10 minutes. The buffer was drained and the coverslip was covered with conjugate, and incubated at 37°C in a humid carbon dioxide incubator for 50 minutes. Thereafter the coverslip was washed twice in TBS, twice in distilled water, and

mounted with buffered glycerine (9 parts glycerine and 1 part PBS) on a microscope slide. The slides were examined with a Leitz Ortholux Fluorescent microscope with BG38 heat suppression filter and KP490 Exciter filters. Photomicrographs were taken on Kodak high speed Ektachrome film at ASA400. Light source was a HBO 200 lamp and the barrier filter was K510.

Growth of Virus:

Plastic petri dishes were seeded with 4×10^5 BT cells contained in 2 mls of growth media. After 24 hours, the dishes were washed twice with PBS and infected with 0.2 ml of 10^{-1} dilution (10^6PFU/ml) of the virus preparation described above). The virus was allowed to adsorb for 1 hour at 37°C, the dishes were washed twice with PBS, and maintenance media added. This was post infection time 0. Samples were harvested at 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, 36 and 48 hours post infection. Samples were removed from incubator and immediately stored at -70°C.

When all samples were harvested, the petri dishes were thawed, and cells were scraped off with rubber policemen, and the samples transferred to test tubes. The samples were frozen and thawed twice (at -70°C and room temperature respectively) to release virus. Log Serial dilutions of samples were made, (all dilutions were made with MEM, and in ice bath) and the virus titer determined by plaque assay. The virus was plaqued on MDBK cells grown in 35 x 10 mm petri dishes.

Results:

The results are shown in Table 1 and in figures 1 to 12 as photomicrographs. Figure 13-I is a graph of IBR infectivity titer.

(1) Specificity of Conjugate:

Uninfected control cells and cells infected with parainfluenza virus or virus of Bovine Virus Diarrhea did not show any characteristic fluorescence (Fig. 1 and 2).

(2) Appearance and Localization of Specific Fluorescence:

Cells harvested at 0 hour post infection (p.i), that is after 1 hour of adsorption, showed a diffuse pale fluorescence of the cytoplasm. This fluorescence was presumed due to virus adsorbed onto the cells. The first specific fluorescence was observed at 2 hour p.i as bright linear area of fluorescence lining the nuclear membrane, and occasionally radiating into the cytoplasm (Fig. 3). Between 5 and 9 hours p.i, nuclear fluorescence was observed. By 5 hours, the nucleus appeared pale greenish colour, and bright spots of apple green fluorescence appeared in the nucleus by 6 and 7 hours p.i. In the 8-9 hour samples, infected cells had granular intranuclear fluorescence, with radiations into the cytoplasm. Between 10 to 12 hours p.i, cytoplasmic fluorescence increased, and the nuclear area was still brightly fluorescent, but it was difficult to determine whether the fluorescence was actually in the nucleoplasm or in the overlapping cytoplasm. (Figs. 4-8). Samples harvested between 16 and 48 hours p.i generally showed bright, often granular cytoplasmic fluorescence, with the nuclear area apparently devoid of fluorescent material. The cells also showed characteristic cytopathic effects--some were elongated and stallate, but most were rounded up and gave the appearance of bunches of grapes. Large fluorescent granules were evident in some nuclear membranes and in the peripheral area of the cytoplasm.

(3) Virus growth:

The titer of virus in samples harvested at various times is shown in Table I and Fig. 13-I. Virus titer began to rise at 12 hours p.i, and rose rapidly to the last sample.

Discussion

The time of appearance and progress of cytoplasmic antigens observed in this study is in agreement with previous reports (2, 11, 20, 35, 39). However, Bindrich (2) observed early intranuclear fluorescence which increased as infection progressed; Jasty et al. (20) reported intranuclear fluorescence that persisted up to 20 hours post infection but other workers (11, 35, 39) did not observe intranuclear fluorescence at any time. Workers with other members of the herpes group have also observed intranuclear fluorescence in infected cells (22, 31, 33). Electron microscopy studies of IBR-virus infected cells (11, 18, 19, 37) as well as herpes simplex infected cell systems (25, 34) have demonstrated viral nucleocapsids inside the nucleus and that the nucleocapsids are assembled in the nucleus and acquire envelopes from the nuclear membranes of infected cells. It appears then that viral antigens must exist in the nucleus. This makes explanation of the contradictory reports on intracellular localization of IBR virus antigens more difficult.

Roane and Roizman (31) suggested that the immunologic specificity of antibodies used in the immunofluorescence studies may be responsible for the observed differences. Antisera made against intact virions may contain antibodies against the outer covering of the virus (i. e. envelope), and very little of antibodies against core nucleocapsids such as found in the nucleus. Roane and Roizman (31, 33) easily demonstrated intranuclear

fluorescence with antisera prepared against 72 hour infected cell stroma rendered free of infectious undenatured virus, but not with antiserum against whole virus. It has also been suggested that viral antigens may change antigenic specificity in moving from cytoplasm to nucleus (and vice versa) (33).

Another explanation that has been offered (39) is that the intranuclear inclusions observed in IBR-Virus infected cells are not rich enough in virus or viral antigens to be detected by immunofluorescence. It was suggested that rapid viral multiplication may result in insufficient accumulation of viral antigens in the nucleus. Lebrun (22) showed that intranuclear antigenic material which is first to appear, gradually decrease until it disappears from the nucleus, while increasing in the cytoplasm. Cells at this stage show typical type A intranuclear inclusions, this inclusion therefore contains little or no virus, represent an "intranuclear scar."

Environmental and culture conditions, strain and dose of virus and cell system used may also influence the production and/or staining of viral antigens.

CONCLUSION

The sequential development of IBR-virus in bovine turbinate cells was studied by immunofluorescence. Specific fluorescence was first observed at 2 hour post-infection, at the perinuclear area. Nuclear fluorescence was observed to first increase and then diminish between 6 and 12 hours post infection. Cytoplasmic fluorescence was brightest between 16 and 24 hours after infection. Infective virus increased rapidly between 12th and 48 hours post infection.

TABLE I: SEQUENTIAL STAINING OF IBRV ANTIGENS IN BT CELLS INFECTED WITH IBR VIRUS

Post Infection Time (Hours)	Virus Titer (pfu/.2ml)	Fluorescence		Remarks
		Nuclear	Cytoplasmic	
Uninfected control	-	-	-	Pale autofluorescence in cytoplasm
PI_3-Infected control	-	-	-	-
IBR: 0	2.4×10^2	-	+-	Pale, diffuse, yellowish green fluorescence
2	0.38×10^2	-	+	Perinuclear fluorescence- Bright ring completely or partially around nuclei
4	1×10^2	(+-)	+	Perinuclear fluorescence
5		+	+	Perinuclear, pale amorphous nuclear, and granular cytoplasmic fluorescence
6	$.4 \times 10^2$	++		Most cells had pale green, amorphous, occasionally granular fluorescent material in the nuclei
7		+	++	Bright, granular cytoplasmic, some cells have gramular nuclear fluorescence
8	$.3 \times 10^2$	++	++	Bright intranuclear fluorescence
9		+	+++	Fluorescence is generally brighter. Large granules appear in cytoplasm
10	$.7 \times 10^2$	(+?)	+++	Diffuse granular cytoplasmic fluorescence. Nuclear area appears bright.
11				Cells appear to string out. Some are rolled up.
12	8.6×10^2		+++	Nuclear area appears paler. Many granules in perinuclear

TABLE I: (continued)

Post Infection Time (Hours)	Virus Titer (pfu/.2ml)	Fluorescence		Remarks
		Nuclear	Cytoplasmic	
16	4.15×10^5	-	++++	Very bright fluorescence. Cells are rolled up. Granular cytoplasm.
20	6.6×10^5	-	++++	Same as above.
24	2.1×10^6	-	+++	Rolled up. Cells have "bunch of grapes" appearance. Fluorescent granules appear in intercellular space.
30	7.3×10^6	-	+++	Fluorescent cells appear paler than previous samples.
36	9.8×10^6	-	++	Fluorescence appears diminished.
48	14×10^6	-	++	Pale fluorescence, granules on the nuclear membrane

Samples from 5 hour to 12 hour were fixed in a 2:1 mixture of Acetone and Methanol, at -20°C. The rest were fixed in Acetone at -70°C. The fixation time was 10 minutes in either case.

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PHOTOMICROGRAPHS SHOWING APPEARANCE OF VIRAL ANTIGENS IN BOVINE TURBINATE CELLS INFECTED WITH IBR VIRUS, AND STAINED BY THE FLUORESCENT ANTIBODY TECHNIQUE. (ALL PHOTOGRAPHS WERE TAKEN AT 250 x MAGNIFICATION, UNLESS OTHERWISE INDICATED).

Fig. 1. Uninfected Control Cells - No Fluorescence.

Fig. 2. Infected Cells - Cells infected with parainfluenza type 3 virus. Absence of fluorescence confirms specificity of conjugate for IBR viral antigens.

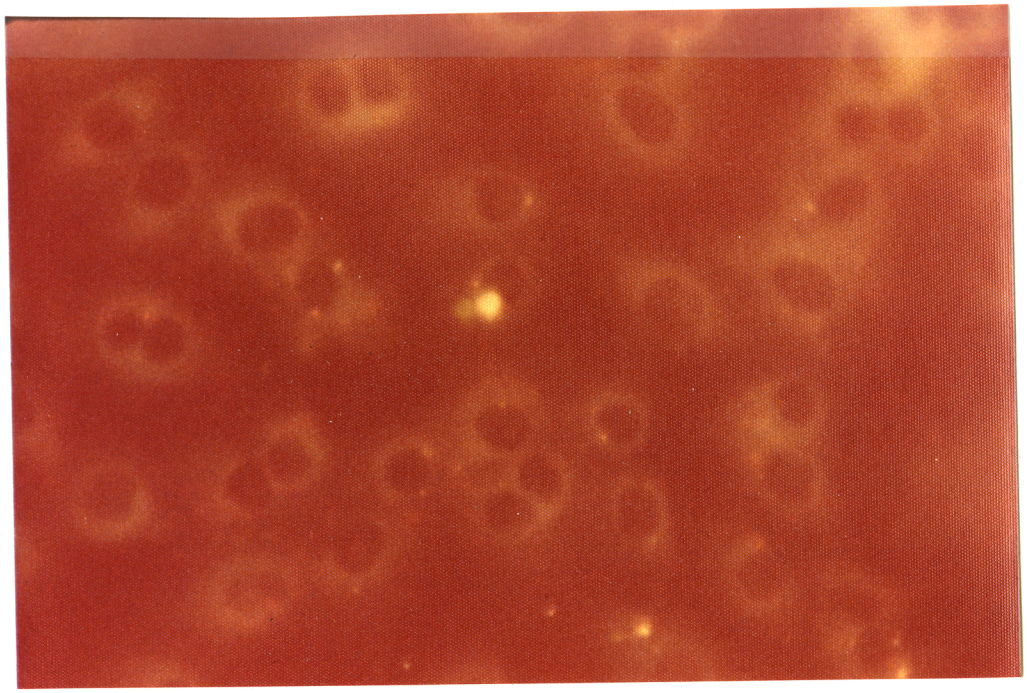


Fig 1

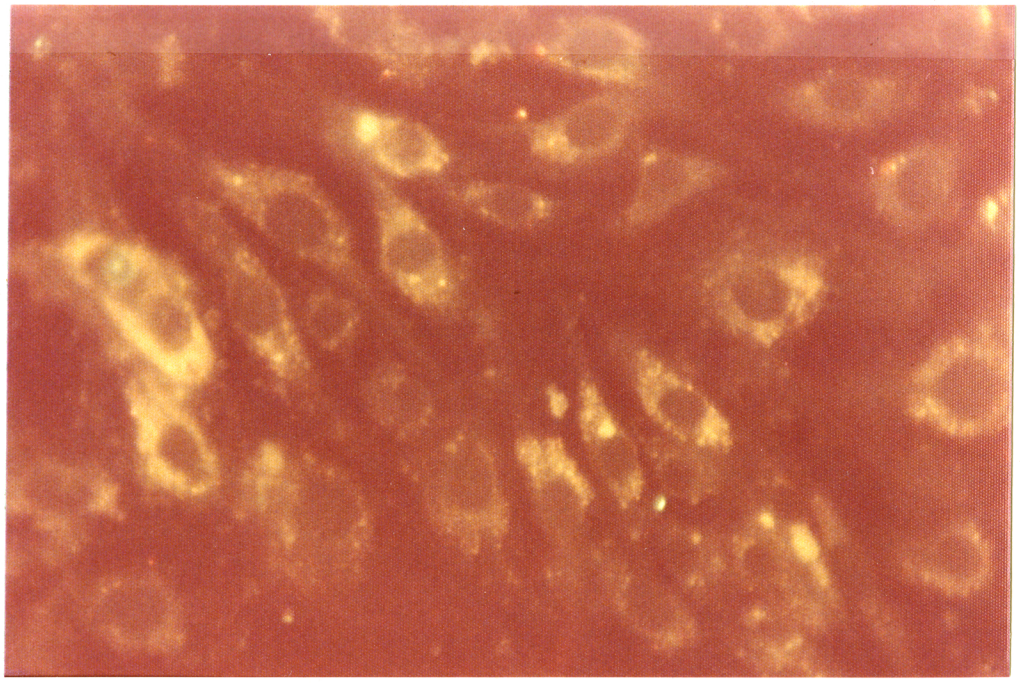


Fig 2

Fig. 3. IBR virus, 2 hours post infection (p.i.). Perinuclear cytoplasmic fluorescence.

Fig. 4. IBR virus, 6 hours p.i. - Intranuclear fluorescence.

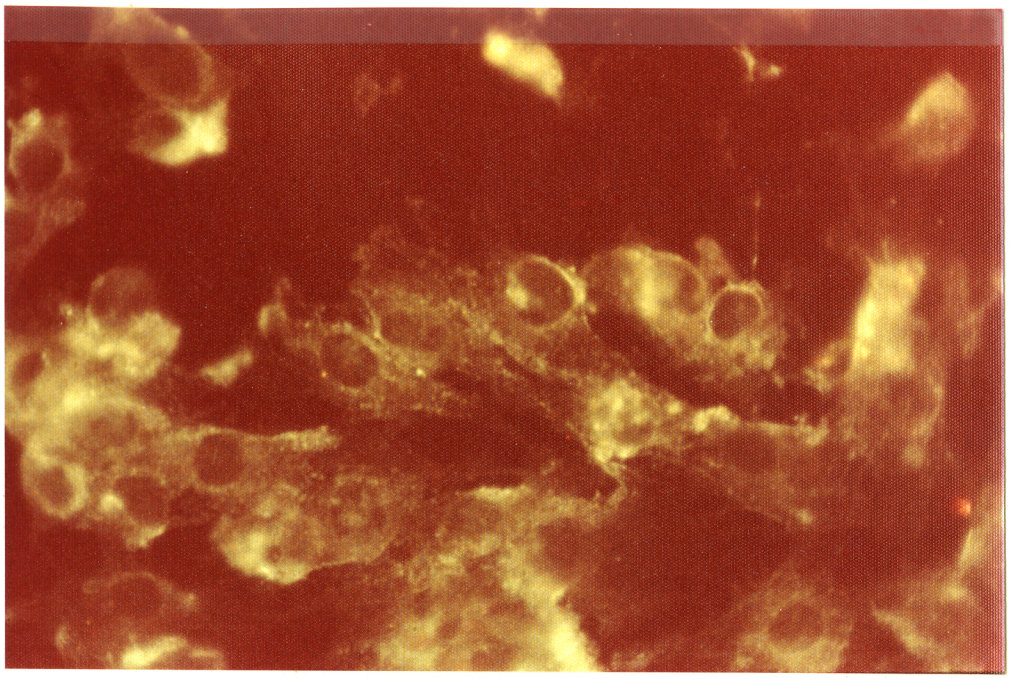


Fig 3

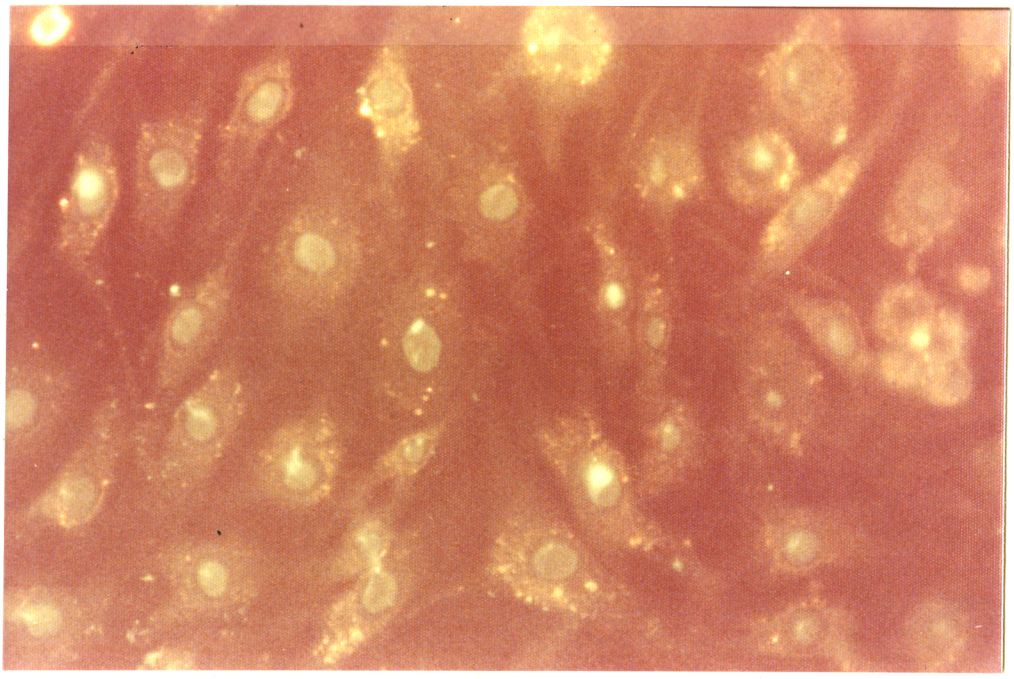


Fig 4

Fig. 5. IBR virus, 6 hours p.i. - Intranuclear fluorescence.

Fig. 6. IBR virus, 7 hours p.i. - Bright Nuclear fluorescence. Some cytoplasmic fluorescence is also evident.

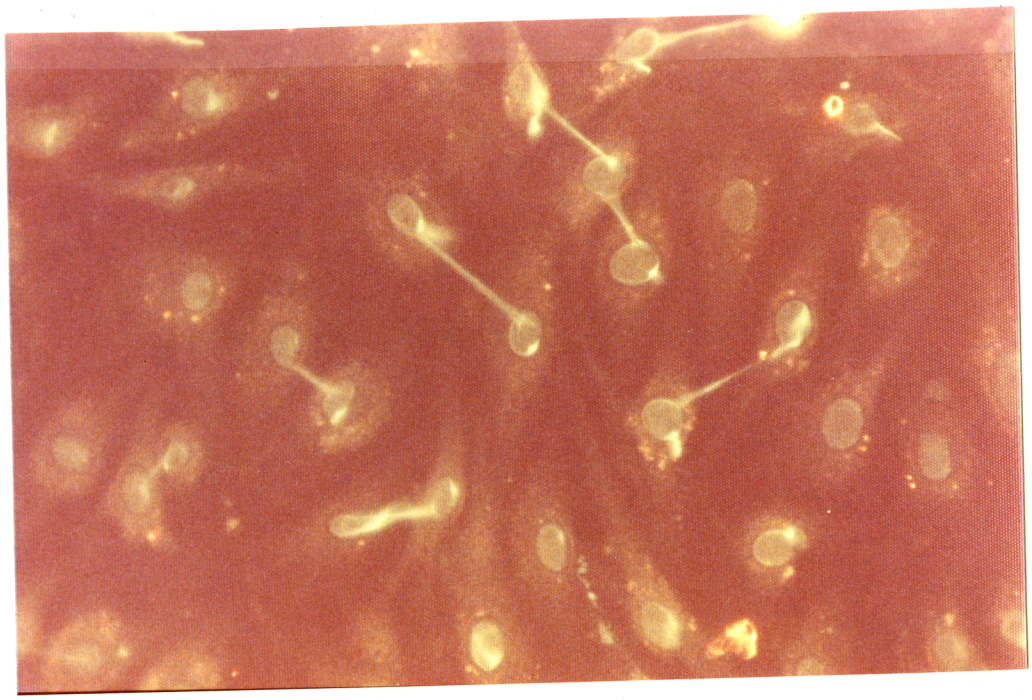


Fig 5

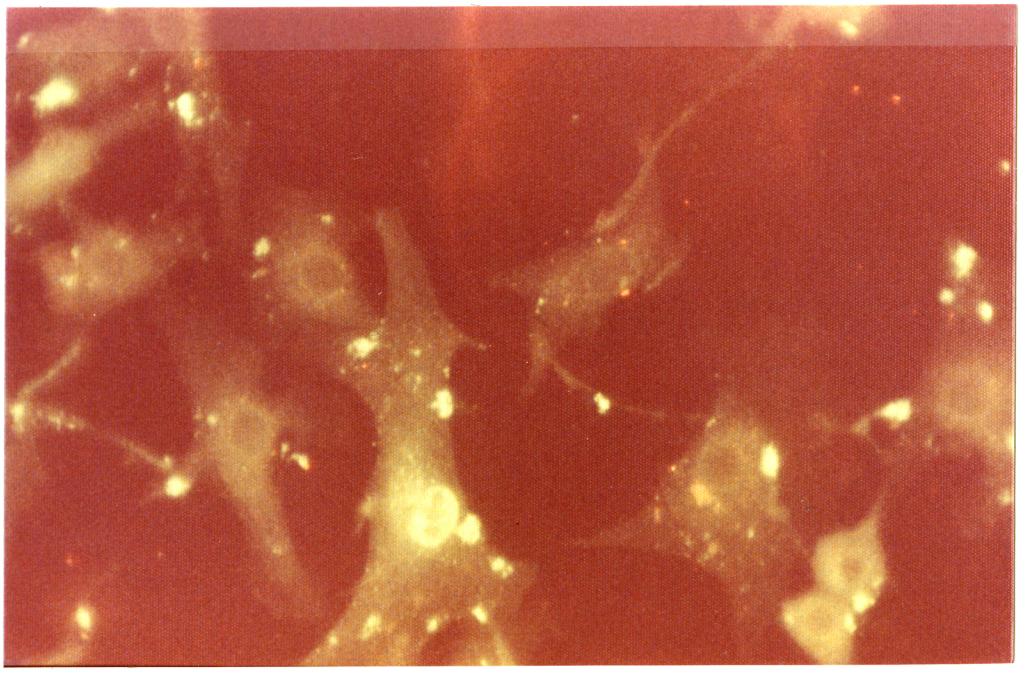


Fig 6

Fig. 7. IBR virus, 8 hour p.i. - Bright nuclear fluorescence, cytoplasmic fluorescence is increasing.

Fig. 8. IBR virus, 12 hour p.i. - Entire cells are fluorescent.

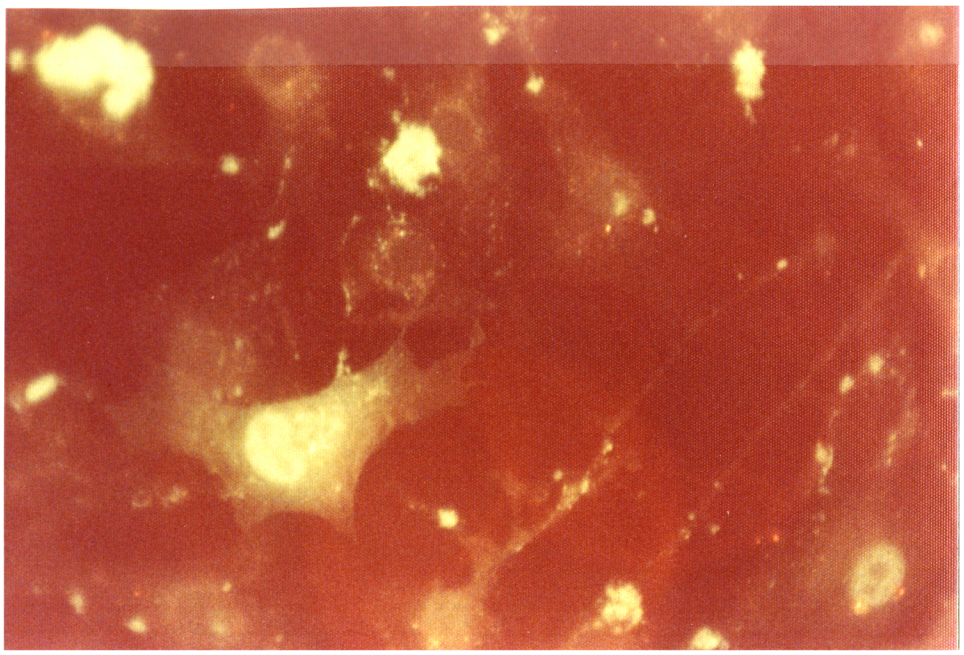


Fig. 7

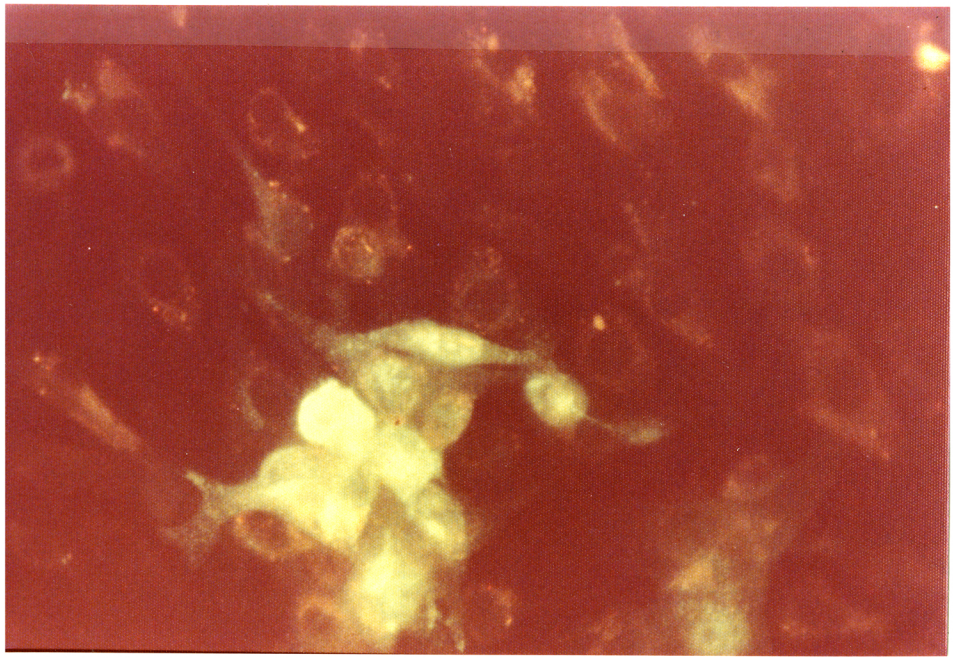


Fig 8

Fig. 9. IBR virus, 16 hour p.i. - Nuclear area appears free of fluorescence.

Fig. 10. IBR virus, 20 hour p.i. - Granular cytoplasmic fluorescence. Fluorescent granules also appear in the intercellular spaces.

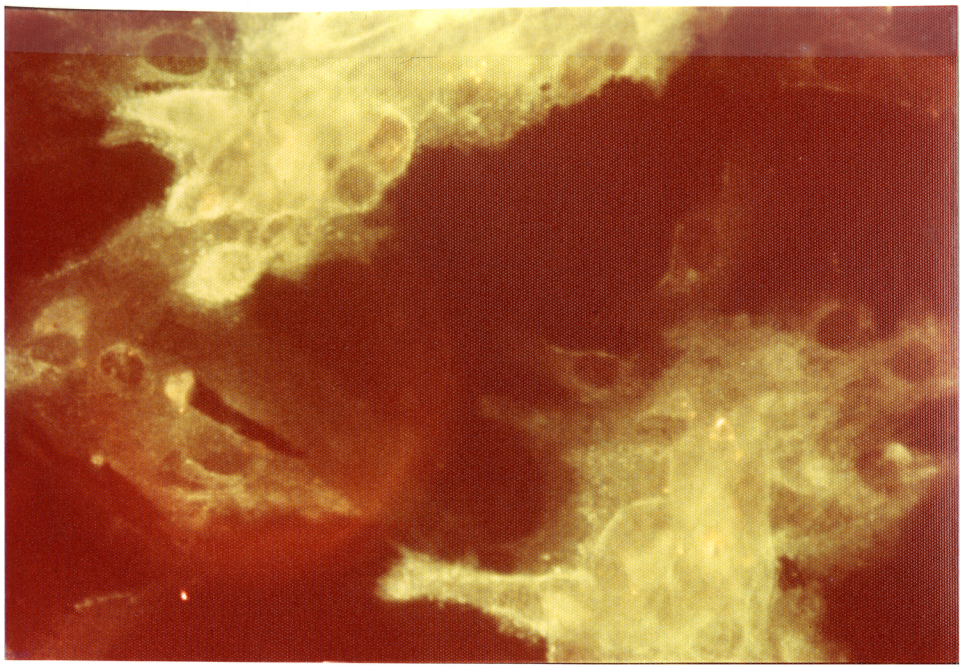


Fig 9

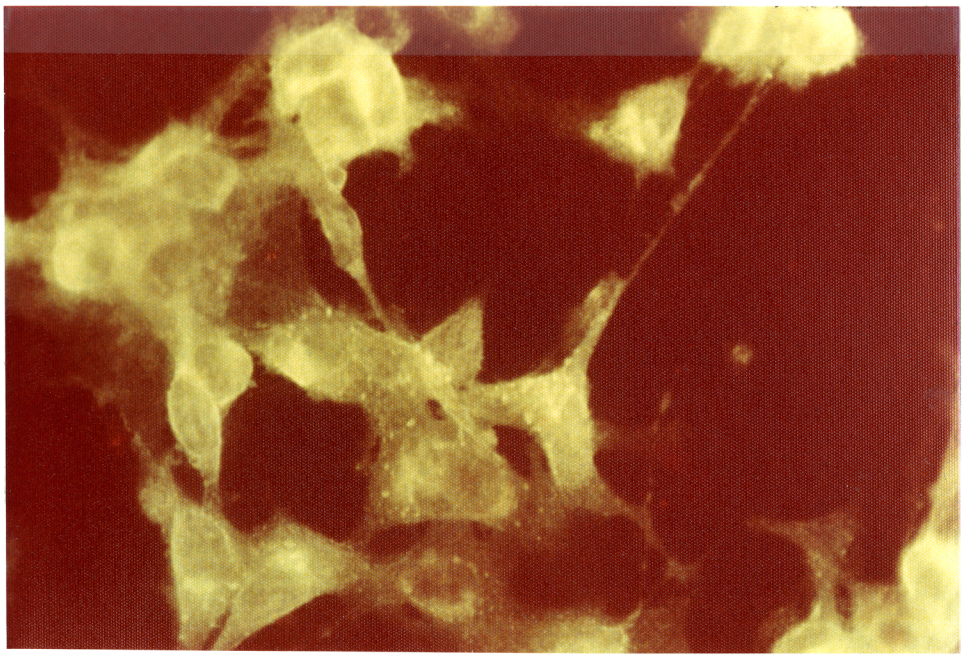


Fig 10

Fig. 11. IBR virus, 24 hour p.i. (x100) - Bunch-of-grapes appearance of fluorescent cells. Nuclear area is totally devoid of fluorescence.

Fig. 12. IBR virus, 48 hour p.i. - Granules appear on cell and nuclear membranes. General decline in brightness of fluorescence.

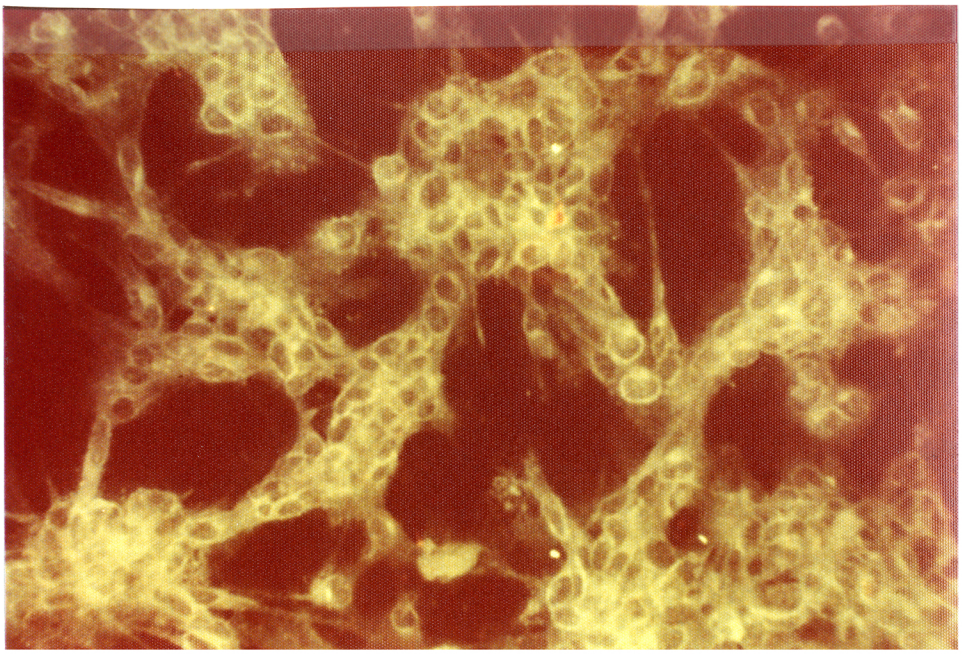


Fig 11

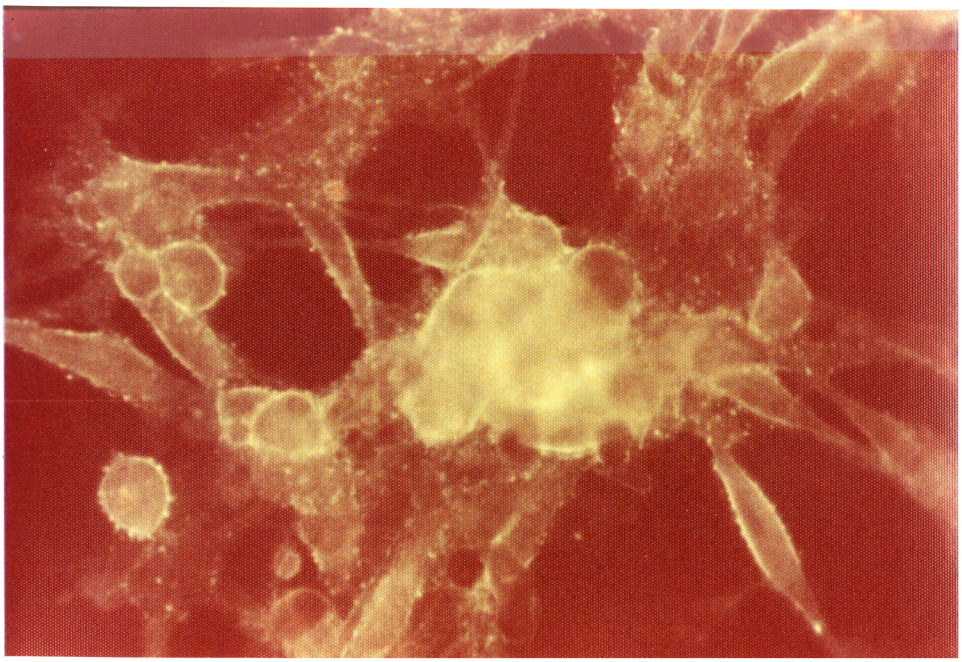
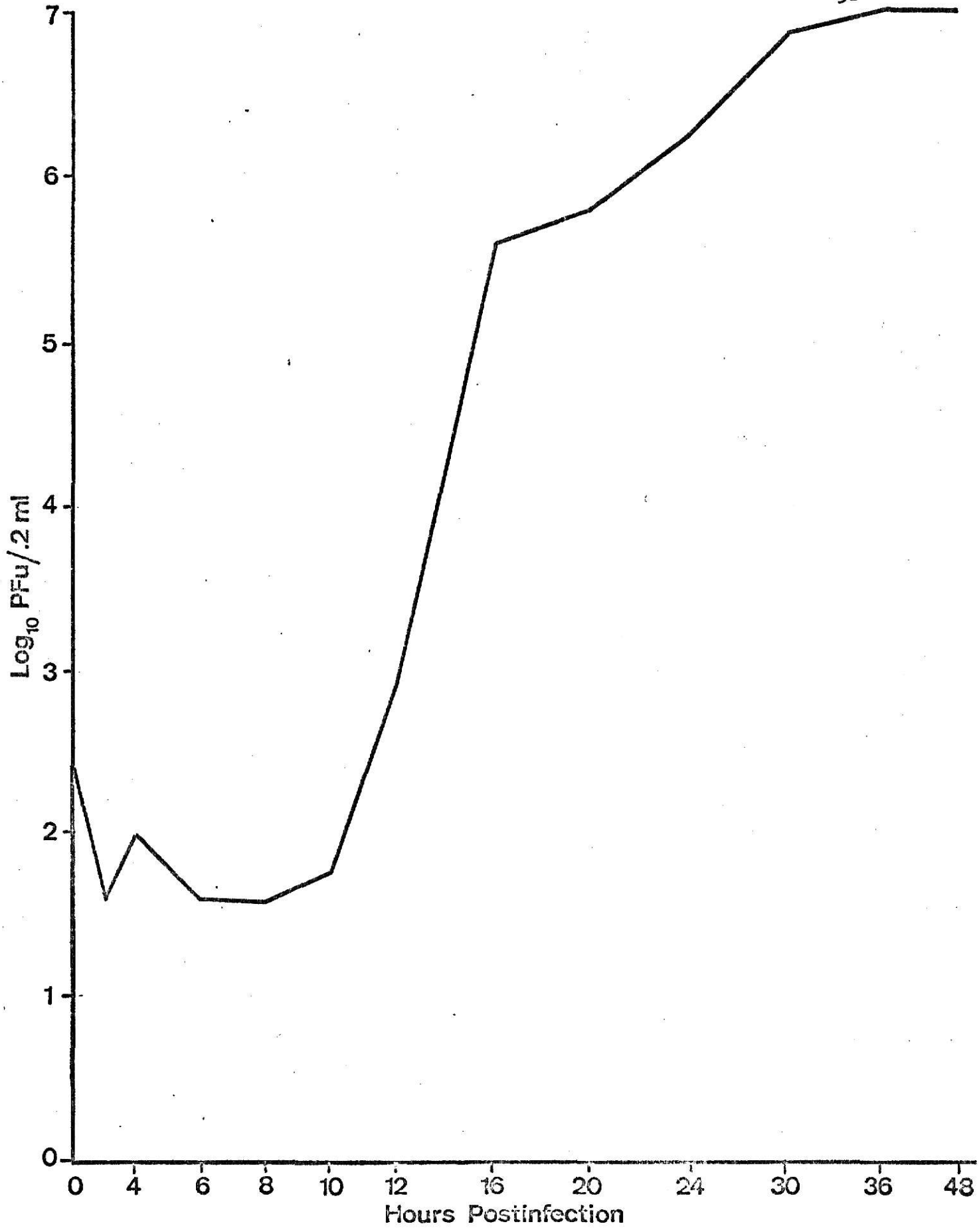


Fig 12

Fig. 13-I. Time course of IBR virus infectivity titer (PFu/.2ml).

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IBR virus infectivity (PFu) in petridish cultures.

Fig.13

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Paper II:

SEQUENTIAL DEVELOPMENT OF BOVINE PARAINFLUENZA TYPE 3 VIRAL ANTIGENS IN BOVINE TURBINATE CELL CULTURES STUDIED BY FLUORESCENT ANTIBODY TECHNIQUE

Summary:

The development of bovine parainfluenza type 3 virus antigens in bovine turbinate cells was studied by immunofluorescence staining. The appearance of specific fluorescence was correlated with appearance of infective virus. Specific fluorescence was first observed in the perinuclear area at four hours post infection. Fluorescence subsequently spread diffusely throughout the cytoplasm. No fluorescence was observed in the nucleus. Infective virus first appeared at 16 hours post infection.

INTRODUCTION

Bovine parainfluenza type 3 virus (PI_3-V) was first isolated in 1955 as shipping fever (SF) virus from cattle afflicted with respiratory disease, and was later identified as a strain of parainfluenze type 3 virus (7, 25). PI_3-V has been isolated from man, cattle, calves, water buffalo, horses and monkeys and aborted fetuses (5, 12, 13, 25). Guinea pigs, hamsters, sheep and swine are susceptible to experimental infection. The bovine strain of the virus is a paramyxovirus closely related but antigenically distinct from the prototype isolate of human origin (7, 25). Serological evidence of infection has been found in man, horses, guinea pigs, swine, deer and sheep. Serological evidence and isolation from widely separated areas indicate that PI_3-Virus is worldwide in distribution (8, 25). Concurrent bovine infection has been found with bovine viral diarrhea-mucosal disease (BVD-MD), Infectious Bovine Rhinotracheitis (IBR), enteroviruses, Pasteurella species, hemolytic cocci, Alcaligenes Spp., Actinobacillus actinoides and Mycoplasma Spp.(18, 19). The virus alone was isolated from 13.2% of 68 sick cattle in the feedlot (25).

Human PI_3-V has been associated with a spectrum of childhood illnesses ranging from pneumonia to afebrile upper respiratory tract illness. In closed populations outbreaks of PI_3-V infection are often explosive and resemble influenza episodes (7). Of all parainfluenza viruses, PI_3-V spreads most rapidly and disseminates most widely among susceptible population. Mufson (14) reported that one out of five uninfected, hospitalized children became infected with PI_3-V. From 70%-90% of human population over six years of age have been infected with the two most prevalent parainfluenza viruses--PI_3-V and PI_4-V (25). Interest in the parainfluenza

group of viruses have also been further inflamed by the recent association of some members of this group with multiple sclerosis (11).

Shipping fever of cattle is a complex disease involving the interaction of viral and bacterial multiplication. The PI_3-V has been regarded as a devitalizing agent which renders the respiratory tissue more susceptible to microbial invasion and multiplication, resulting in pneumonia. Hence the cytopathology of this group of viruses has generated considerable interest.

LITERATURE REVIEW:

The paramyxoviruses are distinguished from the influenza group by their larger size and their ability to cause formation of syncytia in cultured cells. With paramyxoviruses, synthesis of internal antigen is initiated in the cytoplasm in contrast to influenza virus, the soluble antigen of which is first recognized in the nucleus. Both groups mature at the cell surface (6).

Omar (16) conducted a detailed study of the cytopathic effects (CPE) and immunofluorescent pattern of PI_3-V in several tissue culture systems. He reported syncytia formation, spindling and cytoplasmic retraction of single cells, in addition to cytoplasmic and nuclear inclusions as constituting the specific CPE in bovine kidney (BK) cell cultures. In FA studies, he reported cytoplasmic fluorescence observed first at 12 hours after infection. No unequivocal nuclear fluorescence was detected, and he concluded that no apparent correlation occurred between immunofluorescence and cytoplasmic inclusions.

McLean and Doane (13) also reported eosinophilic cytoplasmic inclusions, followed later by eosinophilic nuclear inclusions. By electron

microscopy, they observed aggregates of nucleocapsid-like filaments in both cytoplasm and nucleus. They concluded that bovine PI_3-V differed from other parainfluenza viruses by its ability to produce both nuclear and cytoplasmic inclusions, whereas other parainfluenza viruses characteristically produce cytoplasmic inclusions.

Churchill (3) also demonstrated both nuclear and cytoplasmic inclusions in monkey kidney and Hep-2 cell cultures infected with bovine strain of PI_3-V, but was unable to show similar nuclear inclusions in Hep-2 cells infected with a human strain under the same conditions.

Howe, et al. (6) studied parainfluenza type 2 virus by FA and electron microscopy. With antisera prepared against soluble viral antigens they observed small cytoplasmic fluorescent foci 8 to 10 hours after infection. By 20 to 24 hours, large perinuclear aggregates of fluorescence was observed, and correlated well with eosinophilic inclusions seen in similar preparations stained with azure eosin. However, eosinophilic inclusions were observed much later than fluorescence was detected. With antiserum prepared against concentrated virus, fluorescence was observed at the surface of the infected cells--and indicated sites of virus maturation. No specific nuclear fluorescence or striking tinctorial changes in the nucleus was observed, and syncytia was uncommon.

Sominina et al. (22) in a study of PI_3-V in continuous monkey kidney cells observed a short-lasting nuclear fluorescence 2 to 3 hours post infection. This was followed by accumulation of viral antigens in the cytoplasm. Perinuclear fluorescence was observed 9 to 12 hours post infection. Klein and Collier (9) studied PI_3-V infection of hamster tracheal organ culture and observed bright fluorescence in the epithelial border and in the cytoplasm of many epithelial cells within 24 hours post

infection. Other cellular changes reported were nuclear swelling, chromation migration, cyncytia formation and budding of virions in close association with cilia. Maassab and Loh (12) used monkey kidney cell line and rhodamine isothiocyanate to study PI_3-V, and observed specific viral antigens at 4 hrs. after infection, as fine granules of fluorescence in the cytoplasm, especially in the perinuclear region, of infected cells. Fluorescence increased with time, and by the 12th hour was observed towards the periphery of infected cells. No specific fluorescence in the nuclei, and no evidence of syncytium formation was observed. With acridine orange staining they observed RNA aggregates in the cytoplasm, but no DNA-containing structures were observed in cytoplasm. Tsai and Thompson (23, 24) infected colostrum free and normal calves with PI_3-V and followed its pathogenesis from the trachea to the lungs by several techniques for 5 to 12 days. By electron microscopy of epithelial cells they observed aggregates of virus nucleocapsid in cytoplasm, alterations of cilia and basal bodies, dissolution of cytoplasmic membranes and shedding of virus into luminal spaces. They concluded that epithelial cells of the respiratory tract were the primary target cells for virus replication. In studies with bovine embryonic kidney (BEK) cell cultures, Tsai and Thompson (23) reported intracytoplasmic fluorescence surrounding the nuclei of infected cells by day 2 post infection. From electron microscopy studies they concluded that the first sign of virus development was the appearance of aggregates of viral nucleocapsids in the vicinity of the nucleus. Virus maturation was expressed by budding process through the cell-membrane into oval, round or elongated forms. Viral production from mitotic cells was by a process identical to that in resting cells. They suggested that intranuclear inclusions observed in BEK cells infected with PI_3-V is an

indication of terminal stages of virus infection.

Morphogenesis studies on other viruses of the paramyxoviurs family have been done. Reda, et al. (19) in a study of New Castle disease virus (NDV), observed fluorescence around the nucleus at 3 hours post inoculation in chick embryo cell cultures. Phase contrast microscopy run in parallel suggested that the foci were situated outside the nucleus. However, in another cell system and in later stages of infection, fluorescent foci could be observed in the nucleoplasm. Rapp, et al. (18) studied development of measles virus in human amnion and human epidermoid carcinoma cells. They reported that the fluorescent antigen first appeared either in the perinuclear area or in the nucleus, or both. The fluorescence spread peripherally and finally occurred in the long fusiform or spindle shaped processes that are constant features of measles virus infection. Giant cells were observed, and spread of infection from cell to cell in the monolayer continued even when extracellular virus was inhibited by antibody. Nakai et al. (15) in an electron microscopy study of the same virus, observed viral nucleoprotein tubules first in the cytoplasm of infected cells, and soon after observed similar tubules in the nucleus. No evidence to show that tubules migrated from one site to the other was obtained. Cytoplasmic tubules was associated with active budding and high yields of virus, whereas when intranuclear tubule formation was predominant, little or no budding was detected and viral yields were low. Sattar and Mitchell (20) utilized immune lactoglobulins produced by instillation of PI_2-V into mammary glands of the goat, in the immunofluorescence staining of para-influenza type 2 virus. They observed brightly stained inclusions in the cytoplasm of infected cells. No fluorescence was detected in the nuclei of such cells.

Tsai and Thompson (24) observed that epithelial cells of the respiratory tract were the primary target cells for PI_3-V replication. However, few studies have been reported on respiratory epithelial cells (1, 25). The present study was therefore undertaken to investigate the sequential development of PI_3-V antigens in bovine turbinate (BT) cells by fluorescent antibody (FA) technique.

MATERIALS AND METHODS:

Growth Media: The growth media used was Eagles Minimum Essential Media, with 5% Lactoalbumin hydrolysate, 1% sodium pyruvate, 10% inactivated fetal calf serum and antibiotics (See Appendix).

Petri dish cultures: Polystyrene tissue culture petri dishes (Corning #25000) were seeded with 400,000 bovine turbinate (BT) cells in 2 mls of growth media, and incubated at 37°C in CO_2 incubator till monolayers were formed, (about 48 hrs.). These were used to assay the production of infective virus.

Coverslip cultures: Twenty-two mm square coverslips, acid-washed and autoclaved were placed in 35 mm petri dishes and seeded with 300,000 BT cells in 1½ mls of growth media, and incubated as above for 24 hours.

The coverslips and petri-dish cultures prepared above were infected with 0.2 mls of 10^3 $TCID_{50}$ of a laboratory strain of PI-3 virus obtained from Dr. R. M. Phillips (Diagnostic Laboratory, College of Veterinary Medicine, K.S.U., Manhattan, Kansas). The virus was allowed to adsorb for one hour, each petri-dish was washed twice with PBS and 2 mls of maintenance media was added and incubated at 37°C in a carbon dioxide incubator. This was considered time 0 hour post infection. At previously determined intervals (0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, 36, and 48 hours post infection) one

coverslip was harvested. The coverslip was removed from the incubator, washed twice with Tris-Saline Buffer (TBS), dried at 37°C for 5 to 10 minutes, and fixed in cold acetone (-70°C) for 10 minutes. The fixed slide was air dried and stored at -70°C until stained.

Harvesting of Petri-Dish Cultures: The petri dishes were removed at appropriate intervals and immediately stored at -70°C until further assay.

Staining of Coverslips: Coverslips were taken out of cold storage and allowed to regain room temperature. They were placed in petri-dishes lined with moist filter paper and each coverslip was flooded with TBS for 5 to 10 minutes. The TBS was drained off, and each coverslip was covered with PI3 conjugate, and incubated at 37°C in a CO_2 incubator for 50 minutes. Control uninfected coverslips, and coverslips infected with IBR virus were similarly stained.

After 50 minutes the coverslips were washed twice in TBS, and twice in distilled water, mounted on glass slides with buffered glycerine (9 parts glycerine with 1 part PBS). A Leitz Ortholux Microscope with HBO 200 lamp, BG 38 heat suppression filter, K 510 barrier and KP 490 exciter filters was used to examine the slides. Photomicrographs were taken with a Leica camers, on Kodak EH 135 film (high speed Ektachrome film, processed at ASA 400).

Virus Assay: The petri-dish cultures were frozen and thawed twice (at -70°C and room temperature respectively) to break open the cells. Infectious virus titer was determined by the microtiter and plaque assay technique as described in Appendix A.

RESULTS

Localization of Viral Antigens

Table II summerizes the sequence of appearance and localization of viral antigens as identified by FA staining. Uninfected cells, and cells infected with IBR virus showed no fluorescence or little, grey-green autofluorescence (Fig. 1). At 4 hours post infection, cells with areas of bright granules of apple-green fluorescence skirting parts of the nuclear membrane and radiating into the cytoplasm were observed (Fig. 3). By 10-20 hours post infection the perinuclear fluorescence became very bright and spread throughout the cytoplasm. Large granules of fluorescent material was seen at the periphery of the cells (Fig. 4-6). By 24-30 hours, the granules had coalesced into large clumps of bright fluorescence, granules were seen in the intercellular spaces, and it appeared that new cells were being infected (Fig. 7-8). Between 36 and 48 hrs. the cytoplasmic fluorescence appeared to be fading (Fig. 9-11). No definite fluorescence was observed in the nucleus at any time.

Early in the infectious process, infected cells maintained their normal morphology. A few cells were seen in mitosis, though the cytoplasm was filled with fluorescent material. As infection progressed the cells became elongated and often stellate, and a few became rounded up. From 30 hour post infection on, syncytia formation was a common observation. Infective virus was first detected at 12 hour p.i. and increased rapidly after (Table II).

DISCUSSION

The time of appearance and perinuclear localization of first fluorescein-staining antigens, the absence of intranuclear fluorescence

and the syncytia formation observed in BT cells are in general agreement with other published reports in other cell culture systems (9, 12, 16, 22). The exclusively cytoplasmic immunofluorescent pattern observed here is in agreement with that seen in related viruses--Sendai, New Castle disease and mumps virus (16, 19) and parainfluenza type 2 (20). Measles and canine distemper viruses will induce nuclear fluorescence as well as cytoplasmic fluorescence (16, 18). Otherwise, in the myxovirus group, a cytoplasmic immunofluorescence which is preceeded by a nuclear fluorescence is a property of influenza virus (16).

Some investigators (3, 13, 16) have reported intracytoplasmic and intranuclear eosinophilic inclusions in cell cultures infected with PI_3-V. These inclusions are a constant feature of all the strains of bovine PI-3V thus far isolated, except the Sweedish strain (16). McLean and Doane (13) saw aggregates of nucleocapsid-like filaments in cytoplasm and nuclei of cells infected with PI-3V, and concluded that these aggregates correspond to the eosinophilic inclusions shown by light microscope in the same areas. The failure of the fluorescein-labelled antiserum to stain these nucleocapsids may be compatible with the manner in which the antiserum was produced. Antiserum produced against whole virus would contain antibodies against the outer components of the virus--the hemagglutinins and other envelope components. It is probable that these antigens, rather than viral nucleocapsids were stained by the labelled antiserum in this study.

TABLE II: SEQUENTIAL DEVELOPMENT OF PI_3-V ANTIGENS IN BT CELLS INFECTED WITH PI_3-V.

Time Post Infection	Virus Titre	Fluorescence		Remarks
		Nuclear	Cytoplasmic	
Control		-	-	Autofluorescence
0	-	-	-	Autofluorescence
2	-	-	-	
4	-	-	+	Juxtanuclear fluorescence
10	-	-	+++	Bright clear granular cytoplasmic fluorescence
12	$10^{0.5}$	-	++++	Fluorescence is dispersed all over cytoplasm.
20	$10^{2.5}$	-	++++	Fluorescent material clumping up in the perinuclear area. Large granules at periphery of cells.
24	10^{3}	-	+++	Clumps of fluorescent material appear outside cell. New infection appear in contiguous cells.
30	$10^{3.5}$	-	+++	Syncytia--Cells with multiple nuclei--appeared in infected cells.
36		-	++	One cell with five nuclei
48		-	++	Giant cell--diffuse granular cytoplasmic fluorescence.
60	10^{4}			

IMMUNOFLUORESCENT PHOTOMICROGRAPHS OF BOVINE TURBINATE CELLS INFECTED WITH BOVINE PARAINFLUENZA TYPE 3 VIRUS AND HARVESTED AT VARIOUS TIMES

Fig. 1. Uninfected control cells: grey-green autofluorescence, no specific fluorescence.

Fig. 2. PI_3 virus, 0 hour post infection (p.i.). No specific fluorescence.

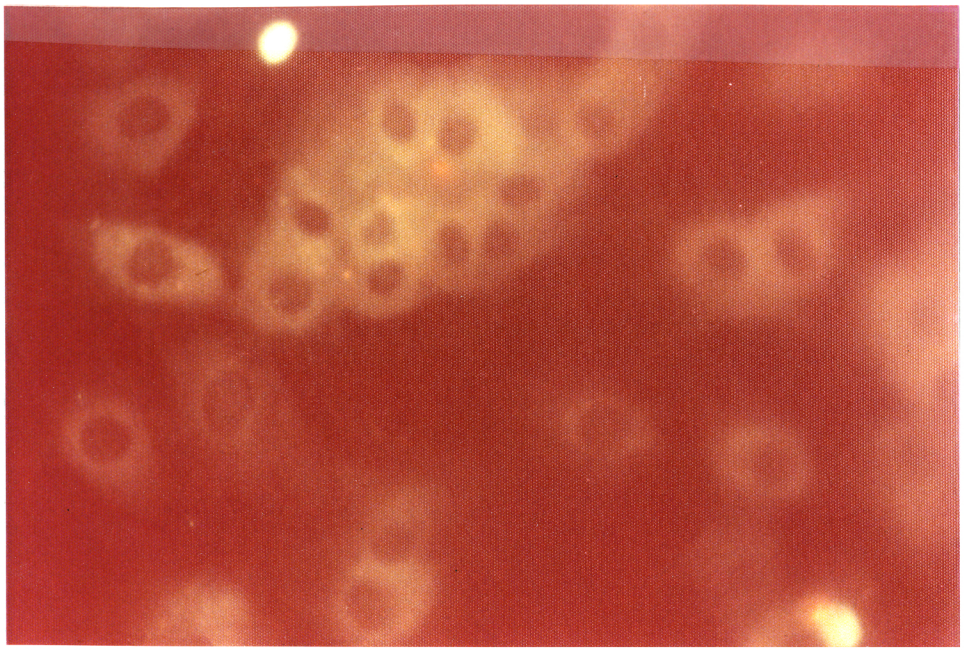


Fig 1

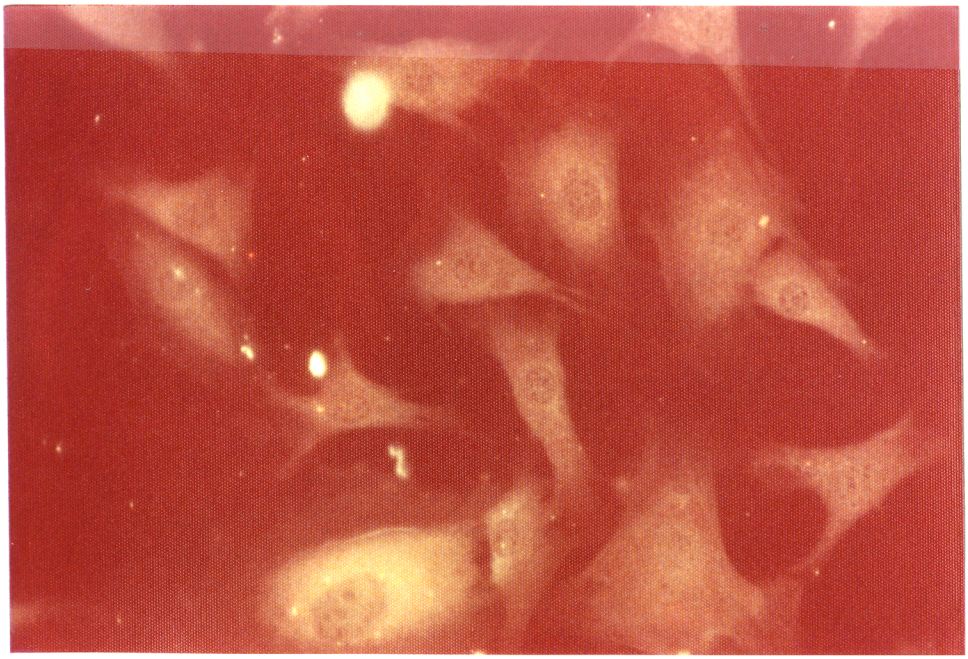


Fig 2

Fig. 3. PI_3 virus, 4 hours p.i. Bright apple-green cytoplasmic fluorescence at the perinuclear region.

Fig. 4. PI_3 virus, 10 hours p.i. Bright cytoplasmic fluorescence. The pale green granules seen in the nuclei of some cells are probably artifacts.

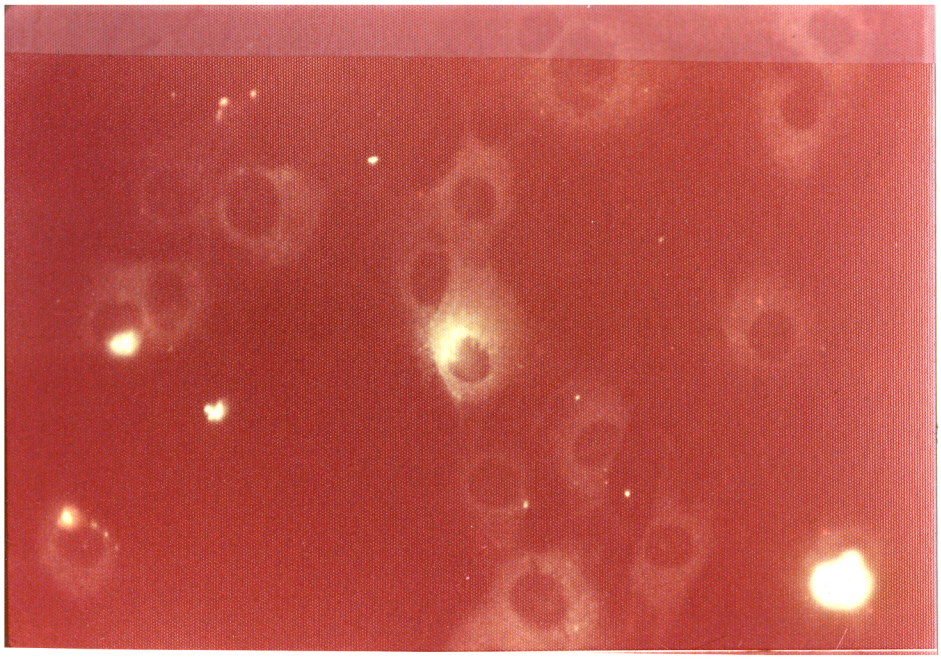


Fig 3

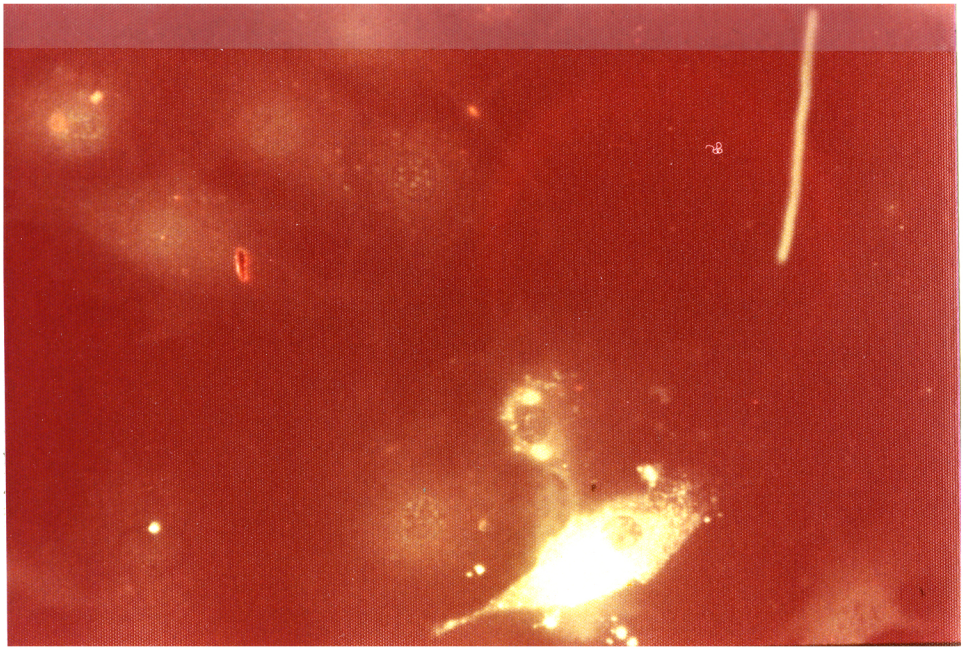


Fig 4

Fig. 5. PI_3 virus, 12 hours p.i. Bright cytoplasmic fluorescence.

Fig. 6. PI_3 virus, 20 hour. Fluorescent granules appear at the periphery of the cells.

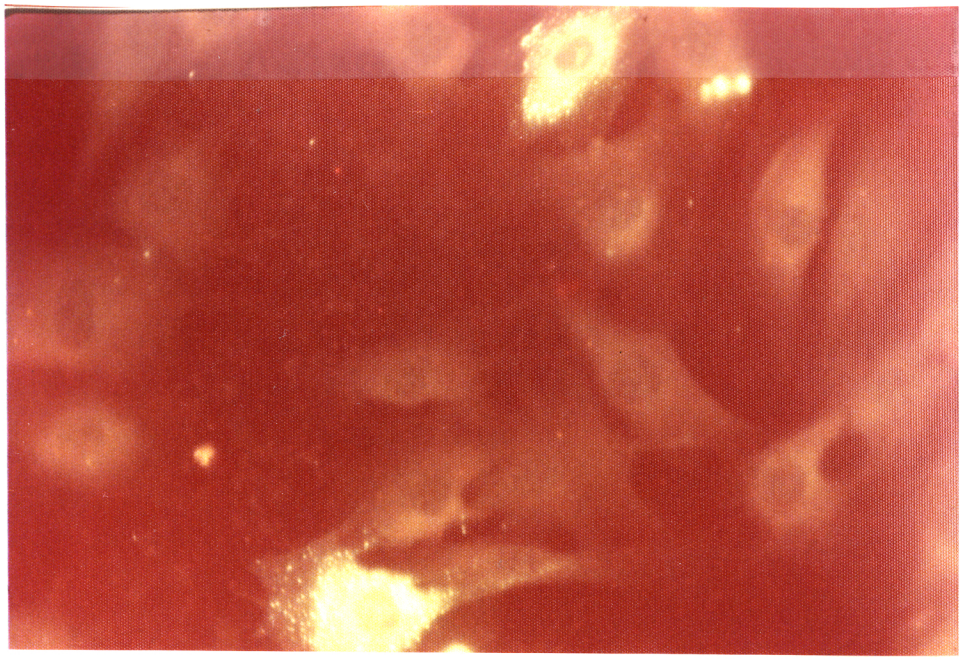


Fig 5

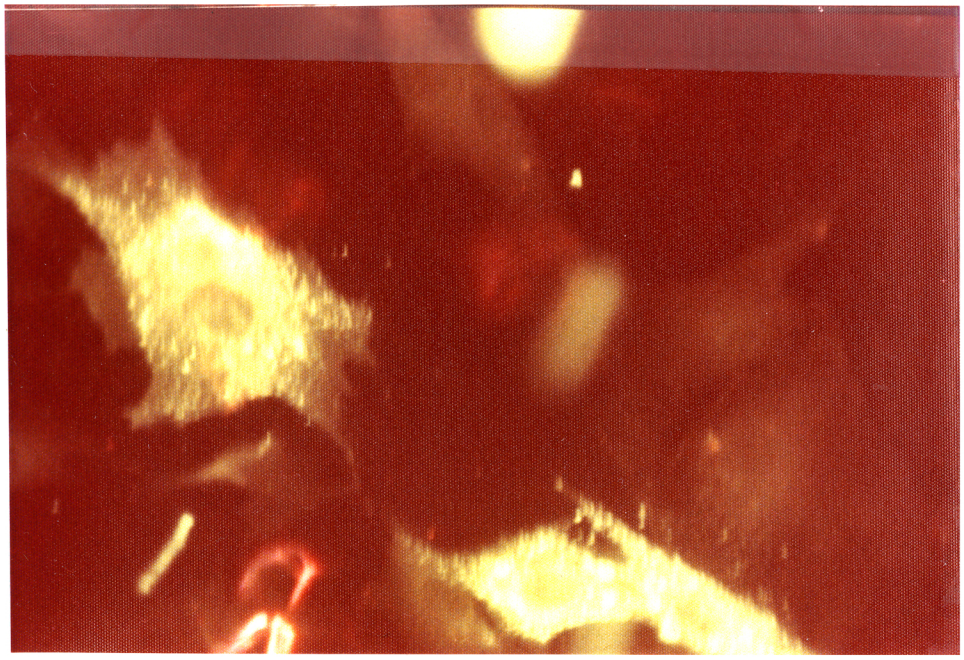


Fig 6

Fig. 7. PI_3 virus, 24 hour p.i. Bright, granular cytoplasmic fluorescence. Stellate appearance of infected cells.

Fig. 8. PI_3 virus, 30 hour p.i. One cell is rounded up. Other contiguous cells show the beginning of infectious process.

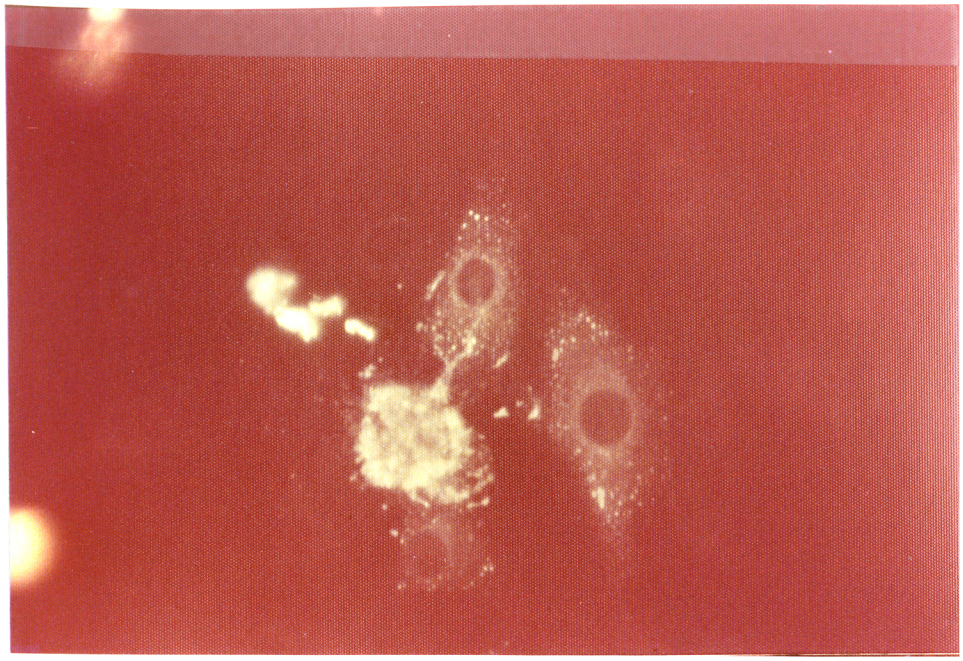


Fig 7

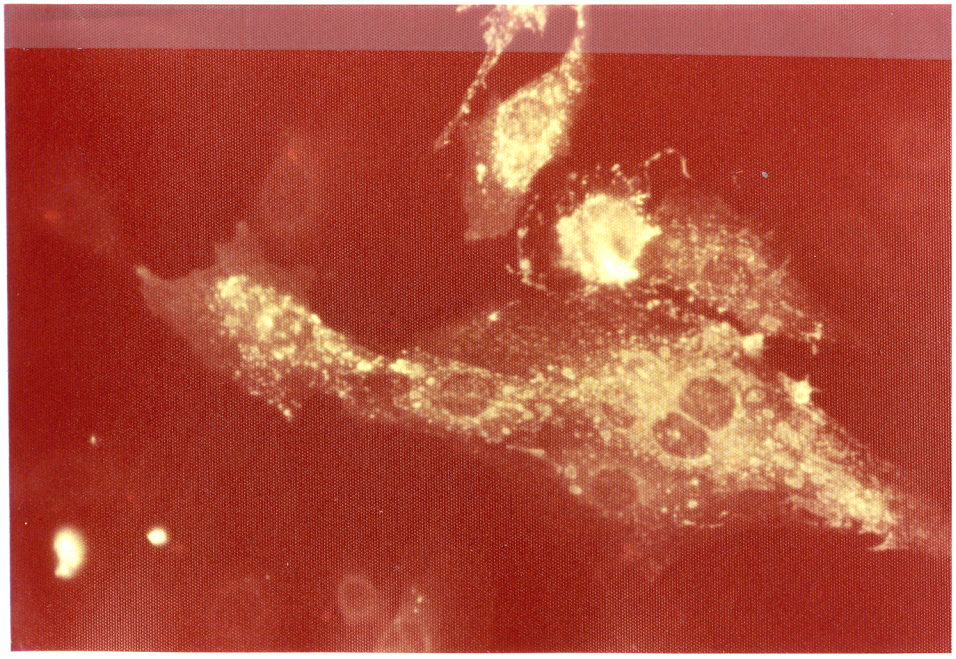


Fig 8

Fig. 9. PI_3 virus, 36 hour p.i. Large clumps of fluorescent material in cytoplasm. Elongated cytoplasmic process.

Fig. 10. PI_3 virus, 36 hour p.i. Granular fluorescence. Syncytia formation as fluorescence appears to fade.

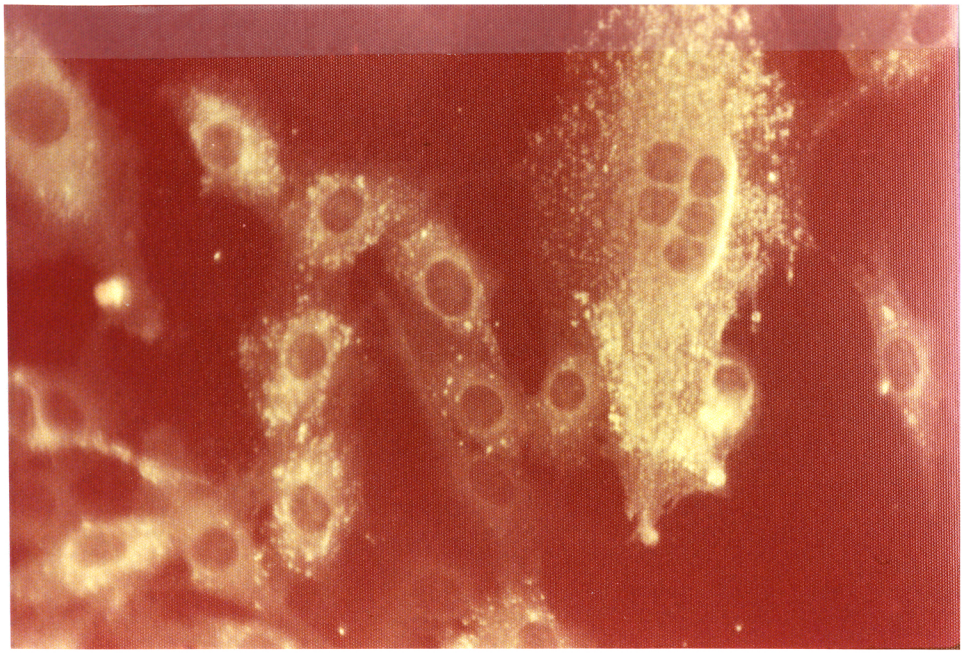


Fig 9

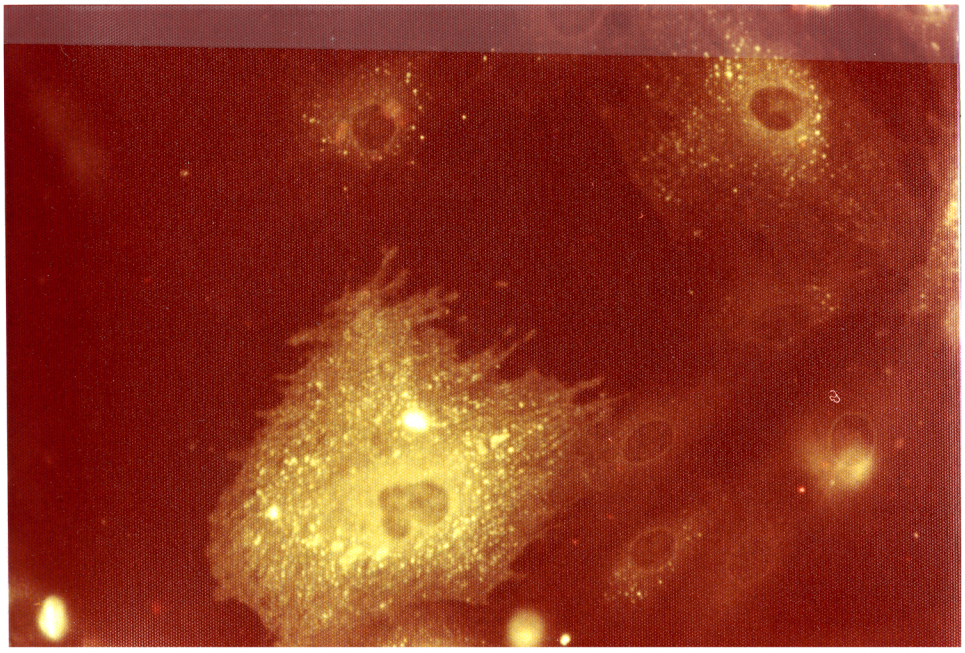


Fig 10

Fig. 11. PI_3 virus, 48 hour p.i. Fluorescence has faded. Large granules at periphery of cells. Syncytia.

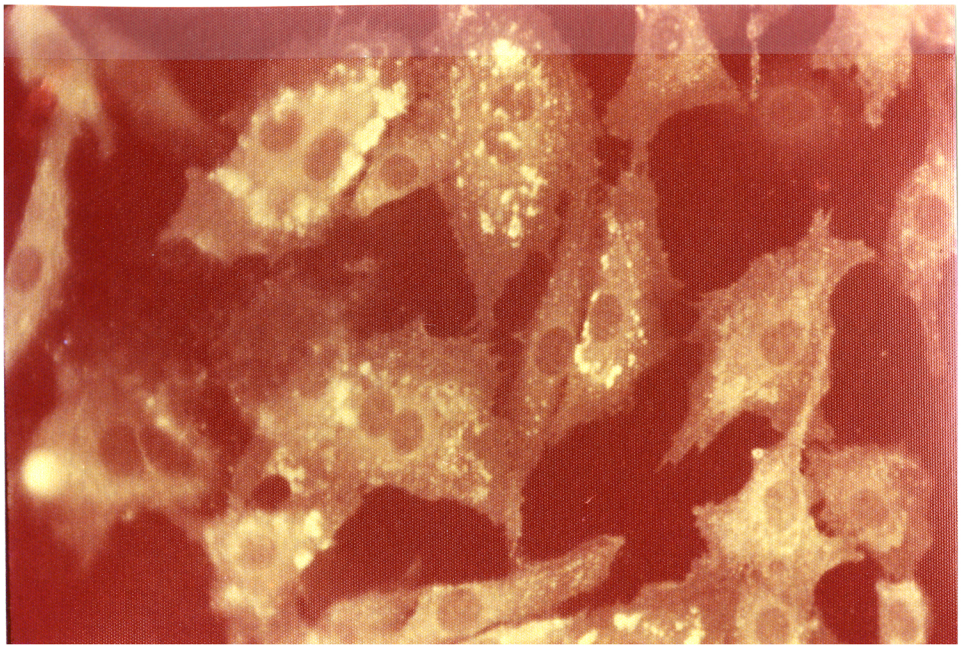
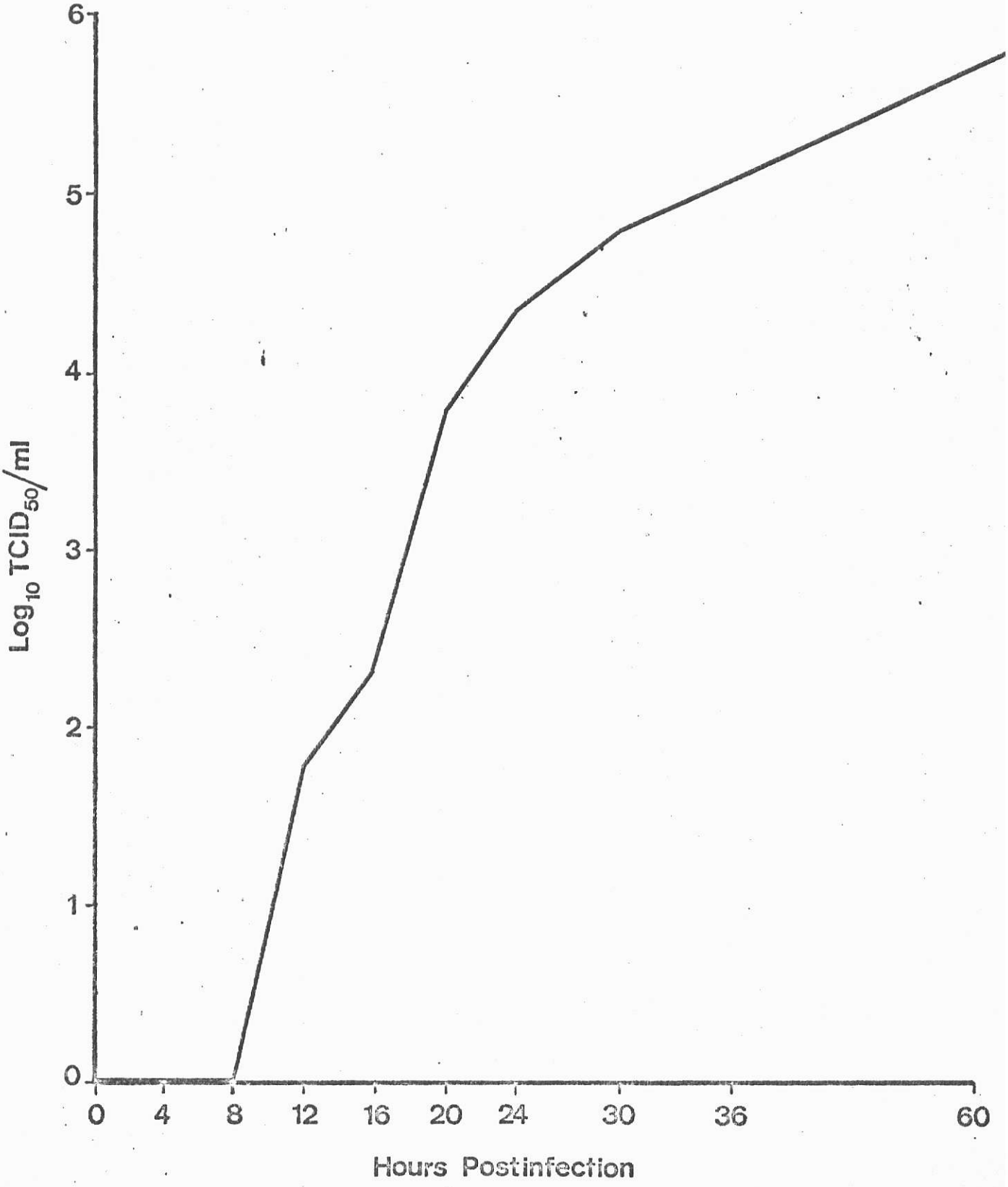


Fig 11

Fig. 12-II. Time course of PI_3 virus infectivity titer ($TCID_{50}$/ml).



PI 3 virus infectivity in petridish cultures.

Fig. 12

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Paper III:

SEQUENTIAL DEVELOPMENT OF BOVINE VIRUS DIARRHEA (BVD) VIRAL ANTIGENS IN CELL CULTURE

Summary

Bovine Embryonic Kidney (BEK) cells infected with NADL strain of Bovine Virus Diarrhea (BVD) virus were harvested at various post infection times and stained with fluorescein-isothiocyanate--conjugated anti-BVD globulins. The sequence of appearance of fluorescent viral antigens was observed. Fluorescence was first observed in the cytoplasm, especially in the perinuclear area and later diffusely throughout the cytoplasm. Fluorescence was not seen in the nucleus. The appearance of fluorescence corresponded approximately with appearance of infective viral particles in the culture.

INTRODUCTION AND REVIEW OF LITERATURE

A new febrile disease of cattle was reported by Olafson, et al. (18) in 1946. They isolated the agent, reproduced the disease experimentally and observed that the disease was a transmissible infection characterized by profuse diarrhea, ulcerations of the mouth which resembled rinderpest, and was transmissible by material which contained no visible organism. They named the disease Virus Diarrhea (VD). A mucosal disease was described by Ramsey and Chivers in Iowa, in 1953, an "X"-disease was reported in Canada in 1946, and an epizootic enteritis occurring in Sweden between 1946 and 1948 was described. All these disease syndromes are related and have a common etiologic agent (15), referred to as Bovine Virus Diarrhea Mucosal Disease (BVD-MD).

Baker et al. (1) isolated the BVD virus from two cows, and used them to study the virus. They gave detailed descriptions of the clinical syndrome caused by BVD virus, and reported on pathologic and serologic studies. They successfully adapted the NY-1 Strain of BVD Virus to rabbit, where the virus showed progressive attenuation, and after 75 rabbit to cow transfers, was so modified in virulence that it could be considered for immunization purposes. Susceptible calves inoculated with the 75th rabbit passage virus showed a slight decrease in leukocyte count and elevated temperatures for one day, and were immunized against fully virulent virus. Fernelius et al. (6, 7, 8) succeeded in adapting a cytopathogenic BVD virus strain (NADL strain) to grow in rabbit, and cell lines. Such rabbit-adapted viruses produced different cytopathic effects in Bovine embryonic kidney cell cultures, as compared with unadapted virus.

Early study of BVD virus was hampered by the fact that the original

isolates (e.g. NY-1, Baker et al. (1)) did not produce cytopathic effects in tissue culture cells. Hence these noncytopathogenic (NCP) strains had to be studied in live cattle. Gillespie et al. (9), in 1960 made a major breakthrough in identification when they isolated a cytopathic (CP) strain from the spleen of a calf that had died of BVD in Oregon. This strain (C24V) has become the prototype virus in many laboratories.

Malmquist (15) has reviewed the etiology and pathogenesis of BVD-MD. He reported that the greatest number of isolations of BVD are from the spleen, blood, or lymph node, although the greatest amount of virus was found in the mucous membranes of the head and intestinal tract, followed by respiratory tract, with the lowest in pools of liver, spleen and kidney.

BVD virus is classified as a togavirus (22), a family taken from the Arbovirus group. Other members of this family include equine arteritis virus, Western, Eastern and Venezuelan encephalomyelitis virus, and hog cholera virus. In addition to the classic febrile disease (diarrhea and mucosal ulceration of cattle) described by Olafson, (18) , Baker, et al. (1) and Ramsey, (15) BVD-MD virus has been associated with lethal and teratologic effects on bovine fetus, including abortion, cerebellar hypoplasia, ocular defects and possibly mummifications (Scott, et al.) (19). The disease is widespread, since over 50% of adult cattle appear to have neutralizing antibody to BVD (15). The disease occurs most commonly as a subclinical infection. In a closed herd of 160 cattle at the National Animal Diseases Laboratory (NADL), there has been only one clinical case of BVD-MD on record, yet about 60% of the animals have serum titers against BVD-virus, a figure that is very close to the incidence reported for most areas of the country (15).

It is possible that a calf may be infected with BVD during intrauterine life (14). Gillespie et al. (11) have isolated a noncytopathic virus, and Scott, et al. (21) isolated a cytopathic strain of BVD virus from bovine fetuses, as well as from maternal and fetal placenta. Some workers (15) have demonstrated BVD virus in fetal calf serum, frequently enough that they assume that all pooled lots of fetal calf serum have the virus.

The early non-cytopathogenic (NCP) strains had to be identified and titrated in live calves. Gillespie et al. (10a) described a method for identification of NCP strains of BVD. This was a plaque inhibition assay method (or interference test), and was based on the principle that tissue culture cells become resistant to a plaque forming CP strain of BVD virus if the NCP strain has been inoculated at least 3 days earlier. Fernelius (4) described a method of detecting and titrating NCP strains of BVD virus by immunofluorescent staining. He found that Fluorescein conjugates of serum prepared in calves against a CP or a NCP strain of virus can be used to identify the NCP viral-infected tissue culture cells, and suggested immunofluorescence staining as a diagnostic test as well as for titration of NCP BVD virus. Gillespie, et al. (9) later described a cytopathic (CP) strain, the C24V, isolated from an Oregon calf.

Fernelius (5) reviewed some studies of development of RNA viral antigen in cell cultures by immunofluorescence. He found that some RNA viruses produce antigens that react with fluorescein conjugated antisera in the cytoplasm only. Other RNA viruses produce early cytoplasmic reactive and late intranuclear reacting antigens. Some RNA viruses cause production of FA-reacting antigens first in the nucleus, and later in the cytoplasm, and still others cause development of FA reacting antigen first in the

cytoplasm then in the nucleus, and again in the cytoplasm. Fernelius, (5) in his study on BVD virus grown in bovine embryonic kidney (BEK) cells reported that viral immunofluorescence antigens appeared first in the nucleus, then in the perinuclear and/or juxta nuclear area, spread throughout the cytoplasm, appeared at the periphery of the cell and finally disappeared.

Mayling (16) conducted FA and histologic studies on tissue specimens from 59 cattle affected by BVD virus. Specimens were from both field and experimental cases. Antisera for FA studies was obtained from goats that have been subjected to a prolonged immunization schedule with BVD virus, from goats exposed to a shorter immunization period, and from convalescent cattle. BVD virus was demonstrated by FA in 85.4% of all tissue examined. Intracellular specific fluorescence was cytoplasmic, and the intensity of staining was highest around the nucleus, where granules were frequent.

Mengeling, et al. (15) demonstrated specific antigen in BEK cell cultures infected with C24V or NADL strains of BVD virus, with fluorescein conjugated swine anti-hog cholera and bovine anti-BVD Sera. Specific fluorescence was diffusely distributed throughout the cytoplasm of most infected cells. As further demonstration of antigenic relationship between hog cholera virus and BVD virus they were able to stain antigens in cells infected with hog-cholera virus with anti-BVD (conjugated globulin as well as antihog cholera conjugate.

MATERIALS AND METHODS:

Coverslip Cultures: 22 mm square coverslips, acid washed and autoclaved and placed in 35 x 10 mm plastic petri dishes were seeded with 300,000 bovine embryonic kidney (BEK) cells in 2 mls of growth media.

After 48 hours the growth media was removed, the cells were washed twice with PBS, and inoculated with 0.2 ml of 10^3 $TCID_{50}$ NADL strain of BVD virus. The virus was allowed to adsorb for 1 hour at 37°C, then the cells were washed twice with PBS, and 2 mls of maintenance media was added. The cultures were re-incubated at 37°C. At predetermined intervals (0, 3, 6, 9, 12, 16, 20, 24, 30, 36, 48 hours post infection) coverslips were harvested, washed twice with TBS, and fixed in an acetone-methanol (2:1) mixture at -20°C, for 10 minutes. The slides were stored at -20°C for further use.

Petri dish cultures: 35 x 10 mm petri dishes were seeded with 400,000 BEK cells, and grown to monolayer. They were infected as described above, and at intervals dishes were removed from incubator and frozen at -70°C until further assay.

Staining of Coverslips: The coverslips were allowed to regain room temperature, wetted with TBS, then stained with BVD conjugate for 50 minutes at 37°C in humid chamber. The coverslips were then washed twice each in TBS and distilled water, mounted with buffered glycerine (9 parts glycerine and 1 part PBS) and examined with a Leitz ortholux fluorescent microscope, equipped with an HBO 200 lamp, K510 barrier filter, KP490 exciter filter and BG38 heat suppression filter. Photographs were taken on Kodak high speed Ektachrome film, at ASA 400, with a Leica camera.

Virus Assay: Each petri-dish culture was assayed for virus titer by the microtiter technique, on BT cells. The microtiter plates were read 6 days after inoculation.

RESULTS

Fluorescent Antibody: Uninfected cells and cells infected with another virus (IBR) showed no specific fluorescence when stained with BVD-conjugate. An occasional bright area of fluorescence was observed in the uninfected cell slide, but these were generally located where the cell monolayer had been torn or disrupted, and the fluorescent dye had lodged underneath the cells. These were not considered specific fluorescence.

The first specific fluorescence was observed at 20 hours post infection--as pale but distinct cytoplasmic fluorescence. Between 12 and 20 hours, some fluorescence was observed, but this fluorescence was not clearly distinquishable from the uninfected cell autofluorescence. At 30 hours the perinuclear area of the cytoplasm was brightly fluorescent. By 36 hours, the cytoplasmic fluorescence appeared to be maximal. Large, fluorescent granules of various sizes were observed in some of the cells. These were considered nucleoli, because similar granules were observed in contiguous cells that appeared uninfected, though no such granules were observed in uninfected control cells.

By 48 hours most of the cells showed typical cytopathic effects (CPE)--cells were elongated and stringy, and most of them showed cytoplasmic fluorescence. A few cells with multiple nuclei were observed.

Virus Assay: Infective virus first appeared at 12 hours post infection and increased rather slowly (Fig. 9).

DISCUSSION

Fernelius (5) reported intranuclear fluorescence in BEK cells

infected with BVD virus. He reported nuclear fluorescence at 6 hours but not 12 hours post infection. Earlier, the same author (16) had reported only cytoplasmic fluorescence. In this study, only cytoplasmic fluorescence was observed. No fluorescence was observed between 6 and 12 hours post infection, as reported by Fernelius (5), who observed the possibility that only cells cracked or damaged by the fixing process in -60°C acetone allowed labelled antibodies to enter the nucleus and tag soluble antigen or other virus proteins being synthesized in the nucleus. Our experiments employed milder fixing procedures--a mixture of acetone and methanol (2:1) at -20°C. It is possible that early in the infectious process (between 0-12 hours post infection) synthesis of viral antigens had not proceeded to a level detectable by the technic employed. Viral antigens may be synthesized entirely in the cytoplasm. Antiserum prepared against whole virus particles will contain a high proportion of antibodies against viral structural proteins, and few antibodies against non-structural or soluble antigens (SA). In order to completely understand the sequential development of BVD viruses in cell cultures, it will be necessary to use antisera against pure SA and structural antigens, to map their respective sites of accumulation.

TABLE III: SEQUENTIAL DEVELOPMENT OF BVD ANTIGENS IN CELL CULTURE

Time Post infection (hours	Virus Titre ($TCID_{50}$)	Fluorescence		Remarks
		Nuclear	Cytoplasmic	
Uninfected Control	-	-	-	Pale autofluorescence
IBR-Infected Control	-	-	-	Evidence of CPE, no fluorescence
BVD virus:				
0 hr.	0	-	-	Pale green autofluorescence
3	0	-	-	Pale green autofluorescence
6	0	-	-	Pale green autofluorescence
9	0	-	-	Pale green fluorescence
12	$10^{0.5}$	-	-	Pale green fluorescence
16	$10^{1.5}$	-	-	Pale green fluorescence
20	$10^{1.5}$	-	+	More distinct cytoplasmic apple green fluorescence
24	$10^{1.5}$	-	+	More distinct cytoplasmic apple green fluorescence
30	-	-	++	Bright perinuclear fluorescence
36	10^{2}	?	+++	Bright cytoplasmic fluorescence. Some nuclei show brightly fluorescent bodies that could be nucleoli. Cells show cytopathic effects.
48	10^{3}	-	+++	Most cells are rounded up and show bright fluorescence, apparently intracytoplasmic. Typical stringy cytopathic effects, with some syncytia observed.

PHOTOMICROGRAPHS SHOWING SEQUENCE OF APPEARANCE OF FLUORESCENT VIRAL ANTIGENS IN BOVINE EMBRYONIC KIDNEY CELLS INFECTED WITH BOVINE VIRUS DIARRHEA VIRUS

Fig. 1. Control - Uninfected cells stained with BVD conjugate.

Fig. 2. Control - Cells infected with Infectios Bovine Rhinotracheitis virus and stained with BVD conjugate. Absence of fluorescence indicates the conjugate is specific for BVD viral antigens.

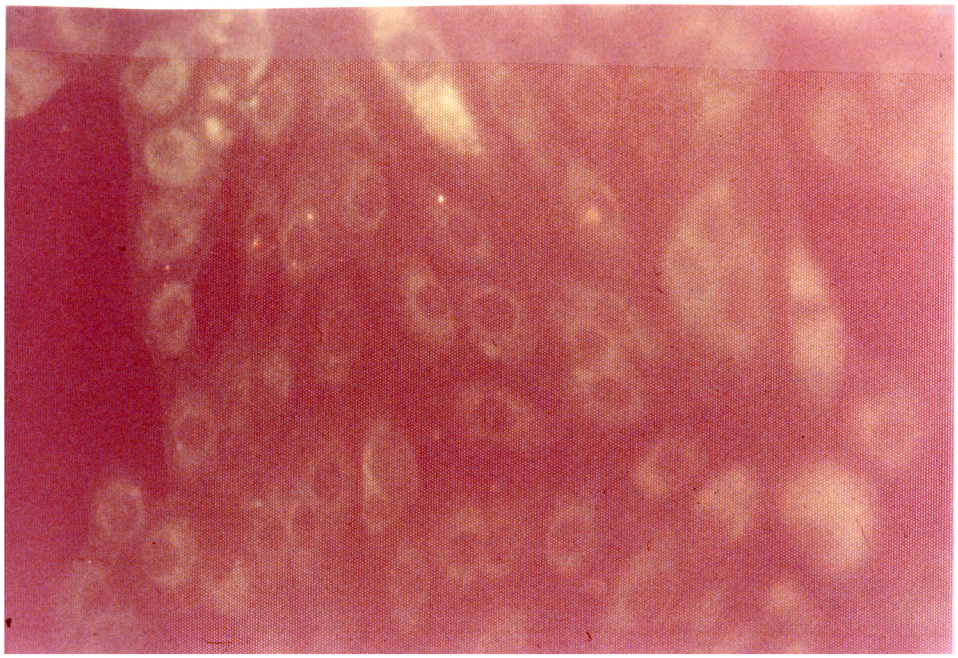


Fig 1

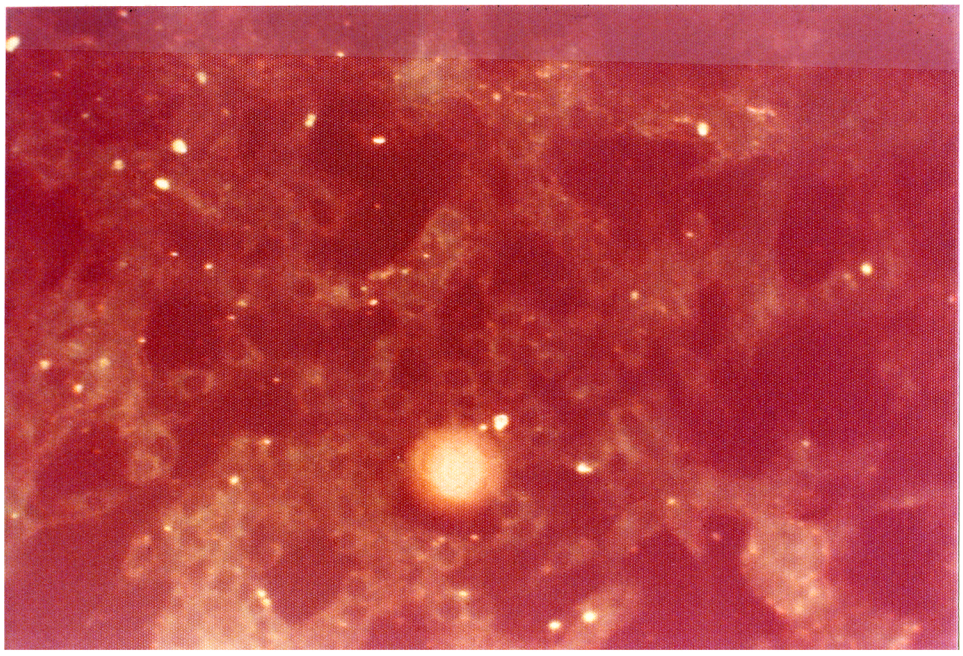


Fig 2

Fig. 3. BVD virus, 16 hours post infection (p.i.). Diffuse cytoplasmic fluorescence.

Fig. 4. BVD virus, 20 hours p.i., cytoplasmic fluorescence.

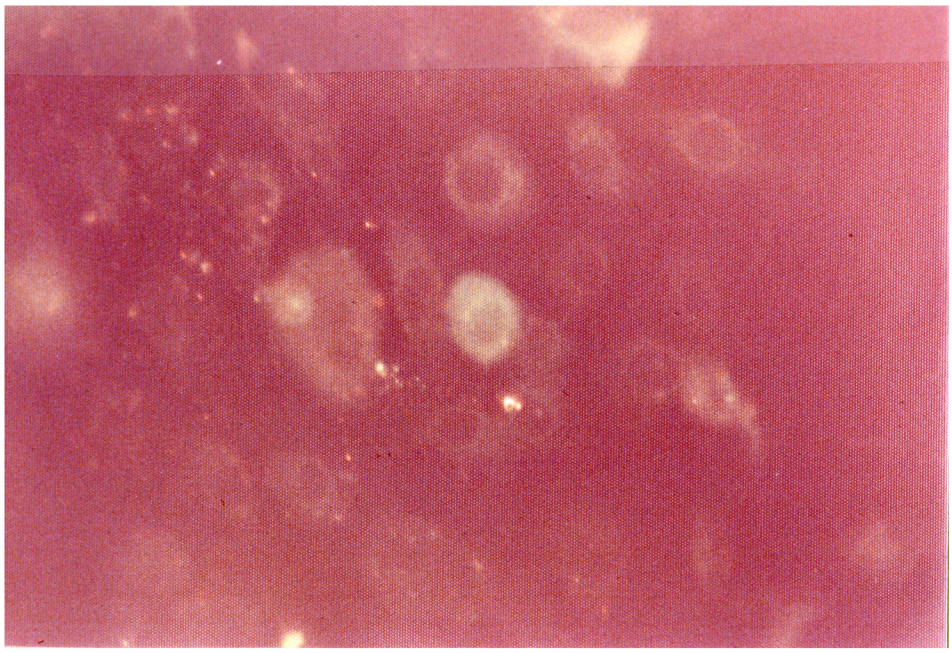


Fig 3

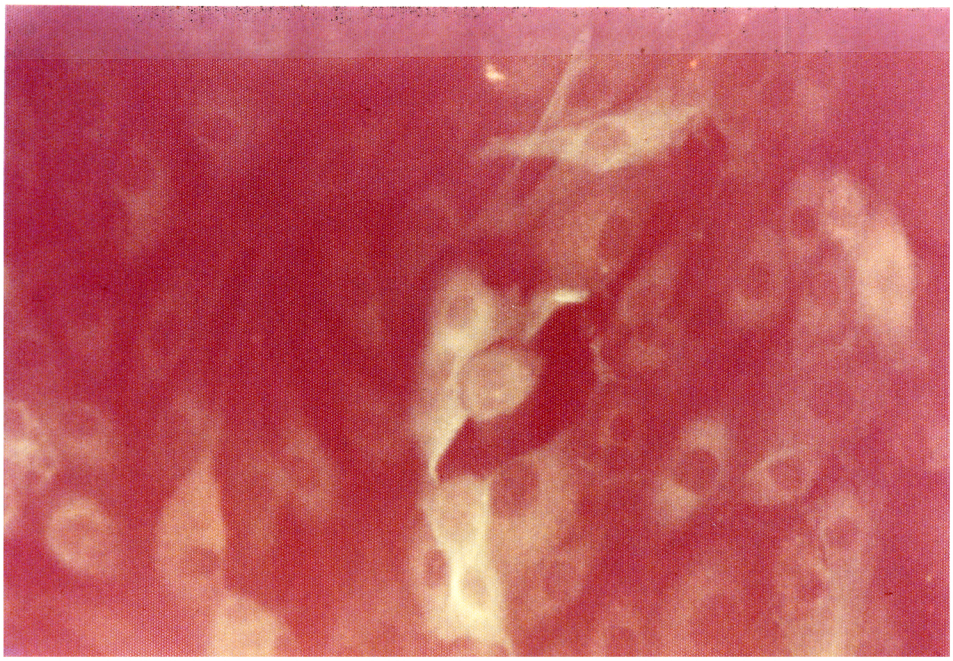


Fig 4

Fig. 5. BVD virus, 24 hours p.i., cytoplasmic fluorescence.

Fig. 6. BVD virus, 36 hours p.i., cytoplasmic fluorescence.

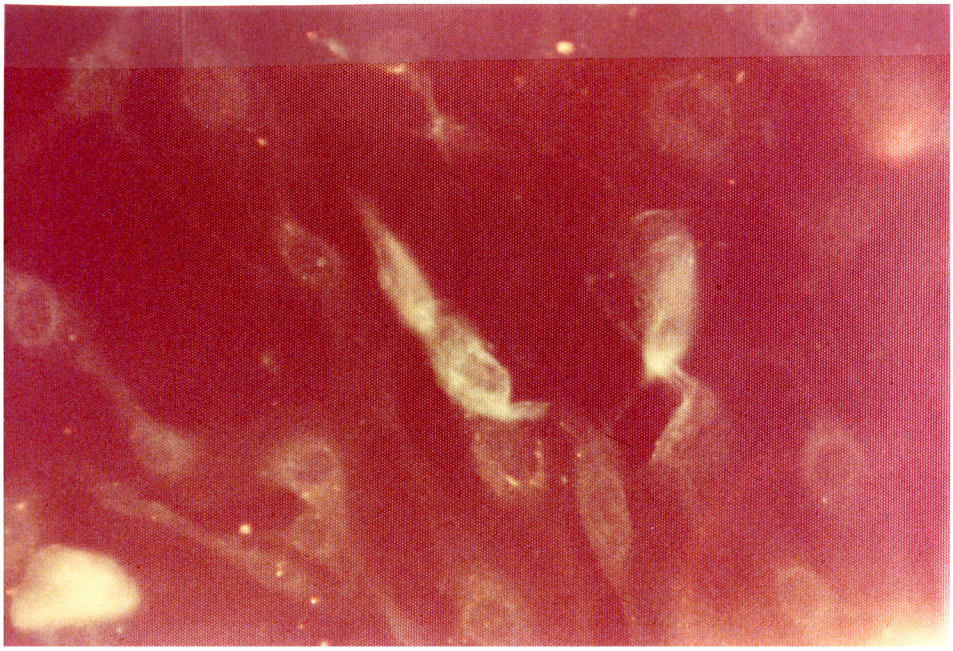


Fig 5

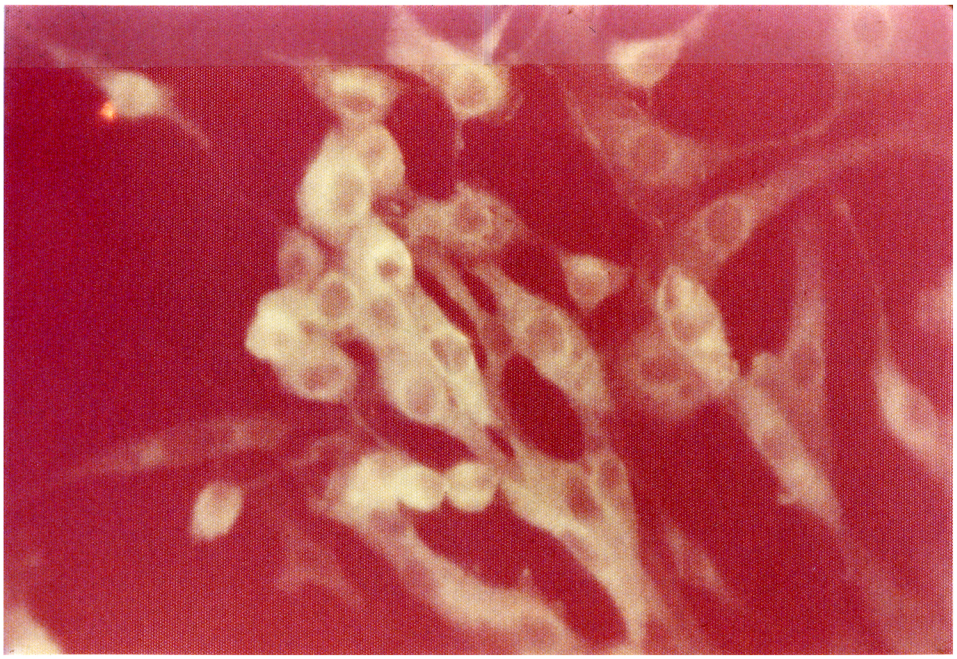


Fig 6

Fig. 7. BVD virus, 36 hours p.i., cytoplasmic fluorescence. Fluorescent granules seen in the nucleus are probably nucleoli, and not specific BVD fluorescence.

Fig. 8. BVD virus, 48 hours p.i., cells show typical cytopathic effect, and cytoplasmic fluorescence.

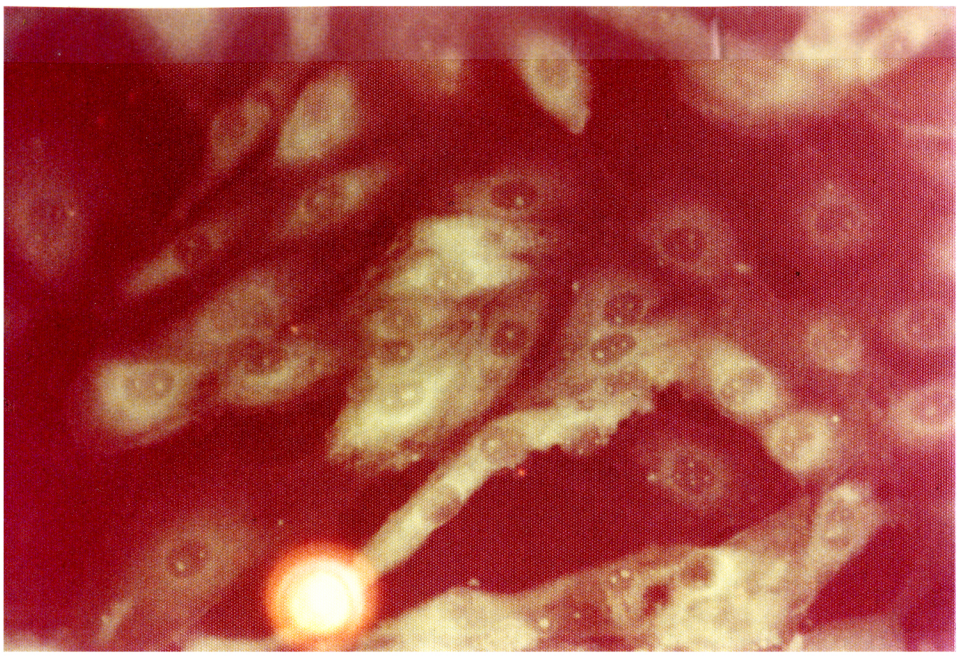


Fig 7

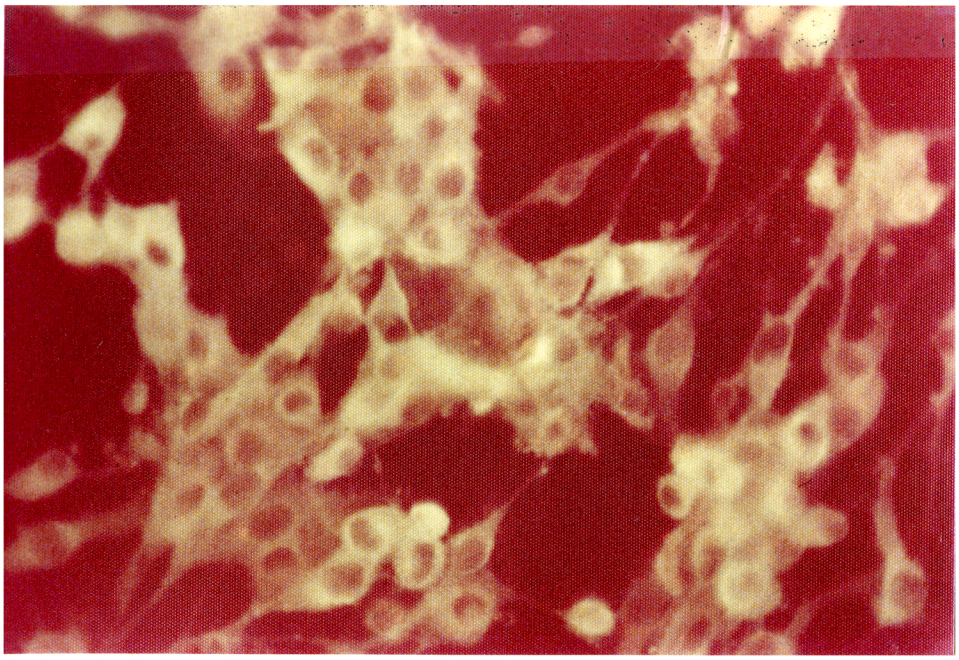
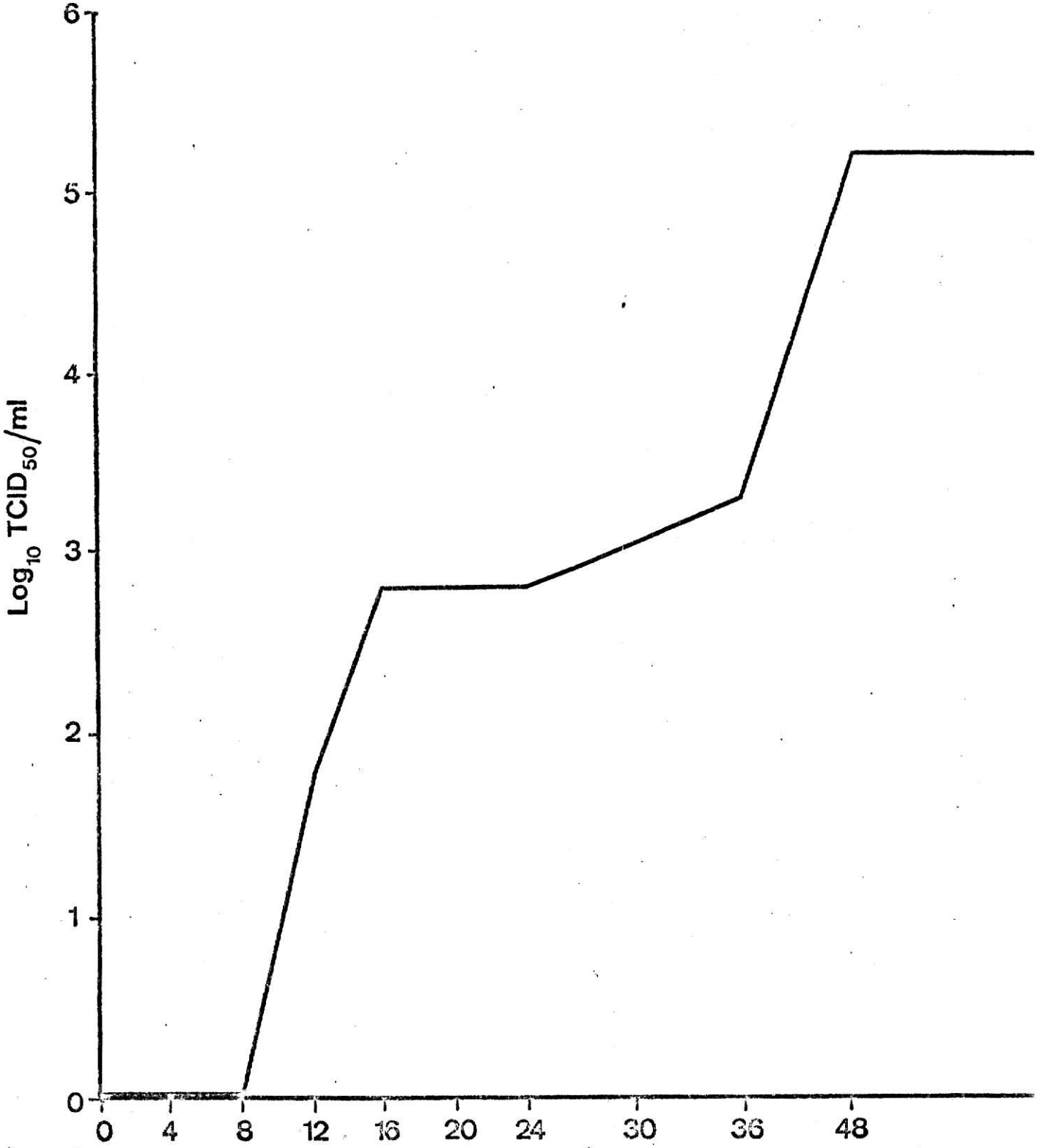


Fig 8

Fig. 9-III. Time-course of BVD virus infectivity titer ($TCID_{50}$/ml).



BVD virus infectivity

Fig. 9

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APPENDIX A: METHODS

(1) VIRUS TITRATIONS

A. Microtiter Technique

(a) Serial log dilutions of the virus were made, usually with MEM.

(i) .025 ml of each dilution was placed in a microtiter well. Four replicate wells were used for each dilution.

(ii) .2 ml of cell suspension (Bovine Embryonic Kidney, BEK) cells containing 50,000 cells per ml, was placed in each well. (Control wells, contained no virus).

(iii) The wells were examined daily for CPE, and scored as + or - according to presence or absence of definite CPE. When there was no further change, TCID virus titer was calculated by the Method of Reed and Muench (1938).
(See diagram)

For titration of PI_3V and BVD V growth-curve samples, Bovine Turbinate cells were used.

B. Plaque Assay Technique:

Carboxymethylcellulose (CMC) was prepared by stirring 3.0 grams of CMC powder in 400 mls of double-glass-distilled water (.75% CMC) overnight at $4^{\circ}C$. When CMC was in solution, aliquots of 100 mls were distributed in bottles, and autoclaved.

35 x 10 mm plastic petri dishes were seeded with 2 mls of Madin-Darby Bovine Kidney (MDBK) cell suspension, containing 150,000 cells/ml in

MEM, containing 5% Lactoalbumin hydrolysate and 10% horse serum, with antibiotics and fungizone, and incubated till monolayer was formed. The growth media was discarded, the monolayer was washed with PBS, and infected with .2 ml of virus. Duplicate plates were infected for each viral dilution. The plates were placed in a CO_2 incubator (37°C) for 1 hour.

The overlay media was prepared by mixing equal parts of the CMC solution and double strength (2x) MEM medium. Horse serum was added to a final concentration of 4%. Two mls of the overlay media was placed in each petri dish, and the dishes were put back in incubator. Positive control dishes were prepared by infecting monolayer dishes with standard laboratory virus. Negative controls were uninfected monolayer dishes. Care was taken not to move the petri-dishes. The positive (and negative) control dishes were examined daily, under an inverted microscope. When plaques were observed in positive control dish, the rest of dishes were stained and plaques counted.

Staining: The overlay media was removed by vacuum suction. Two mls of 10% Formalin was added to each dish and allowed to fix the cells for 30 to 45 minutes. Then about 2 mls of crystal violet solution (containing crystal violet, 7.5 gm and 50 mls of 70% ethanol in a liter made up with distilled water) was added to each dish. The stain was removed after 30 seconds to 1 minute, excess stain was washed out with tap water, and the plaques, which were white (unstained) on a deep purple background, were counted.

The initial titration of IBRV, BVD was done by plaque assay, but 2% Agar was substituted for CMC. In this case, the agar was autoclaved at 250°C for 20 minutes, and placed in water bath at 50°C. The 2X media was

also warmed to 50°C for 10 minutes before mixing with Agar. Staining of plaques was by Neutral red (.00125% or 1.25 ml a 1% soln of neutral red per 100 ml of Media-Agar mixture). The neutral-red was incorporated in Media-Agar mixture, placed on top of the previous agar layer. Plaques were read 12 to 24 hours later.

2. PREPARATION OF CONJUGATE:

A: Fractionation of Serum

Bovine anti-BVD antisera was obtained from Dr. R. M. Phillips, and rabbit anti-IBRV antisera was obtained from Dr. H. C. Minocha. Fractionation of gamma globulin from other serum proteins was according to the method described by Herbert (1) and Herbert, et al. (2):
Stock Saturated Ammonium Sulfate (SAS) Solution ($(NH_4)_2SO_4$) was prepared by dissolving 55 grams of ammonium sulphate in 100 mls. of distilled water. The solution was allowed to stand for several days at room temperature. Working solution was prepared fresh by mixing 70 mls. of stock SAS with 30 mls. of distilled water, to make a 70% SAS solution. To 20 mls. of antiserum in a Volumetric flask immersed in icebath, 20 mls. of working solution (70% SAS) was added slowly, dropwise with constant agitation of the mixture. The mixture was allowed to stand at room temperature for 4 hours. Then it was centrifuged at 4°C, for 30 mins., at 2,800 rpm (1440 g). The supernate was discarded, the sedimented globulin was dissolved in 20 mls. distilled water. The globulin was again re-precipitated as described above, with 20 mls. of 70% SAS, and immediately centrifuged as above. The resulting sediment was dissolved in water, and reprecipitated a third time. After the third precipitation, the globulin sediment was dissolved in 7 mls. of distilled water, placed in a semipermeable membrane and dialysed against

frequent changes (after the first hour, then after every 12 hours for 48 hours) of .85% saline solution (pH8). The absence of sulphate from the globulin was determined by mixing equal volumes of saturated barium chloride with the saline dialysate. When no cloudiness resulted, the globulin solution was considered essentially free of sulphate.

B: CONJUGATION OF GLOBULIN

The dialysis Method, described by Herbert, et al. (2) was used.

1. Globulin

The protein concentration of the globulin was determined with a TS meter[b]. All reagents were stabilized at room temperature (25°C). 5 mls of globulin was placed in a flask, and 2.5 mls of 0.2 M Na_2HPO_4, (28.4 gm per liter, pH 9) was added slowly, while mixing. The pH[a] of this mixture was brought to 9.5 by dropwise addition of 0.1M Na_3PO_4 (38 gm $Na_3PO_4.12H_2O$/liter dist. water). The final volume of globulin mixture was brought to 10 mls with 0.85% NaCl solution.

2. FITC Solution

18.8 mg of Fluorescein isothiocyanate (FITC) powder[c] was weighed out and dissolved in 120 mls of 0.1M Na_2HPO_4. The pH was raised to 9.5 by dropwise addition of 0.1M Na_3PO_4, and the volume was brought to 150 ml with 0.1M Na_2HPO_4, (to make a final concentration of 0.125 mg FITC/ml buffer). The globulin mixture was placed in a 20 mm dialysing tubing[d]

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a. pH was determined with Model 265 pH meter, Instrumentation Laboratory Inc., 113 Hartwell Avenue, Lexington, Mass. 02173.

b. TS Meter-Model 10400 American Optical Company, Buffalo, New York 14215.

c. Fluoresceinisothiocyanate on Celite, 10%: Calbiochem. La Jolla, California 92037.

d. Dialysing tubin, Scientific Products, Division of American Hospital Supply Corporation, 1430 Waukegan Road, McGaw Park, Illinois 60085.

and tied in such a way to remove all air bubbles. The sac was then submerged in the FITC solution, placed in a dark shelf and the reaction allowed to proceed for 4 hours at room temperature.

At the end of 4 hours, the sac of labelled globulin was removed, rinsed under cool tap water, and placed in a container of PBS, pH 7.6 at 4°C for dialysis. The conjugate was dialysed against PBS for 3 days, with the dialysis fluid changed every 12 hours. At the end of 3 days, the dialysis fluid showed no fluorescence when placed under an ultraviolet lamp.

The conjugate was removed from the bag, centrifuged at 1500 rpm for 10 mins, and stored for further processing. Merthiolate (Thiomesol) was added to a final concentration of 1:10,000, as a preservative.

C: PREPARATION OF BOVINE KIDNEY POWDER

(a) Fresh kidneys obtained from slaughterhouse were cleaned of perirenal fat, cut up, washed for about one hour, and minced in a Waring blender (3 parts kidney to 1 part water, minced at high speed for 6 to 8 minutes).

(b) 3 volumes of acetone to each volume of kidney mince was added, and the slury filtered through cheese cloth. After the filterate had settled, the supernate was siphoned off and the sediment was once again precipitated with acetone and filtered through cheese cloth.

(c) The filtrate was filtered through the Buchner funnel containing #3 filter paper, and washed with acetone till the wash no longer had a greenish tinge. The suction pump was continued, for about 30 minutes, to allow air pass through the cake in the Buchner funnel.

(d) The cake was removed, spread on tin-foil and placed to dry in an

incubator for 48 hours. After 48 hours, the dried cake was placed in a Waring blender and rendered to powder.

D. ADSORPTION OF CONJUGATE

37 mg of kidney powder was weighed out (20 mg of kidney powder per 1 mg of protein in conjugate), and mixed with .1 ml PBS (2.5 ml PBS per gram of powder). 3 mls of the conjugate was added and the mixture stirred overnight at 4°C. The mixture was cleaned by centrifugation at 3000 rpm for 20 minutes, followed by 30,000 rpm for 1 hour in a Beckman Ultracentrifuge.[a] The supernate was filtered through a .45 microns millipore filter.

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a. Beckman Ultracentrifuge Model. L 2-65-B

APPENDIX B- SOLUTIONS

1. Growth Media: Eagles Minimum essential Media 5% Lactoalbumin hydrolysate, 1% (v/v) sodium pyruvate, penicillin, and 10% Fetal Calf Serum.

2. Maintenance Media: Eagles Minimum Essential Media, 5% Lactoalbumin hydrolysate, 1% (v/v) sodium pyruvate, antibiotics and 10% (v/v) horse serum.

3. Tris-buffered Saline (TBS)

	per liter
Sodium chloride (NaCl)	8.00 gm.
Potassium Chloride (KCl)	0.37 gm.
Sodium diphosphate, anhydrous (Na_2HPO+)	0.1 gm.
Glucose	1.0 gm.
Tris (Trishydroxymethylaminomethane)	3.0 gm.
10 N. Hydrochloric acid (HCl)	1.8 mls.

qs - Sterile distilled water to 1000 mls.

4. PBS (Phosphate Buffered Saline)

Concentrated (10 x) stock solution:

Na_2HPO_4, anhydrous, reagent grade	12.36 gm.
$NaH_2PO_4.H_2O$ Reagent grade	1.80 gm.
NaCl Reagent grade	85.00 gm.

Distilled water - to a final volume of 1000 ml.

Working Solution (pH 7.2):

Stock solution 100 ml.

Distilled water - to a final volume of 1000 ml.

5. Antibiotics--Stock Solutions:

Penicillin - 1,000,000 units/100 ml stock

Streptomycin - 1 gm./100 ml stock

Kanamycin - 50 mg/100 ml

Media-contained 10 ml antibiotic stock per liter,

Fungizone- 0.5 Mg/ml. 1 ml. Fungizone was added per liter of media.

6. Conjugation Regents:

(a) 0.2 M Na_2HPO_4, pH 9

(Dissolve 28.4 gm in 1 liter of distilled water.)

(b) 0.1 M Na_2HPO_4, pH 9 (14.2 gm/liter of water).

(c) 0.1 M Na_3PO_4 (16.4 gm/liter of water

of 38.0 gm of Na_3PO_4 .12 H_2O/liter of water).

(d) 0.85% NaCL (8.5 gm/liter of water).

The protein content of the globulin was determined with a TS meter method and the globulin was adjusted to 10 mg/ml of protein.

APPENDIX C- RESULTS

Table A-a: Initial Titration of IBR, BVD by Plaque Assay

Virus	Log Dilution 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	PFU/.2 ml	PFU/ml
BVD (C24V)			TNTC	19	1		1.87×10^6	9.35×10^6
	Ly	PLY	TNTC	18	2			
IBR (Los Angeles Strain)			TNTC	16	2			
		PLY	TNTC	15	0	1	2.0×10^6	1.0×10^7

LY = Lysis
PLY = Partial Lysis
TNTC = Too numerous to count

Table A-1

ERUM NEUTRALIZATION ICROTITER TECHNIQUE — Initial Microtiter Titration of the Three Viruses — DATE IN: 5/28 DATE OUT: 6/3

	a			b			c						
A Serial 10-1	+	+	+	+	+	+	+	+	+	+	+	+	Titres/.05 ml.
dilution B -2	+	+	+	+	+	+	+	+	+	+	+	+	
C -3	+	+	+	+	+	-	-	-	+	-	+	+	a = 10^5 $TCID_{50}$
D -4	+	-	+	+	-	-	-	-	-	-	-	-	b = $10^{2.67}$ $TCID_{50}$
E -5	+	-	+	-	-	-	-	-	-	-	-	-	c = $10^{3.33}$ $TCID_{50}$
F -6	+	-	-	-	-	-	-	-	-	-	-	-	
G -7	-	-	-	-	-	-	-	-	-	-	-	-	
H Control	-	-		-	-	-	-	-	-	-	-	-	

\+ = CPE

-) = No CPE

a = IBR Virus, Cooper Challenge Strain
b = BVD, NADL Strain
c = PI_3-V Lab. Strain

ERUM NEUTRALIZATION
ICROTITER TECHNIQUE

DATE IN: _____
DATE OUT: _____

A													
B													
C													
D													
E													
F													
G													
H													

Table A-2

ERUM NEUTRALIZATION ICROTITER TECHNIQUE — Final Titration of PI_3-V — DATE IN: 7/5/76 DATE OUT: 7/9/76

	Time of Harvest (hours post infection)												
	0	4	12	16	20	24	30	36	50	60	C_1	C_2	
A Log dilution 0	-	-	+	+	+	+	+	+	+	+	-	+	
B 0	-	-	+	+	+	+	+	+	+	+	-	+	
C -1	-	-	-	+	+	+	+	+	+	+	-	+	
D -1	-	-	-	-	+	+	+	+	+	+	-	+	
E -2				-	+	+	+	+	+	+	-	+	
F -2				-	+	+	+	+	+	+	-	+	
G -3					-	+	+	+	+	+	-	+	
H -3					-	-	-	+	+	+			

Table A-3

ERUM NEUTRALIZATION ICROTITER TECHNIQUE — Final Titration of BVD (virus) — DATE IN: 7/5/76 DATE OUT: 7/11/76

	Time of Harvest (hours post infection)													
	0	4	8	12	16	20	24	30	36	50	60	72	
A Log dilution 0	-	-	-	+	+	+	+	+	+	+	+	+	
B 0	-	-	-	+	+	+	+	+	+	+	+	+	
C -1	-	-	-	-	+	+	+		+	+	+	+	
D -1	-	-	-	-	-	-	-		+	+	+	+	
E -2	-	-	-	-	-	-	-		+		+	+	
F -2									-			+	
G C_1	-	-	-	-								+ -	-3
H C_2	+	+	+	+								-	-3

(+) = CPE
(-) = No CPE
C_1 = Uninfected Controls
C_2 = Infected Controls

Table A-4: IBRV Titration By Plaque Assay

Time of Harvest (hours post-infection)	No of Plaques per Log Dilution						Titer/.2 ml
	0	-1	-2	-3	-4	-5	
0	TNTC	26					2.4×10^2
	TNTC	25	2				
2	38	2	0				$.38 \times 10^2$
	37	5	0				
4	60	9	0				$.88 \times 10^2$
	70	12	1				
6	40	6	0				$.4 \times 10^2$
	42	6	0				
8	30	4	0				$.3 \times 10^2$
	22	3	0				
10		6	0	0			$.7 \times 10^2$
		7	0	0			
12		84	8	1			8.6×10^2
		75	9	0			
16			LY	TNTC	38	5	4.15×10^5
				TNTC	48	3	
20			LY	TNTC	80	6	6.6×10^5
				TNTC	75	5	
24			LY	PLY	132	23	21×10^5
				PLY	TNTC	27	
30			LY	PLY	TNTC	76	73×10^5
				PLY	TNTC	70	
36			LY	LY	PL	100	98×10^5
				LY	TNTC	96	
48			LY	PLY	TNTC	104	140×10^5
				PLY	TNTC	122	

LY = Lysis
PLY = Partial Lysis
TNTC = Too numerous to count

APPENDIX: REFERENCES

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THE SEQUENTIAL DEVELOPMENT OF THE ANTIGENS OF SOME BOVINE VIRUSES IN CELL CULTURES STUDIED BY THE FLUORESCENT ANTIBODY TECHNIQUE

by

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AN ABSTRACT OF A MASTER'S THESIS

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The viruses of Infectious Bovine Rhinotracheitis (IBR), bovine parainfluenza type 3 (PI-3) and Bovine Virus Diarrhea (BVD), individually or in combination, are responsible for a wide spectrum of bovine diseases, from respiratory, enteric to genital and cerebellar diseases. Even though these viruses infect susceptible animals most commonly via the oral-nasal route, few studies of their morphogenesis in oral-nasal epithelial cells have been reported. The present study was designed to investigate the sequence of development of viral antigens in bovine turbinate (BT) cells (for IBR and PI-3), and bovine kidney cells (BK), by the fluorescent antibody (FA) technique.

Coverslip cultures were infected with virus and harvested sequentially, at predetermined intervals. The harvested coverslips, fixed in acetone or acetone-methanol mixture, were stained with fluorescein-isothiocyanate-conjugated globulin specific for each virus. The preparations were examined under a Leitz microscope equipped for fluorescent antibody procedures. Production of infective virus was studied concurrently, and virus titers were determined by plaque assay and microtiter techniques.

Fluorescent IBR-viral antigens first appeared in the perinuclear region, then in the nucleus, spread throughout the cytoplasm and finally disappeared. The antigens of PI-3 virus also made initial appearance in the juxtanuclear area, and also spread throughout the cytoplasm. Nuclear fluorescence was not prominent, though some granules were observed inside the nucleus. With BVD, fluorescing antigens were also confined to the cytoplasm, first in the perinuclear area and later diffusedly in the entire cytoplasm. In all cases, appearance of infective virus approximated the appearance of fluorescent antigens.