IMMUNOFLUORESCENCE OF AVIAN INFECTIOUS BRONCHITIS VIRUS AND NEWCASTLE DISEASE VIRUS IN SINGLY AND DUALLY INFECTED CELL CULTURES

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ABSTRACT

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By

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On the basis of fluorescence microscopy, avian infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) were specifically and differentially identified in single and dual infections of primary chicken embryo kidney cells (CEKC). In singly infected CEKC cultures, the size and number of foci of viral infected cells was directly related to the viral concentration of the inoculum and the time of viral replication. Evidence of interference of NDV by IBV was observed in dual infections of CEKC. Replication of NDV occurred equally well in baby hamster kidney cells (BHK-21) as in CEKC, but there was no evidence that IBV infected BHK-21 cells.

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INTRODUCTION

Study of viruses using a living indicator system requires cognizance of the host cell's susceptibility to a virion and recognition of any interrelationship between different viral agents present in the host cell population. Infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) are of particular interest in this regard because the chicken is a natural host for both viruses and there is a high probability of their coexistence under both laboratory and environmental conditions.

The capacity of IBV to exert interference upon NDV replication has been reported and has even been used as a means of assaying IBV (6). However, the interference phenomenon could mask the detection of a low concentration of NDV in the presence of a high concentration of IBV. Embryonating chicken eggs and avian cell cultures are commonly used for growth of IBV and NDV. To assure the complete purity of viral stocks, it is essential to know if small amounts of NDV or IBV can be detected in the presence of the other virus and their reciprocal influence.

Using the fluorescent antibody technique, this study was designed to demonstrate if low levels of each virus could be detected in the presence of large numbers of the other, the time of viral development, the cytopathic effect, and possible differences in the fluorescence of the two viruses during synthesis within the cell.

LITERATURE REVIEW

Avian infectious bronchitis virus (IBV) has an average diameter of 80 to 100 mμ with distinctive surface projections 20 mμ long, club-shaped in outline, and attached to the viral surface by a narrow stalk (11, 12). It has an ether sensitive lipoprotein coat (29) and a ribonucleic acid core (2). It is presently not classified in any recognized major viral group (44), but recognition by Almeida and Tyrrell in 1967 (3) of the similarity between IBV and recently isolated human respiratory viruses, B814 and 229E, indicated the existence of a previously unrecognized viral group. Additional work has confirmed these findings (7, 28, 31), and it was also pointed out that mouse hepatitis virus (MHV) possessed similar properties. This has prompted a group of virologists to propose that IBV, several human respiratory viruses, and MHV are members of a unique viral group (4). Because their electron microscopic appearance resembles the solar corona, they have suggested that this group be called the coronaviruses, with IBV as the prototype.

Infectious bronchitis virus can be propagated readily in embryonating chicken eggs, the most usual route being the allantoic

cavity (16). Propagation in cell culture is possible with several primary avian cell types (17), but one of the most sensitive and frequently used is the primary chick embryo cell (CEKC) (27). Infection begins when viral particles adsorb to the outer membrane of the CEKC. There is a 4 hour eclipse period. Maximal viral yield is reached in 48 hours, maintained through 72 hours, and then gradually declines (2, 17).

Newcastle disease virus (NDV), a member of the paramyxovirus group (44), is an RNA-containing, enveloped, roughly spherical pleomorphic particle, with a diameter of approximately 100-200 mμ (19, 22). The helical nucleocapsid is enclosed by an ether sensitive lipopritein coat with surface spikes about 8mμ in length and spaced about 8-10 mμ apart (42).

Newcastle disease virus multiplies to high titer when inoculated into the embryonating chicken egg (25) and grows in a wide variety of cells of avian and mammalian origin (24). In chicken embryonic lung cell cultures, NDV has a latent period of 2 hours and a period of viral release of 4 hours, with approximately 50 plaque-forming units released from each cell (20).

Separation and characterization of virus-specific antigens is reported for both IBV and NDV. Three antigenically distinct soluble antigens are associated with IBV and can be differentiated

on the basis of thermostability, size, buoyant density, and sensitivity to enzymes (40). Newcastle disease virus possesses two distinct and separable type-specific antigens, a surface lipoprotein antigen (hemagglutinin or V-antigen) and an internal or ribonucleoprotein antigen (nucleocapsid or S-antigen) (38). The antigens can be differentiated on the basis of enzyme activity, hemagglutinating, complement-fixing, and enzyme sensitivity.

The earliest report of detection of IBV by fluorescence microscopy indicated the presence of intranuclear IBV antigen (29). Later work by Lukert has contradicted these findings (27). Using IBV-infected chicken embryo kidney, liver, lung, and fibroblast cell cultures, no nuclear fluorescence was detected. Development of IBV antigen was essentially the same in all cell types. The first fluorescence occurred 4 hours after infection, primarily in the nuclear region, and progressed peripherally until the cytoplasm contained diffuse and granular fluorescence. Syncytia developed within 7 to 8 hours and reached their maximum size at 18 to 20 hours.

Fluorescence microscopy has been used to study cellular multiplication of NDV (14, 35, 37, 43, 45). Using specific antibody against S- and V-antigens by the direct test, Reda, Rott, and Schafer (35) reported that distinct fluorescent foci appeared around

the nucleus in about 3 hours. These foci increased in size and brightness with time and occurred throughout the cytoplasm. The V-antigen was detectable shortly after the S-antigen and at about the 7th hour it was distributed throughout the cytoplasm.

Viral interference occurs when two viruses or two strains of a single virus infect a single cell, and one virus interferes with the growth or replication of the other (36). Interference of IBV with NDV has been demonstrated in chickens (21), embryonating chicken eggs (33), and chick embryo kidney cells (7). Regardless of the test system used, interference was dependent upon an excess of IBV. Interference apparently occurs intracellularly because when NDV was inoculated into developing chicken embryos 4 hours prior to the administration of IBV, interference was still demonstrated. The interference mechanism does not appear to involve blocking of cellular receptor sites or to be influenced by interferon.

MATERIALS AND METHODS

Viruses

The Beaudette strain of avian infectious bronchitis virus (IBV-42) adapted to chick embryo kidney cells (CEKC) (18) was used and assayed in primary CEKC cultures as plaque-forming units (PFU) (16). Primary CEKC in petri dishes were inoculated with 2.2×10^5 PFU of the 130th passage of IBV. At 48 hours the extracellular fluid was harvested, pooled, and distributed in 1 ml quantities into glass vials for storage at -90 C. The virus contained 2.5×10^7 PFU per ml.

Newcastle disease virus (NDV), Texas-GB strain, 1.2×10^6 PFU in 0.1 ml, was used to inoculate 10-day-old embryonating chicken eggs via the allantoic cavity. All embryos died within 28 hours and were chilled at 4 C for 6 hours prior to collection of allantoic fluid. The fluid was dispensed in 1 ml amounts into glass vials and stored at -60 C. The titer of the virus was 2.8×10^9 PFU per ml assayed in CEKC (16).

Cell Cultures

Primary CEKC prepared from 17-day-old chicken embryos (16) were suspended in growth medium consisting of Medium 199 with

2 mM L-glutamine, Grand Island Biological Company (GIBCO), fortified with amino acids and vitamins of Eagle's basal minimum essential medium (GIBCO), 0.1% sodium bicarbonate, 100 units/ml penicillin, 100 μg/ml dihydrostreptomycin, and 50 units/ml Mycostatin (Squibb). Newborn calf serum, 5% final concentration, was then added. For plaque assay, a 1:100 suspension of packed cells (approximately 10^7 cells/ml) was dispensed in 4 ml quantities into 15 × 60 mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.). Leighton tubes containing a coverslip (9 × 22 mm) were seeded with 1 ml of a 1:400 suspension (approximately 2.5×10^6 cells/ml) of packed cells. All cell cultures were incubated in an atmosphere of 8% CO_2 and 80-85% relative humidity at 37 C. Monolayers formed in the Leighton tubes within 24 hours and in the petri dishes within 48 to 72 hours.

Baby hamster kidney cells (BHK-21) (Microbiological Associates, Bethesda, Md.) were also used starting with the 150th passage. The cells were propagated in milk dilution bottles with screwcaps as a closed system. The growth medium was Eagle's minimum essential medium with Hanks' solution (HMEM) and 2 mM L-glutamine, without sodium bicarbonate (GIBCO), 50 mcg/ml aureomycin, 100 units/ml tylosin tartrate, and adjusted to pH 7.4 with 7.5% sodium bicarbonate.

After development of a confluent monolayer in 3 to 4 days, the cells were rinsed twice with 5 ml of phosphate buffered saline (PBS) without Ca^{++} or Mg^{++} (39). The PBS was decanted and approximately 4 ml of buffered 0.25% trypsin containing 6.8 g/L sodium chloride, 0.4 g/L potassium chloride, 1 g/L glucose, 2.2 g/L sodium bicarbonate, and 0.005 g/L phenol red (GIBCO Solution A) at room temperature was added to the cells. In approximately 2 minutes, the trypsin solution was decanted and the bottle was inverted for another 2 minutes or until the cells detached from the surface. The cells were then suspended in growth medium at a volume 3 times that of the original medium and agitated vigorously. Fetal calf serum was added to a final concentration of 10%.

The suspension of cells was dispensed in 15 ml amounts into bottles and incubated at 37 C. The medium was replaced at approximately 2 day intervals within 7 days with equal parts of the growth medium and nutrient mixture F10 with 2 mM L-glutamine (GIBCO), 100 units/ml penicillin, 100 μg/ml dihydrostreptomycin, and 0.0075% sodium bicarbonate. There was a final concentration of 5% fetal calf serum.

Leighton tubes were seeded with 1 ml of trypsinized BHK-21 resuspended in growth medium at the original volume. Monolayers were formed within 24 hours.

Inoculation of Cells in Leighton Tubes

The extracellular fluid from CEKC was decanted and the cells were washed with PBS without Ca^{++} or Mg^{++}. Dilutions of the viruses to the PFU desired were made at approximately 0 C in PBS without Ca^{++} or Mg^{++} but containing 2% newborn calf serum. An inoculum of 0.2 ml was pipetted onto each coverslip culture. The cultures were reincubated for 60 to 90 minutes and then 2 ml of equal parts Medium 199 and F10 containing 2% newborn calf serum was added.

Tubes of BHK-21 were handled similarly except that fetal calf serum was substituted for the newborn calf serum in the diluent. In the maintenance medium HMEM replaced Medium 199.

The cells were first examined by bright field microscopy at 12 hour intervals for cytopathogenic effects. The coverslips were then removed for staining and examination by fluorescent antibody microscopy.

Antisera

The IBV antisera from specific pathogen free chickens was kindly supplied by Dr. D. D. Oshel, Agricultural Research Service, Ames, Iowa. The neutralization index against IBV-42 was 6.3×10^5 as determined by the embryo lethal $dose_{50}$ (ELD_{50}) in chicken embryos.

Dr. M. S. Hofstad, Iowa State University, Ames, Iowa, kindly supplied NDV-antiseria from specific pathogen free turkeys. The neutralization index ELD_{50} was greater than 5.6×10^6 against Texas-GB and less than 10^1 against IBV-42.

Conjugation of Antiserum

The antisera were conjugated according to a method described by Lukert (27). Serum was fractionated by the dropwise addition of one volume of saturated ammonium sulfate into two volumes of serum with constant mixing in an ice bath. Precipitated globulins were centrifuged at 2000 g. for 15 minutes and redissolved in 0.85% NaCl solution equal to the original volume of serum. This procedure was repeated twice. The final precipitate was restored to one-half the original volume and dialyzed at 4 C against at least 100 volumes of 0.85% NaCl. The saline solution was changed 3 times within 24 hours or until it was free of SO_4^{++}. Any precipitate in the immunoglobulin fraction at the last dialysis was removed by centrifugation.

The concentration of protein in the immunoglobulin solution was determined (26) using crystalline bovine serum albumin as the standard. The solution was then diluted to contain 10 mg protein per ml of 0.85% NaCl.

A volume of 0.5 M sodium carbonate-sodium bicarbonate buffer solution, pH 9.0, equal to 1/10 the immunoglobulin volume was used to dissolve crystalline fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratory, Cockeysville, Md.) at the rate of 25 μg FITC per mg of protein in the immunoglobulin solution. The FITC-carbonate buffer solution was then added to the immunoglobulin solution and stirred at 4 C for 16 to 20 hours. The mixture was then added to a column of Sephadex G-25, coarse (Pharmacia Fine Chemicals, Uppsala, Sweden) in optimum amounts for maximum differential elution of the FITC labeled globulin from free FITC and eluted with PBS. The column of Sephadex was prepared with 0.01 M PBS, pH 7.2, and had a height-to-diameter ratio or 15:1.

The elute was added to a PBS-rabbit liver powder (RLP) (Baltimore Biological Laboratory) slurry (2.5 ml : 1 mg) to give a final ratio of 1 mg of protein in the immunoglobulin solution to 20 mg of RLP in the slurry. This mixture was stirred constantly 16 to 20 hours at 4 C. RLP was sedimented by centrifugation at 2000 g. for 30 minutes followed by 45,000 g. for 90 minutes at 4 C. The supernatant fluid was passed through a filter (Millipore Filter Corp., Bedford, Mass.), pore size 0.30 microns, and stored in glass vials at -20 C. Just prior to use, the conjugate was thawed, diluted 1:4 with PBS, pH 7.2, and centrifuged at 2000 g. for 15 minutes to remove any precipitate.

Fluorescent Antibody Staining of Coverslip Cultures

Staining was also by the method of Lukert (27). Appropriate coverslip cultures from the Leighton tubes were washed in PBS, pH 7.2. While wet, they were momentarily immersed in acetone for partial dehydration. They were then fixed in another container of acetone for 3 to 5 minutes at room temperature and air dried. A few drops of labeled antibody were placed on the coverslips which were incubated for 45 minutes in a moist chamber at 37 C. The coverslips were immersed in tap water at room temperature for 3 minutes with gentle mixing, after which they were air dried. A drop of equal volumes glycerin and PBS, pH 7.2, was used for mounting the coverslips on a slide.

Microscopy

Cells were examined with an AO-Spencer "Microstar" microscope illuminated with an Osram HBO-200 mercury vapor arc lamp. The excitation filter was the Schott BG-12 and the barrier filter the Schott OG-1. Photomicrographs were made with high speed Ektachrome daylight film using exposure times of 2 to 5 minutes.

RESULTS

Relationship Between Concentration of Virus and Time of Development of CPE

Infectious bronchitis virus

There was direct relationship between the number of PFU of IBV in the inoculum and the time when cytopathic effects (CPE) were present (Table 1). With 250 PFU, CPE was observed as early as 36 hours, but with the more dilute inoculum, -1.8 log units, CPE was not evident in some cultures until as late as 84 hours. There was no CPE in dilutions greater than -1.8 log units indicating that infectious virus was not present in the inoculum. Characteristic cell alterations were the loss of cytoplasmic detail, formation of circumscribed cellular aggregates and syncytia, followed by lysis of cells and their detachment from the surface of the tube.

Newcastle disease virus

The CPE of cultures inoculated with 280 PFU of NDV and with dilutions as high as -1.8 was first detected at 36 hours (Table 2). However, with -2.1 to -3.0 dilutions of virus the time of development

Table 1. -- Development of CPE in CEKC infected with various dilutions of IBV.

Log dilution of 250 PFU IBV inoculated onto each of 4 Leighton tube cultures	Hour post-inoculation						
	12	24	36	48	60	72	84
0			+	+++			
-0.3			+	+	++		
-0.6					++++		
-0.9					++++		
-1.2					+	+	++
-1.5					+	+	++
-1.8							++
-2.1							

+ = number of cell cultures with CPE.

Table 2. --Development of CPE in CEKC infected with various dilutions of NDV.

Log dilution of 280 PFU NDV inoculated onto each of 4 Leighton tube cultures	Hour post-inoculation								
	12	24	36	48	60	72	84	96	108
0			++++						
-0.3			++++						
-0.6			++++						
-0.9			++++						
-1.2			++++						
-1.5			++++						
-1.8			++++						
-2.1			+	+			+		
-2.4				+			+		+
-2.5					+				
-3.0					+	+			
-3.3									

+ = number of cell cultures with CPE.

of CPE ranged from 36 to 108 hours. At dilutions greater than -3.0, CPE did not develop, indicating that infectious virus was not present in the inoculum. With the exception of a more extensive lysis of the monolayer by NDV, the CPE produced by NDV was similar to that produced by IBV.

Fluorescence of IBV as the Single Inoculum in CEKC

At 12 hours fluorescence was present in widely separated individual cells or in 2 or 3 adjoining cells in cultures inoculated with 125 or 250 PFU (Figure 1). By 24 hours, fluorescence was also evident in cultures infected with 63 PFU (Figure 2). Both the size and number of fluorescent foci increased with time, indicating the rate of replication and release of new viral infectious units was directly dependent upon the concentration of virus in the inoculum (Figures 3, 4, 5).

During replication of the virus, the first evidence of cellular infection was the development of a diffuse, perinuclear antigen which gradually spread peripherally with subsequent development of small, spheroid packets throughout the cytoplasm (Figures 6 and 7). Fluorescence was not observed in the nucleus.

Fluorescence of NDV as the Single Inoculum in CEKC

Between 12 and 24 hours after infection, the size and number of fluorescent foci increased and were directly related to the viral concentration of the inoculum and the length of time of viral replication (Figures 8 and 9). With all NDV infected cultures, the inevitable outcome was the development of fluorescence within essentially 100% of the cells comprising the monolayer. There was an ultimate total disintegration and lysis of infected cells (Figure 10).

The earliest evidence of NDV replication was small, brilliantly fluorescing, segregated cytoplasmic bodies near the nucleus. These bodies subsequently developed into large polymorphous forms (Figures 11 and 12). Shortly following the first appearance of the segregated bodies there was development of a diffuse fluorescence throughout the cytoplasm.

Fluorescence of IBV and NDV as Dual Inoculum in CEKC

When cultures were dually infected with PFU combinations of IBV 10:NDV 10, 10:100, and 100:100, respectively, there was no significant difference in the time of appearance of fluorescence as compared to that when the cultures were singly infected (Table 3). However, the time required for complete cell destruction differed

Table 3. -- Development of fluorescence and CPE in CEKC infected with various PFU of IBV and NDV, singly and in combinations.

Hours after infection	Number of PFU and virus											
	10 IBV	100 IBV	10 NDV	100 NDV	10 IBV	10 NDV	10 IBV	100 NDV	100 IBV	10 NDV	100 IBV	100 NDV
12		+		+		+		+				
24		+	++	++		++		++	+	++	+	++
36	++	+++	+++ *	++++ **	+	+++ *	++	++++ **	+++	++	+++	++++ **
48	++++ *	++++ **	++++ **	####	++	++++ **	+++ ***	++++ ****	++++ *	++ **	++++ ****	++++ ****
60	++++ *	++++ **	####		####	####	####	####	++++ ****	+++ ****	####	####
72 to 96	++++ **	++++ ***							####	####		

+ = intensity of fluorescence, +----++++, for entire cell culture.

* = *----****, for entire cell culture.

= Necrosis of cell culture.

from that when either virus was replicating in a singly infected culture. In the mixture of IBV 10:NDV 100, NDV infected and lysed cells throughout the culture by 60 hours, which was before IBV had infected a number of cells comparable to its concentration. The viruses at IBV 10:NDV 10 and 100:100 completely destroyed the cells in 60 hours, but at IBV 100:NDV 10 the cells were not destroyed until 72 hours.

Fluorescence in cells infected with IBV 100:NDV 10 indicated that IBV interfered with the replication of NDV. At 24 hours, NDV was apparent within several small clusters of cells and in cells radiating from the clusters (Figure 13). At the same time, IBV-induced sycytia were present. Following this, IBV was more pronounced than NDV and at 48 hours IBV completely dominated the cell culture except for scattered areas of NDV infected cells (Figures 14, 15, 16).

Fluorescence of IBV and NDV as Single Inoculum in BHK-21 Cells

Infectious bronchitis virus, 5×10^5 PFU, did not infect the cells based on the absence of CPE or fluorescence, up to and including 60 hours after inoculation.

Newcastle disease virus replicated equally as well in BHK-21 as in CEKC (Table 4). The relationship between the time

Table 4. -- Development of CPE in BHK-21 infected with various dilutions of NDV.

Log dilution of 250 PFU NDV inoculated onto each of 3 Leighton tube cultures	Hour post-inoculation					
	12	24	36	48	60	72
0		+	++			
-1.0			+++			
-2.0			+	++		
-3.0				+		++
-4.0						+
-5.0						

+ = number of cell cultures with CPE.

of development of CPE observed by bright field microscopy and the manner of replication observed by fluorescence microscopy was essentially the same as in CEKC (Figure 17).

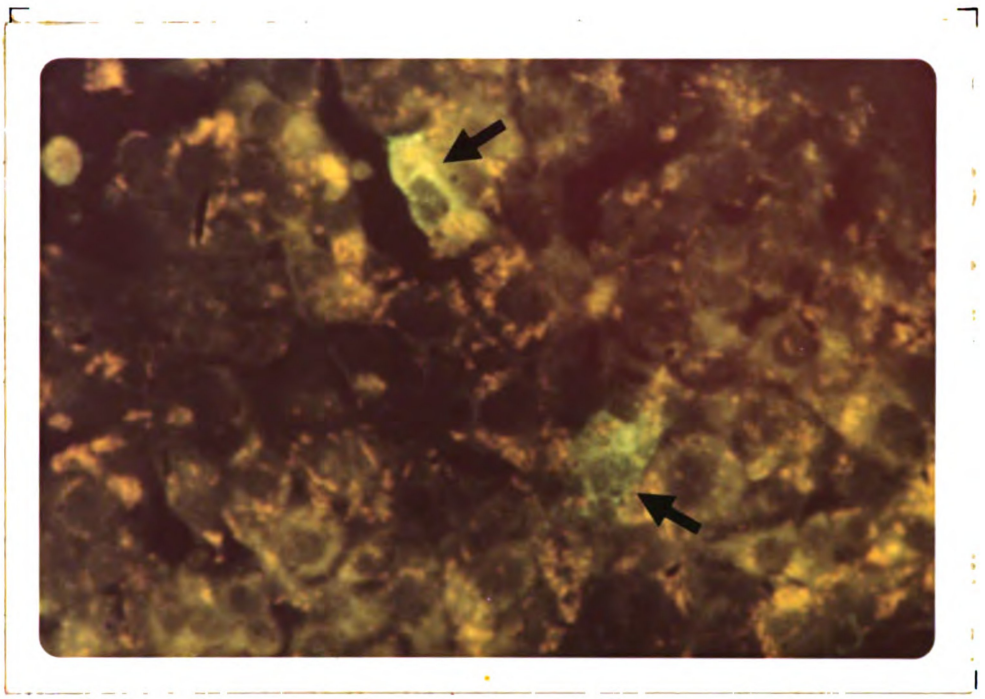

Fig. 1. -- Chicken embryo kidney cells 12 hours after infection with 250 PFU of IBV. Two isolated cells (arrows) are infected with IBV. ×500.

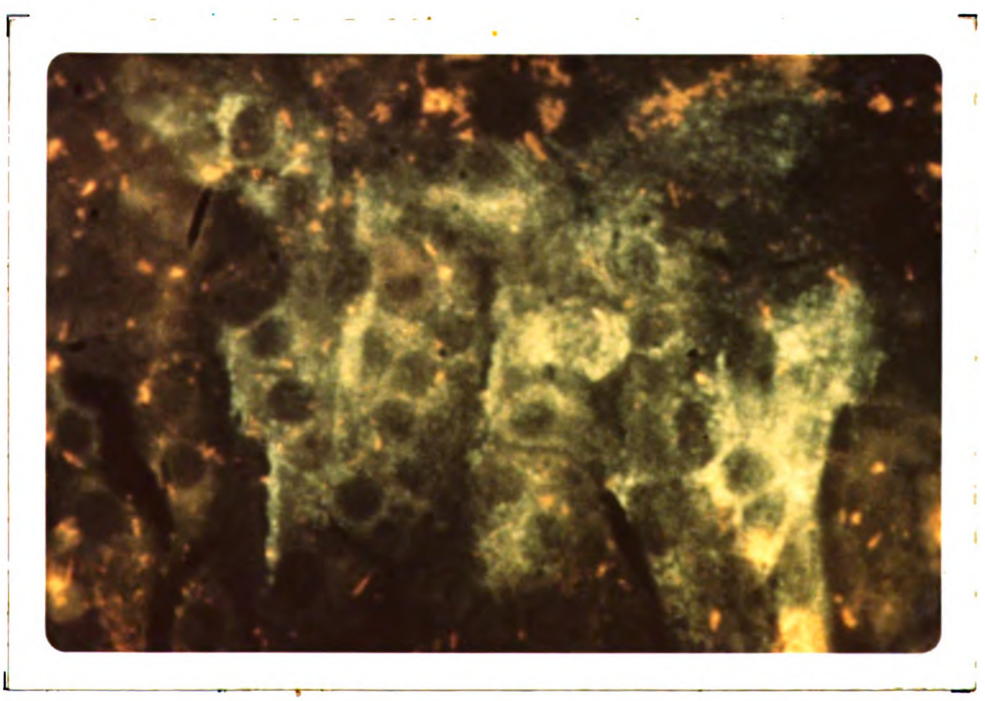

Fig. 2. -- Chicken embryo kidney cells 24 hours after infection with 250 PFU of IBV. ×500.

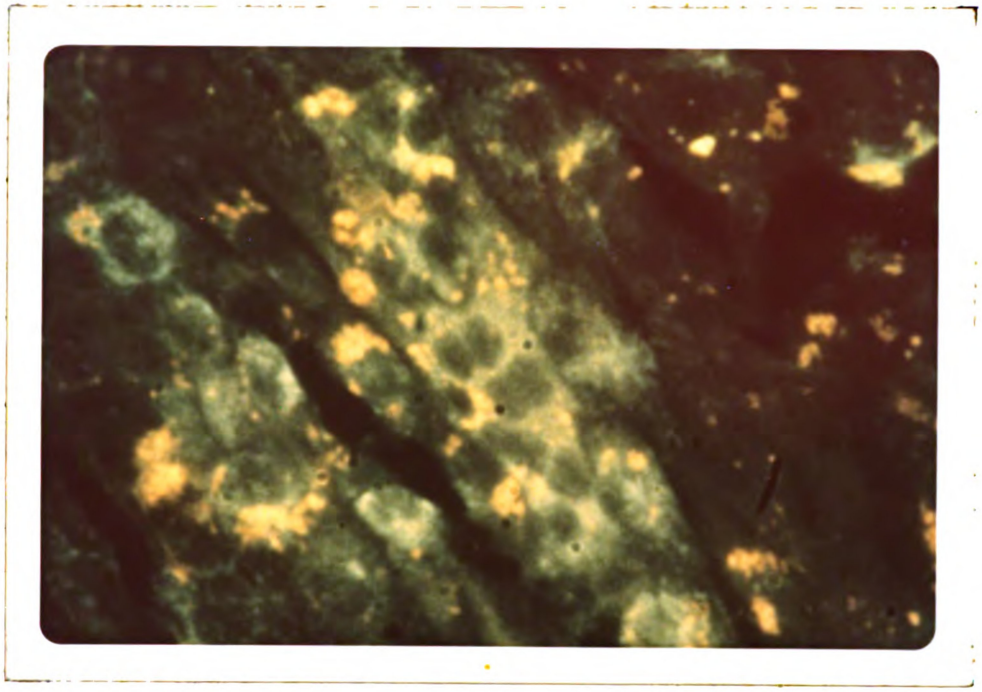

Fig. 3. -- Chicken embryo kidney cells 72 hours after infection with 16 PFU of IBV. ×500.

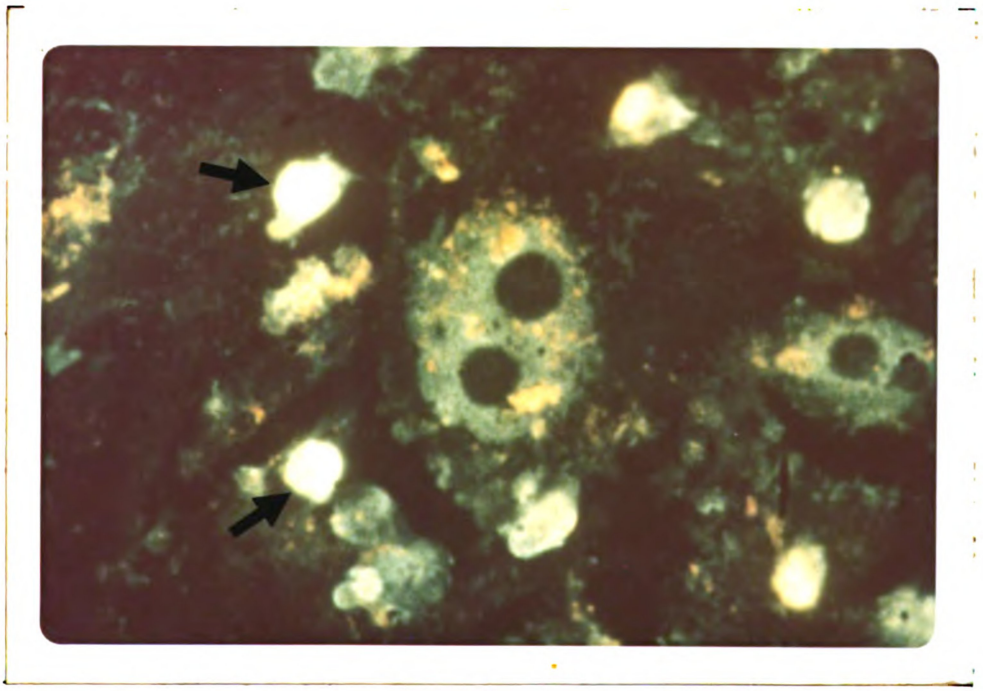

Fig. 4. -- Chicken embryo kidney cells 60 hours after infection with 100 PFU of IBV. The large cytoplasmic fragments (arrows) were characteristic of the CPE produced by IBV. ×500.

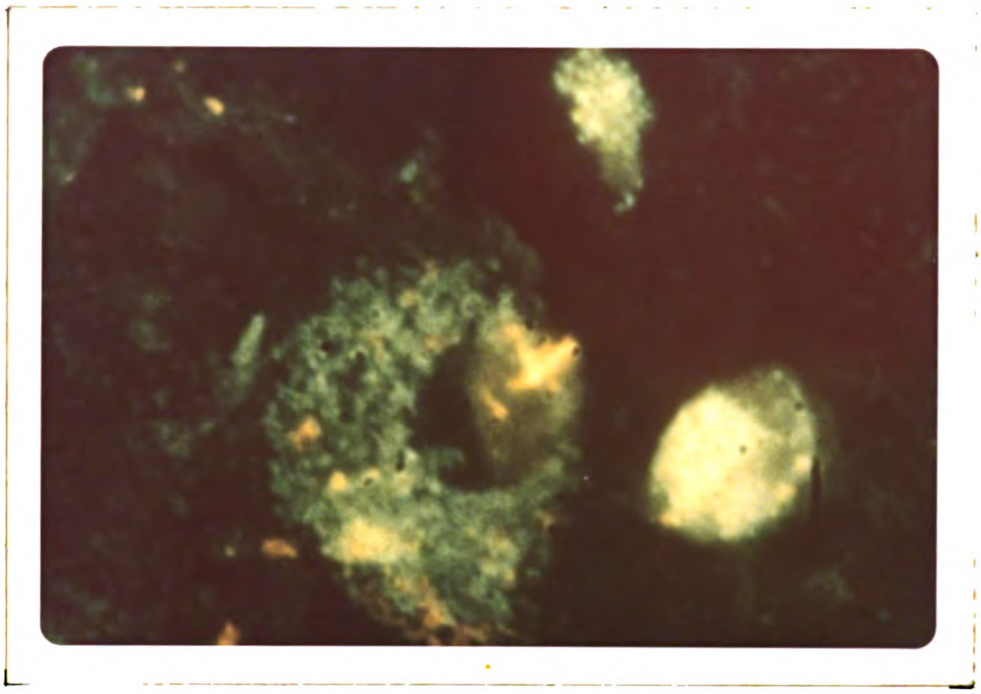

Fig. 5. -- Chicken embryo kidney cells 72 hours after infection with 100 PFU of IBV. ×1000.

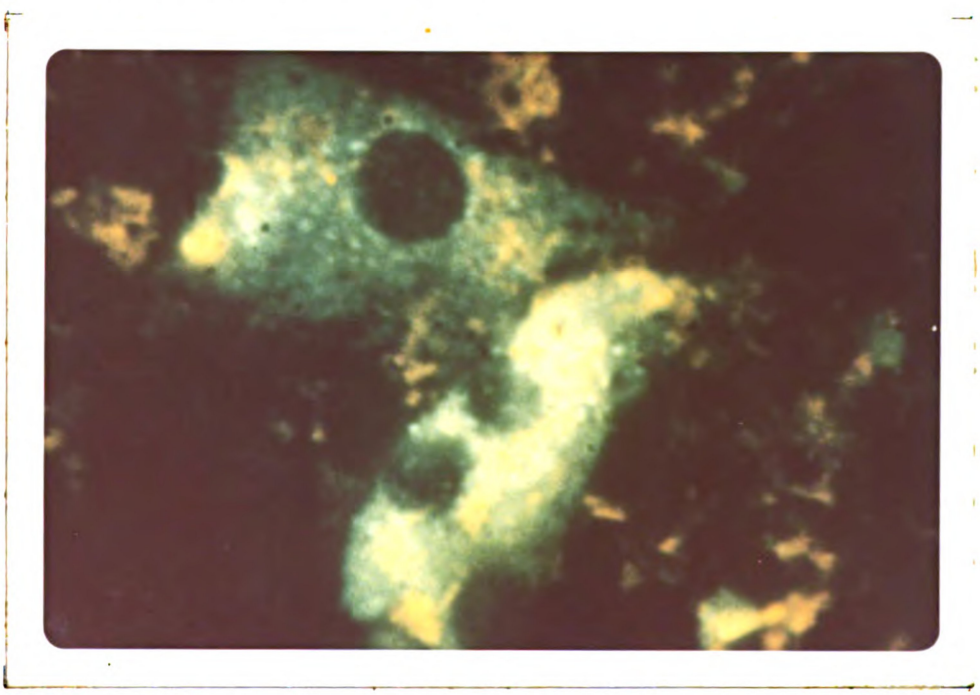

Fig. 6. -- Chicken embryo kidney cells approximately 12 hours after infection with 250 PFU of IBV. Note the antigen is distributed both diffusely and within spheroid granules. ×1000.

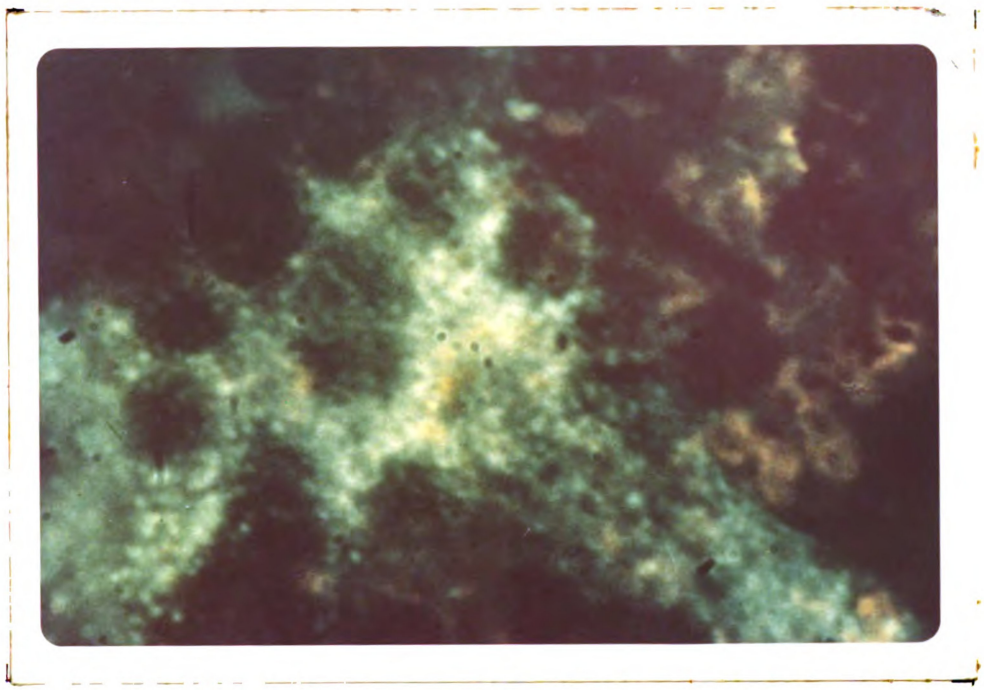

Fig. 7. -- Small syncytium in CEKC 24 hours after infection with 125 PFU of IBV. ×1000.

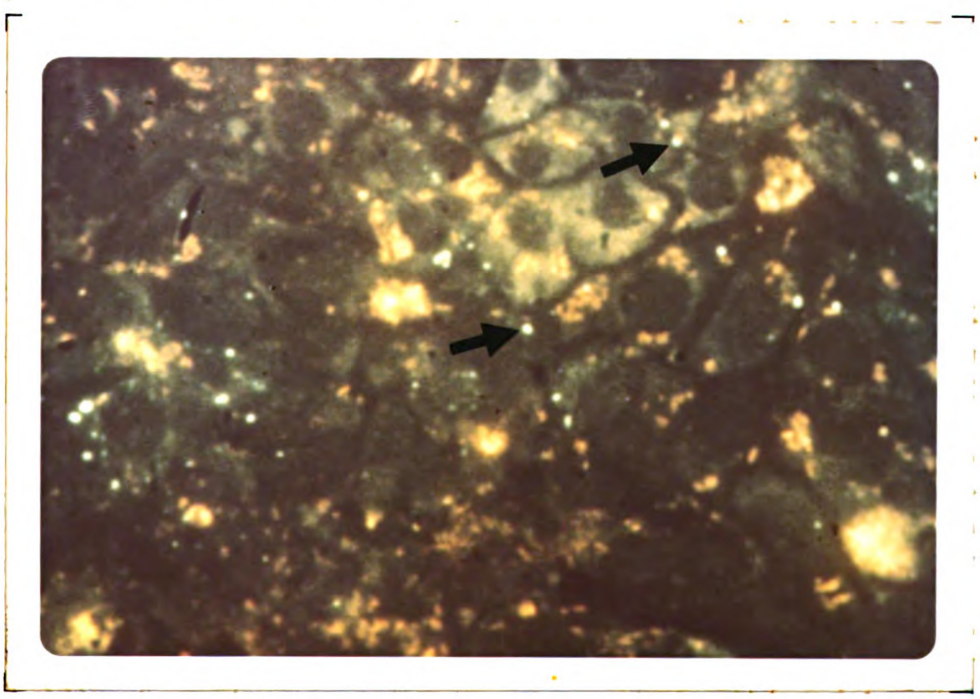

Fig. 8. -- Chicken embryo kidney cells 12 hours after infection with 10 PFU of NDV. Small spherical foci of early NDV replication (arrows). ×500.

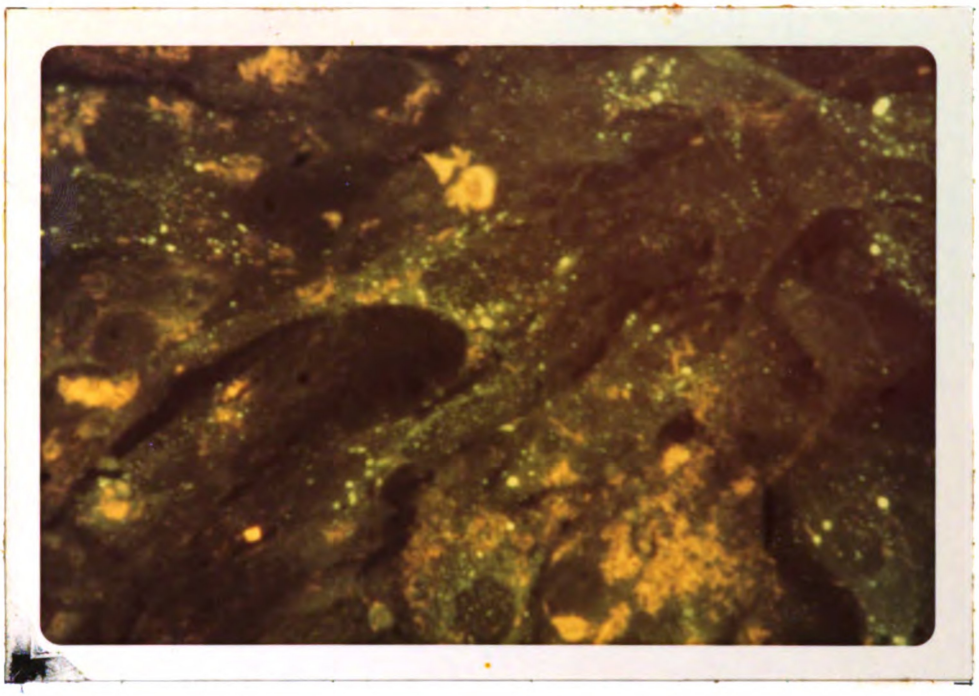

Fig. 9. -- Chicken embryo kidney cells 24 hours after infection with 70 PFU of NDV. The yellow autofluorescence is apparently not viral related as it is also present in non-infected controls. ×500.

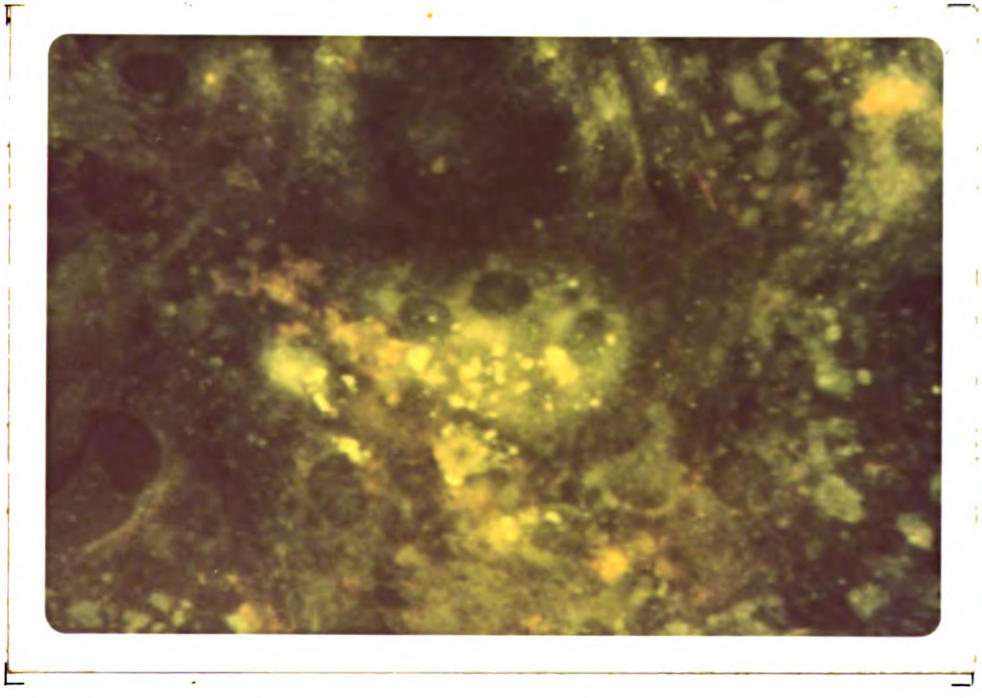

Fig. 10. -- Chicken embryo kidney cells 36 hours after infection with 70 PFU of NDV. ×500.

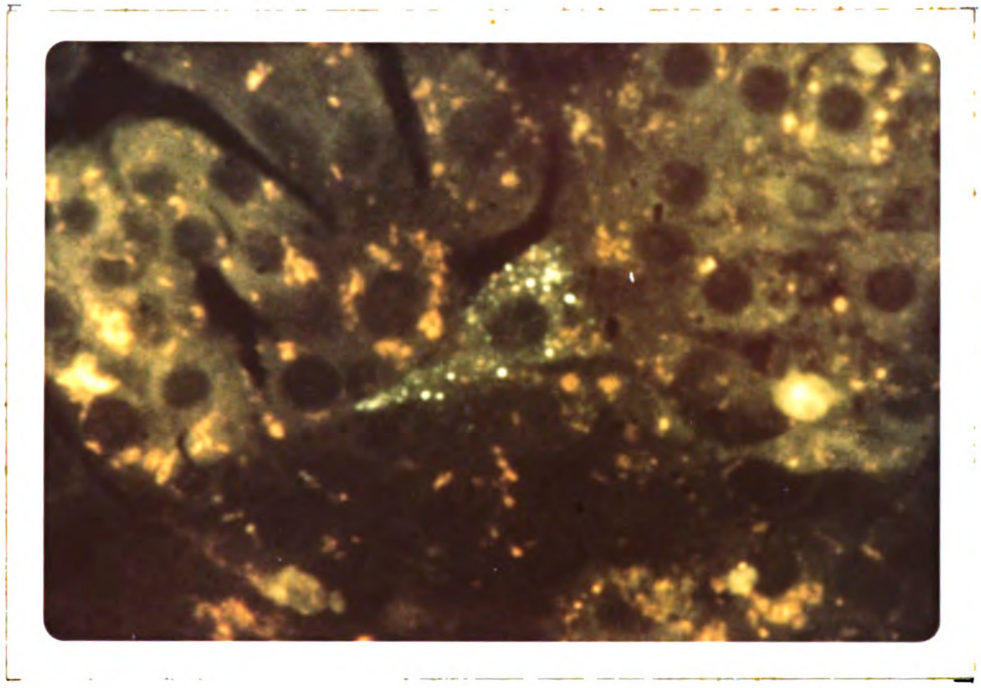

Fig. 11. -- Early stages of replication of NDV in CEKC 36 hours after infection with 35 PFU of NDV. ×500.

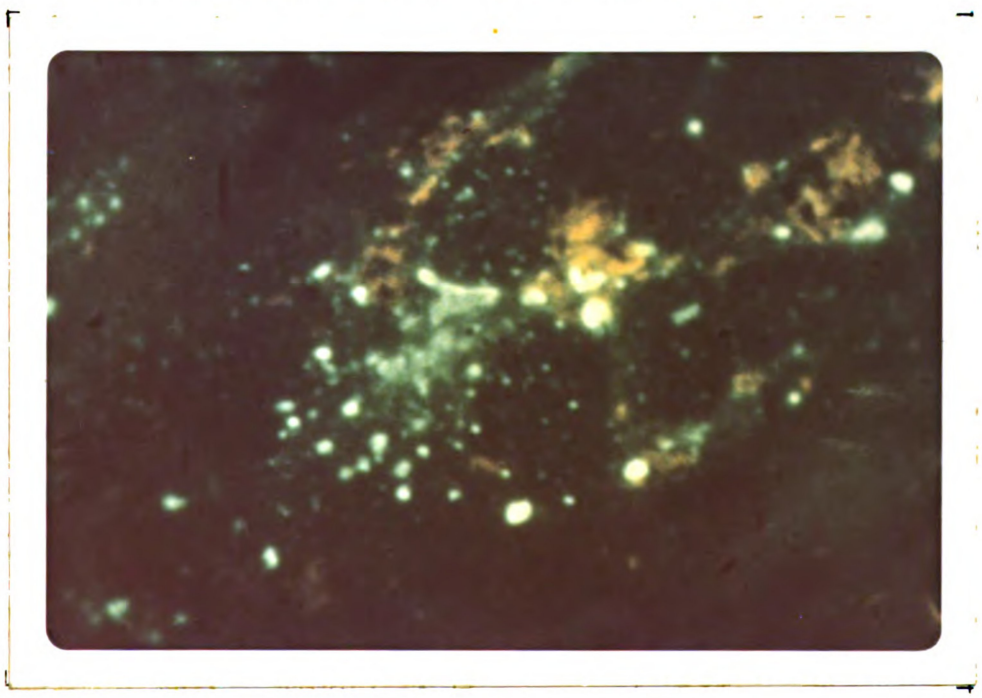

Fig. 12. -- Terminal stages of NDV replication in CEKC 24 hours after infection with 140 PFU of NDV. ×1000.

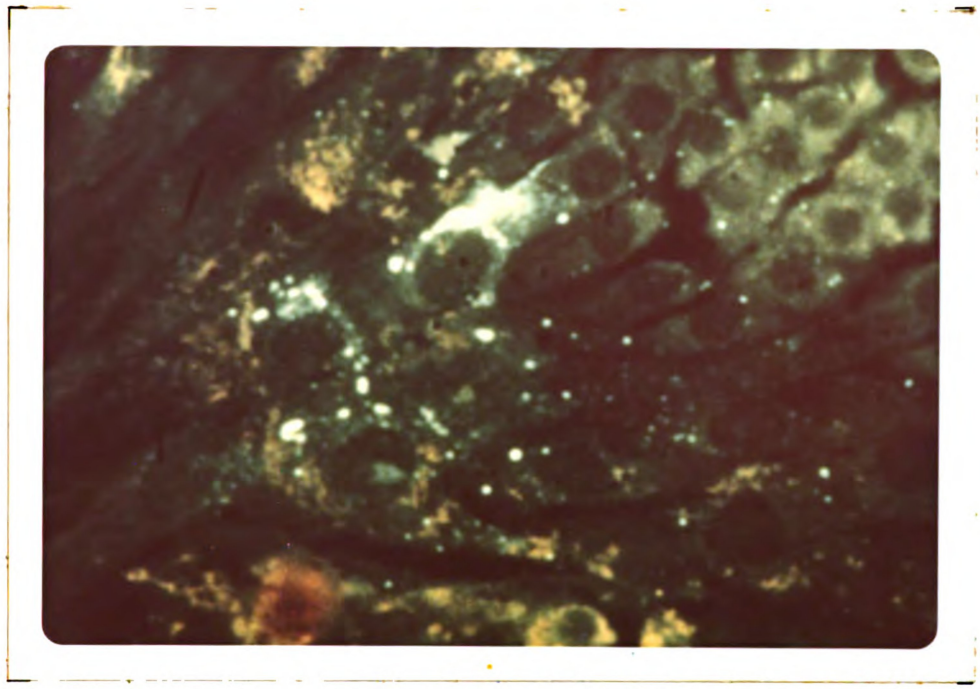

Fig. 13. -- At 24 hours after infection with IBV 100:NDV 10, NDV-specific fluorescent antibody indicates NDV within small clusters of cells and in cells radiating from the clusters. ×500.

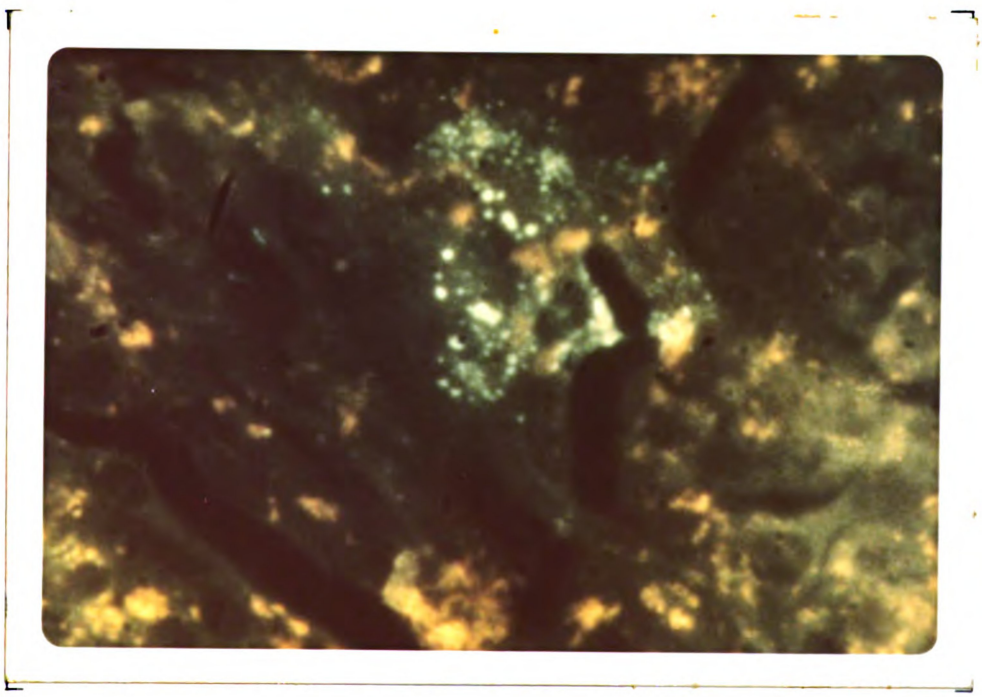

Fig. 14. -- At 36 hours after infection with IBV 100:NDV 10, NDV-specific fluorescent antibody indicates interference in replication of NDV. Infection has failed to spread from the clusters observed at 24 hours. ×500.

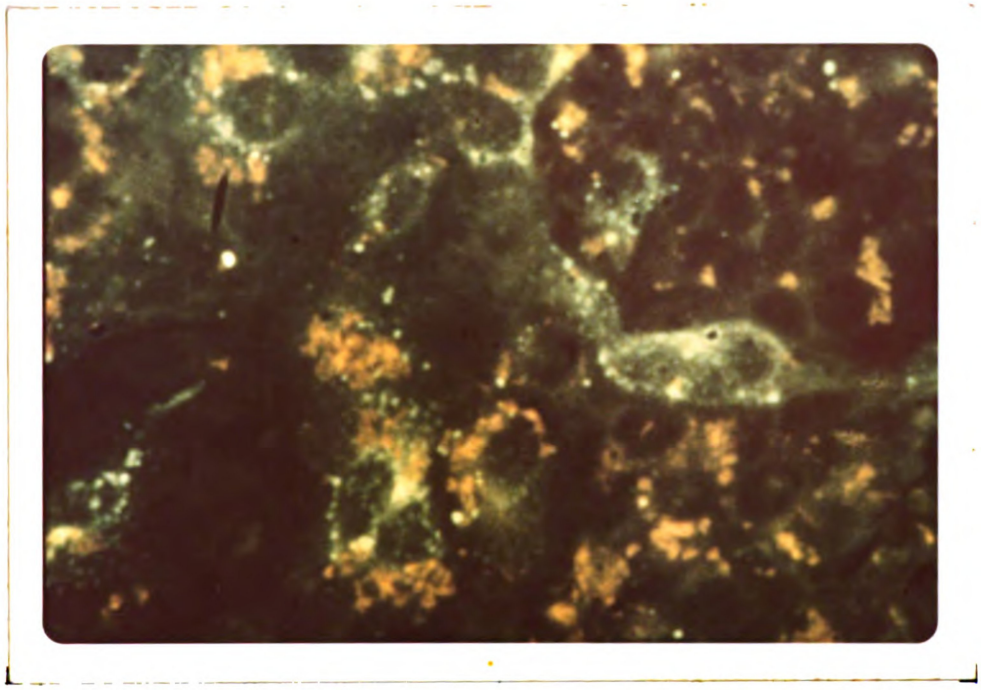

Fig. 15. -- At 48 hours after infection with IBV 100:NDV 10, NDV-specific antibody indicates replication of NDV has been limited to a small segment of the cell population. ×500.

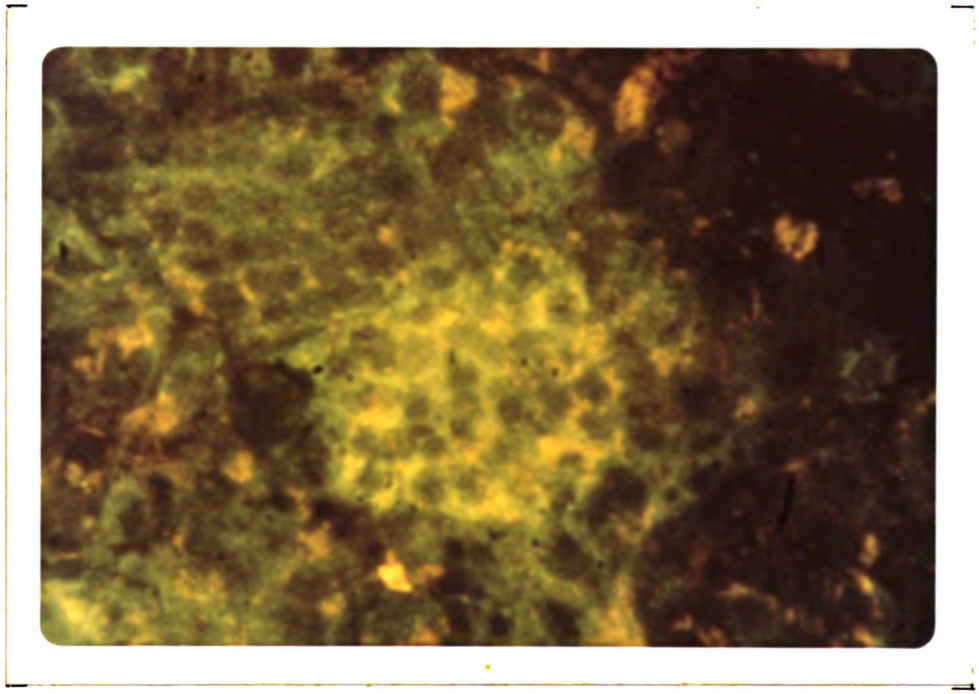

Fig. 16. -- At 48 hours following infection with IBV 100:NDV 10, IBV-specific antibody indicates most cells are infected with IBV. ×500.

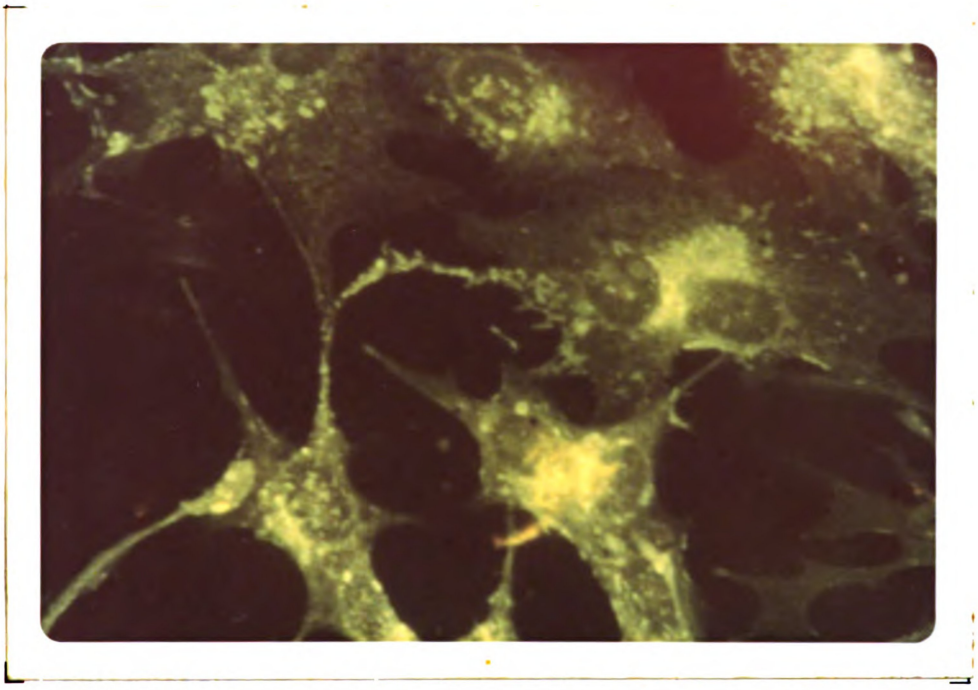

Fig. 17. -- Baby hamster kidney cells 8 hours after infection with 560 PFU of NDV. ×500.

DISCUSSION

According to electron microscopy, morphogenesis of IBV in the chorioallantoic membrane of chicken embryos (9) and in chicken embryo fibroblasts (31) appeared to occur within the cytoplasm by budding of viral structures into cytoplasmic vesicles or cisternae. Avian cells infected with IBV were demonstrated by fluorescence microscopy to contain diffuse and granular intracellular antigen (27). When stained with fluorochromes (2, 10), the fluorescence was from IBV ribonucleoprotein developing as intracytoplasmic granules. The intracytoplasmic fluorescence observed in the present study further supports these reports.

Using electron, phase, and bright field microscopy (1, 13, 15), intracytoplasmic inclusion bodies were detected in cells infected with NDV. Other reports of multiplication of NDV, studied by fluorescence microscopy (14, 32, 35, 41, 43, 45) describe intracytoplasmic fluorescence similar to that observed in the present study. Reda, Rott, and Schafer (35) determined that the nucleoprotein or S-antigen was localized within the segregated fluorescent cytoplasmic bodies, and the hemagglutinin or V-antigen

was diffusely located within the cytoplasm. The large masses of S-antigen appeared as inclusion bodies when examined by phase microscopy. In the present study, inclusion bodies were the most characteristic fluorescence feature of NDV replication.

On the basis of the time of appearance of CPE in CEKC, the replication cycle of NDV is much shorter than that of IBV and lesser amounts of NDV can be detected.

Neutralizing antibody was used in this study and the fluorescence in infected cells was considered to be related to a viral V-antigen-antibody complex, although S-antigens might have been present.

It was clearly evident that IBV and NDV could be specifically and differentially identified by fluorescence microscopy in single and dual infections of the same culture. There was no non-specific fluorescence.

Of particular importance was the evidence of interference of NDV by IBV when the ratios were 100:10 PFU, respectively. This confirms previous reports of interference of NDV by IBV using chickens, chicken embryos, and cell cultures as the indicator host (5, 7, 8, 21, 33). So far as known, this has not been reported on the basis of fluorescence microscopy. Normally, NDV alone would have infected all cells, but instead, IBV was dominant. Another

manifestation attributable to IBV interference was that destruction of cells in dually infected cultures occurred later than in singly infected NDV cultures of equal viral concentration.

The precise mechanism of IBV interference is not understood, but most work implies intracellular interference with no demonstrable involvement of interferon (33, 34). Intracellular interference is also indicated in this study. The invasiveness of NDV and rapid replication of infectious virions was greater than that of IBV; therefore, it does not seem feasible that the more slowly replicating IBV could dominate a cell culture by simply blocking viral cellular receptor sites. In infections in which there was delayed cellular destruction, NDV had initially infected the cells, but IBV altered the further synthesis of NDV.

The results obtained with BHK-21 were similar to those reported using African green monkey kidney cells (23), in that BHK-21 supported replication of NDV, but not IBV. Therefore, BHK-21 provides another means of differentiating IBV and NDV using a non-avian system.

The diffuse and granular fluorescence associated with IBV infection suggests a similarity to the fluorescence of S- and V-antigens within cells infected by NDV. Further studies using purified S- and V-antigens of IBV should provide an answer.

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