FLUORESCENT ANTIBODY STUDIES OF INFECTIOUS BRONCHITIS VIRUS

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FLUORESCENT ANTIBODY STUDIES OF INFECTIOUS BRONCHITIS VIRUS

By

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INTRODUCTION

Previous studies (Nazerian, 1960; Muldoon, 1960; and Spring, 1960) of the interaction of infectious bronchitis virus with chicken erythrocytes and chicken embryo kidney cell cultures prompted this investigation.

The purpose of the present investigation was to observe, by fluorescent microscopy, chicken embryo kidney cell-infectious bronchitis virus interaction, and the chicken erythrocyte-infectious bronchitis virus interaction using normal and trypsinized virus.

The objective was to determine the possible site of infectious bronchitis virus multiplication in chicken embryo kidney cell cultures.

REVIEW OF LITERATURE

Infectious bronchitis, a specific respiratory disease of chickens, is caused by a virus having an average diameter of 60 to 80 mu (Reagan et al., 1948; Reagen et al., 1950; Reagan and Brueckner, 1952; Nazerian, 1960). The virus (IBV) can be propagated readily in chicken embryos by various routes of inoculation. With serial passage in embryonating chicken eggs, the virus increases in virulence for the chicken embryo, and decreases in virulence and antigenicity for the chicken (Cunningham, 1957). The Beaudette, chicken embryo-adapted, strain of IBV can be readily cultivated in chicken embryo kidney cell cultures (CEKC) (Chomiak et al., 1958; Spring, 1960), whole and minced chorioallantoic membrane (CAM) (Hanks, 1949; Ferguson, 1958; Ozawa, 1959), and in chicken embryo liver cells (Fahey and Crawley, 1956), or HeLa cells (Davis, 1956).

Hemagglutination does not occur with unmodified IBV. If the virus is modified with trypsin, hemagglutination occurs with chicken and turkey erythrocytes (Corbo and Cunningham, 1959). The degree of hemagglutination is greatest with low egg-passage virus. The Beaudette chicken embryo-adapted

strain has little or no hemagglutinative activity. Enzymatic activity is not generally considered to be associated with hemagglutination using trypsin modified IBV; since virus which has been inactivated by heat prior to trypsinization can agglutinate chicken erythrocytes. Erythrocytes from which the virus has been eluted are reagglutinable (Muldoon, 1960).

On the basis of thermal inactivation, a thermostable Original (O) phase and a thermolabile Derivative (D) phase of IBV have been demonstrated. Only the O phase is antigenic and pathogenic for chickens (Singh, 1960).

Neutralizing antibodies can be produced in chickens using virulent IBV, trypsin modified, and heat inactivated IBV (Muldoon, 1960; Oshel, 1961).

On the basis of reciprocal neutralization tests, two sero-types of IBV, designated Massachusetts and Connecticut, have been identified (Jungherr et al., 1956; Hofstad, 1958).

Heidelberger et al. (1933); Hopkins and Wormall (1933); Marrack (1934); and Fieser and Creech (1939), coupled chemicals such as sodium salts of benzidine, phenyl isocyanate, anthracene, and 1,2-benzanthracene series with egg albumin, normal horse serum, anti-typhoid serum, and amino

acids. The compounds fluoresced, and were macroscopically and microscopically visible when excited with polychromatic light.

Coons et al. (1941) investigated the use of colored compounds to study antigen-antibody reactions, but found the color intensity of the conjugates to be very weak when illuminated with polychromatic light. From these studies the use of a compound which would fluoresce in ultraviolet light was evolved. The fluorescent antibody, or immunofluorescent, technique subsequently developed by Coons, et al. (1942) is based on the conjugation of an antibody with a compound which will emit visible light when illuminated by ultraviolet rays. This is accomplished by passing polychromatic light, emitted by a mercury vapor lamp, through an exciter filter, which prevents passage of light rays which have a wave length below 300 mu and above 400 mu. The specificity of the reaction between anti-soluble pneumococcal polysaccharide serum prepared in rabbits, and tissues from pneumococcal infected mice was demonstrated by this technique (Coons et al., 1942) and many others.

Conjugation of either whole serum, or globulin obtained by fractionation of serum by 50 per cent saturated ammonium

sulfate, 23 per cent sodium sulfate, cold alcohol or resin absorption (Coons et al., 1942; Hobson and Mann, 1957; Cohn et al., 1950; Chadwick et al., 1958), depends upon the reaction of the globulin fraction with a fluorescent compound. The reaction is thought to occur when the free amino groups of the protein react with the fluorescent compound to form a carbamide linkage (Coons et al., 1942) as follows:

Coons et al. (1942) conjugated globulin by the reaction of an isocyanate derivative of fluorescein with the free amino groups of the protein using acetone, dioxane, carbonate buffer (pH 9) and phosgene. Hiramato et al. (1958) used the same procedure (Coons et al., 1942) to conjugate globulin with tetramethylrhodamine by way of the isocyanate linkage. Goldman and Carver (1957) modified the conjugation procedure (Coons et al., 1942) by omitting acetone and dioxane. According to Marshall et al. (1958) conjugates prepared without acetone had brighter fluorescence at the same wave length of light than those prepared with acetone.

Riggs et al. (1958) conjugated globulin with an isothiocyanate derivative of fluorescein which eliminated the use of phosgene. This reaction with the protein molecule is presumably similar to, if not the same as, the reaction described by Coons et al. (1942) and Hiramato et al. (1958). The intensity of emitted light with isothiocyanate derivatives was four fold greater than with isocyanate derivatives of fluorescein (Marshall et al., 1958).

Weber (1952) used 1-dimethyl naphthalene to label globulin, but the compound was of limited value due to the low intensity of the fluorescence. Uehleke (1958) demonstrated that the light intensity of globulin conjugated with 1-dimethyl-5-naphthalene sulfonic acid was about one-third that of fluorescein, when excited by a wave length of 365 mu. The emission intensity was three times greater, with 3 hydroxypyrene-5,8,10-trisulfonic acid, than that of fluorescein when a wave length of 530 mu was used. The reaction of the sulfonyl chloride derivatives with protein was essentially the same as described by Coons et al. (1942).

Derivatives of fluorescein isothiocyanate, and rhodamine isothiocyanate are commonly employed to conjugate globulins. The isothiocyanate derivatives are highly stable, and readily

available from commercial sources; while the isocyanate derivatives are not stable and are difficult to obtain. The isothiocyanate derivative of fluorescein has a yellow-green fluorescence with a maximum intensity at a wave length of 550 mu, while the isothiocyanate derivative of lissamine rhodamine B 200 (Rhodamine B) has a red to orange fluorescence with a maximum intensity at 640 mu. These derivatives of fluorescein and rhodamine have approximately the same intensity of emitted light at a wave length of 365 mu, but the eye is more sensitive to the green of fluorescein, than to the red of rhodamine; and red autofluorescence is more commonly encountered than green autofluorescence.

Labeling of globulin with fluorescein does not alter the ability of the protein to be used in other serological tests (Mayersbach and Pearse, 1956), but an increased electrophoretic mobility of the fluorescein-labeled gamma-two globulin has been reported by Curtain (1958) and Uehleke (1959).

With the direct method (Coons et al., 1942), known antiserum conjugated with a fluorescent compound is placed directly on the specimen to be examined for the antigen. This method is rapid; sensitive and nonviable pathogens

may be used. The major disadvantage of the direct method is that the conjugate can only be used with the homologous antigen. The indirect method (Weller and Coons, 1954), consists of forming an antigen-antibody complex using homologous antigen and antibody; and then adding a fluorescent anti-two-globulin conjugate to the complex. This method has the same general advantages and disadvantages as the direct method. There is the added advantage that the conjugate can be used with a large number of antigens, as long as the specific antibody will react with the fluorescent anti-two-globulin conjugate. The major disadvantage of the indirect method, is that non-specific reactions are increased; but these can be reduced by using as pure a conjugate as possible, and including sufficient controls in the test procedure. Goldwasser and Shepard (1958) described the complement fixation staining procedure, which used the same technique described by Weller and Coons (1954).

Coons et al. (1942), and Coons and Kaplan (1950), demonstrated optimal reactions with a fluorescent labeled antiserum when 5 mg of fluorescein isocyanate or isothiocyanate per 100 mg of crude globulin protein were used. The ratio of molecular fluorescein to protein was about 2 to 1 when conjugation was complete.

Non-specific staining may be encountered with some conjugated globulins. According to Curtain (1958) serologically active and inactive components of fluorescein labeled antiserum fractionated by convection electrophoresis may produce non-specific staining. Non-specific staining can be reduced by adsorption of the conjugate with acetone dried tissue powder (Coons et al., 1951), adsorption and elution of the conjugate on diethylamino-ethyl cellulose (Riggs et al., 1960), or pretreatment of the specimen with alkaline buffers, and/or rhodamine conjugate of normal serum globulin. Also freeze drying and formalin fixation of the conjugate aid in preventing non-specific reactions.

The fluorescent antibody technique has been used by a great number of investigators in immunological studies and in host-cell interactions. This technique has been successfully used to study the host-cell reaction with measles virus (Cohen et al., 1955 and Enders, 1954), the foamy agent of monkey kidney cell cultures (Carski, 1959), poliomyelitis (Buckley et al., 1955), psittacosis (Buckley, 1956), and type 4 adenovirus (Boyer et al., 1959).

Host-cell interactions can be followed using the fluorescent antibody technique as demonstrated with influenza virus

(Boand et al., 1959), canine distemper virus (Hanson et al., 1957), Newcastle disease virus (Liu and Coffin, 1957), measles virus (Prince and Ginsberg, 1957), and mixed infections with mumps and influenza viruses (Watson, 1956; Rapp et al., 1959).

This technique has been used for the diagnosis of canine distemper, rabies, and human influenza virus infections (Coffin and Liu, 1957; Goldwasser and Shepard, 1958; Goldwasser et al., 1959 and Liu, 1956).

MATERIALS AND METHODS

VIRUSES

The Beaudette strain, IBV42, adapted to chicken embryo kidney cells (CEKC), (Spring, 1960), was used to study the multiplication of the virus in CEKC. The Massachusetts strain, IBV41-7 (Van Roekel et al., 1950) was used to study the virus-chicken erythrocyte reaction employing trypsinized virus. The strains of IBV are identified by the North Central Repository Code Number, and the number of chicken embryo or cell culture passages; e.g. IBV41-7 indicates the 7th egg passage of IBV41, and IBV42-91C indicates the 91st CEKC passage of IBV42.

Strain IBV41-7 was obtained by inoculating 0.1 ml of a 10^{-2} dilution of IBV 41-6 into the allantoic cavity of ten-day-old embryonating chicken eggs. The infected allantoic fluid was collected on the third post inoculation day, pooled, dispensed into screw cap vials, and stored at -60 C. Cultures of 24-hour-old CEKC in tubes were used to propagate IBV42-91C. The cultures were inoculated with 0.1 ml of a 10^{-1} dilution of IBV42-90C. The infected extracellular fluid was collected at 24 hours, pooled, dispensed

into screw cap vials, and stored at -60 C. At the time of use, the fluids were thawed at room temperature, and centrifuged at 437 X G at 4 C for 10 minutes to sediment the debris from the cell cultures, or the precipitate formed on freezing and thawing of the allantoic fluid.

Brewer Thioglycollate Medium (Difco) was used for bacteriological sterility tests.

PREPARATION AND INOCULATION OF CHICKEN EMBRYO KIDNEY CELLS

The kidneys from 16 to 20-day-old chicken embryos were removed asceptically and washed in Hank's Balanced Salt Solution (BSS) in a petri dish. The kidneys were minced into pieces approximately 0.5 to 1.0 mm^3 and then washed with several changes of BSS. The pieces were then transferred to a 250 ml, fluted Erlenmyer flask containing a "Teflon" covered magnet, and 0.25 per cent trypsin (Difco 1:250), pH 8.0 to 8.6, was added. For each pair of kidneys, 10 ml of trypsin were used. The flask was placed over a "Magnestir," and after trypsinization at 37 C for 30 minutes, the cell suspension was filtered through eight layers of sterile cheese cloth. The cells were centrifuged at 437 X G for five minutes at 4 C. The supernatant fluid was decanted and the packed cells were resuspended in BSS. This procedure

was repeated three times. The packed cells from the last washing were resuspended and diluted 1 to 400. There were approximately 1 X 10^6 cells per ml as determined by a cell count employing a hemacytometer. The growth medium contained 0.5 gm of lactalbumin hydrolysate, 5.0 ml of bovine serum, 25,000 units of penicillin and 0.5 mg of dihydrostreptomycin in 100 ml of BSS. Leighton tubes, 16 X 125 mm, containing number 1 glass cover slips, 11 X 22 mm, were seeded with 1 ml of the cell suspension per tube, sealed with a white rubber stopper and incubated at 37 C in a horizontal position.

The cell cultures were incubated for 22 to 28 hours, at which time the monolayers were about 50 to 75 per cent complete. Each test procedure was run in duplicate which required sixteen cell culture tubes, and tests were run at seven time intervals. The growth medium was removed, and fifty-six cell-culture tubes were inoculated with 0.1 ml of undiluted IBV42-91C and allowed to adsorb for 20 minutes. After the 20 minute adsorption period sixteen cell culture tubes; eight inoculated and eight uninoculated cell cultures, were washed three times with BSS, and fixed with acetone; and the virus was stained by the indirect fluorescent antibody technique. All remaining cell culture tubes were

washed three times with BSS; and 1.0 ml of maintenance medium, containing 0.5 gm of lactalbumin hydrolysate, 2.0 ml of bovine serum, 25,000 units of penicillin, and 0.5 gm of dihydrostreptomycin in 100 ml of BSS; was added to each cell culture tube. At time intervals of 30, 60, 120, 240, 360, and 720 minutes post adsorption, sixteen cell cultures, eight inoculated and eight uninoculated, were washed three times with BSS; fixed with acetone; and the virus was stained by the indirect fluorescent antibody technique.

TITRATION OF IBV41-7 IN EMBRYONATING CHICKEN EGGS

The embryo infective dose$_{50}$ (EID_{50}) of IBV41-7 was determined by inoculating ten-day-old chicken embryos with serial ten-fold dilutions of the virus prepared in nutrient broth. All ingredients were kept in an ice bath during the preparation of the dilutions to prevent possible thermal inactivation of the virus. Five embryos were inoculated per dilution using 0.1 ml per embryo. A positive response was one or more of the following: embryo mortality, curling of the embryos, deformed feet compressed over the head, thickened amnionic membrane or urates in the kidneys (Cunningham, 1960). All embryos were examined on the 5th post-inoculation day.

TITRATION OF IBV42-91C IN CHICKEN EMBRYO KIDNEY CELLS

Serial ten-fold dilutions of the virus were prepared using BSS containing 0.5 per cent lactalbumin hydrolysate as the diluent (Cunningham, 1960). All ingredients were kept in an ice bath during the preparation of the dilutions to prevent possible thermal inactivation of the virus. Each dilution of virus was used to inoculate three cell cultures, 0.2 ml per culture. The cultures were incubated at 37 C for 48 hours, and examined microscopically for cytopathic effects (CPE). The end point of the viral activity was considered to be the highest dilution of virus in which two of the three cell cultures demonstrated CPE.

HEMAGGLUTINATION

Blood was collected by cardiac puncture from Single Comb White Leghorn cockerels, and dispensed into test tubes containing 1 ml of a 0.5 per cent sodium citrate solution for each 6 ml of blood. The erythrocytes were washed three times by centrifugation at 437 X G in 0.85 per cent saline. The packed erythrocytes from the third washing were diluted to a two per cent suspension with 0.85 per cent sodium chloride.

One ml of a one per cent solution of trypsin (Difco

1:250) was mixed with two ml of IBV41-7 infected allentoic fluid. After incubation at 37 C in a water bath for three hours, one ml of egg white trypsin inhibitor (Nutritional Biochemical Co.) was added (Corbo and Cunningham, 1959; Muldoon, 1960). The trypsinized virus was dispensed into screw-cap vials and stored at -30 C until used.

To a 12 X 75 mm tube was added 0.25 ml each of 0.85 per cent sodium chloride, trypsinized virus, and 0.5 per cent washed chicken erythrocytes. The tube was well shaken to mix the ingredients thoroughly. The mixture was incubated at room temperature for 30 minutes at which time hemagglutination was complete. The agglutinated cells were then removed with a capillary pipette, and spread thinly on a 22 X 22 mm number 1 coverslip. The cells were air dried, fixed with absolute methanol, air dried, and stained for fluorescent microscopy by the indirect procedure.

FLUORESCENT MICROSCOPY

An American Optical Company "Fluorestar" microscope equipped with a vertical monocular body, 5X eyepiece, achromatic N.A. 1.40 condenser and darkfield stop; a 97X achromatic objective, N.A. 1.25, containing a darkfield funnel stop was used. A Mer-Arc Illuminator (Nems Clarke

Co.) power unit, and an Osram HBO-200 mercury arc lamp were used for ultra violet radiation. A heat absorbing filter was inserted between the illuminator and the exciter filter. An exciter filter, Corning 5840 (55 per cent transmission of the ultra violet rays at a wave length of 365 mμ) was inserted between the heat absorbing filter and the mirror of the microscope. A Corning 5860 (21 per cent transmission of the ultra violet rays at a wave length of 365 mμ) was used in the early stages of the investigation. Fluorescence observed with this filter did not have the desired intensity, and the filter was not used for any of the results reported. A Wratten 2B glass barrier filter was inserted between the objective and the eyepiece of the microscope.

PREPARATION OF ANTI-IBV41 CHICKEN SERUM

Single Comb White Leghorn cockerels were placed in individual Horsfall-Baur type stainless steel isolation units; 0.2 ml of a 10^{-2} dilution of IBV41-7 in nutrient broth was administered by the intranasal and ocular routes. Inoculations were made on day 0, 14, 63, and 91 post isolation. On the 119th day after the first inoculation the chickens were exsanguinated and the sera pooled, filtered through a

Seitz filter, and dispensed into two dram vials and stored at -30 C until used.

NEUTRALIZATION TEST

Serial ten-fold dilutions of IBV41-7 were prepared using nutrient broth as the diluent. Anti-IBV41 chicken serum, 0.3 ml, was mixed with 0.3 ml of each dilution of virus, and 0.1 ml of each virus serum mixture was inoculated into ten-day-old chicken embryos--five embryos per dilution. All ingredients were kept in an ice bath during the preparation of the dilutions to prevent possible thermal inactivation of the virus. Titration of IBV41-7 was carried out at the same time as the neutralization test by using each virus dilution to inoculate five ten-day-old chicken embryos with 0.1 ml per embryo. The neutralization index (NI), a measure of the reduction of viral activity by neutralizing antibody, is the difference between the virus titer and the serum titer (Cunningham, 1960).

STAINING PROCEDURE

Attempts to prepare a satisfactory conjugated globulin for use in the direct staining technique were unsuccessful due to the extreme amount of non-specific fluorescence

present. Attempts to eliminate the non-specific fluorescence by absorbing the globulin with activated charcoal were unsuccessful.

Fluorescein isothiocyanate conjugates of rabbit anti-chicken globulin, goat anti-chicken globulin, and rhodamine bovine albumin (Microbiological Associates, Inc., Bethesda, Maryland) were used. The rabbit anti-chicken globulin conjugate was used initially, but the supply became exhausted and resort was made to goat anti-chicken globulin conjugate which was on hand.

The indirect staining procedure was as follows:

1. The specimen is fixed to the slide, 0.8 to 1.0 mm thick or to a number 1 coverslip, with acetone for 5 minutes.
2. Air dry for 30 minutes at 37 C, or at room temperature for 1 hour.
3. Unlabeled specific IBV antiserum from chickens is applied to the fixed specimen for 30 minutes in a humid atmosphere at room temperature.
4. The specimen is washed in 0.1 M phosphate buffered saline at pH 7.2 for 30 minutes with 3 changes of buffer solution.
5. The specimen is covered with rabbit or goat anti-chicken globulin conjugate and placed in a humid atmosphere for 30 minutes at room temperature.

6. The specimen is washed in 0.1 M phosphate buffered saline at pH 7.2 for 30 minutes with 3 changes of buffer solution.
7. Counter stain the specimen with rhodamine bovine albumin, 1 to 100 dilution, for 5 minutes to reduce non-specific staining. Remove excess counter stain with 0.1 M phosphate buffered saline at pH 7.2 for 10 minutes with 3 changes of buffer solution.
8. Mount the coverslip with the specimen on a glass slide, or place a coverslip over the specimen if originally fixed on a slide, using phosphate buffered glycerol, ph 7.0.
9. Examine by fluorescent microscopy.

Goat or rabbit anti-chicken globulin conjugated with fluorescein isothiocyanate produces a yellow-green fluorescence of high intensity when added to the specific antigen-antibody reaction. The counter stain, an isothiocyanate conjugate of rhodamine bovine albumin, produces a light red to pink non-specific fluorescence.

Controls consisting of the following combinations of reagents were used with each test procedure.

1. Cell-virus complex to which normal goat or rabbit serum, goat or rabbit anti-chicken globulin conjugate, and rhodamine bovine albumin counter stain had been added.
2. Cell-virus complex to which normal chicken serum, goat or rabbit anti-chicken globulin conjugate, and rhodamine bovine albumin counter stain had been added.
3. Uninfected cells to which anti-IBV41 chicken serum, goat or rabbit anti-chicken globulin conjugate, and rhodamine bovine albumin counter stain had been added.
4. Uninfected cells to which normal chicken serum, goat or rabbit anti-chicken globulin conjugate, and rhodamine bovine albumin counter stain had been added.
5. Uninfected cells to which normal goat or rabbit serum, goat, or rabbit anti-chicken globulin conjugate, and rhodamine bovine albumin counter stain had been added.
6. Uninfected cells to which goat or rabbit anti-chicken conjugate, and rhodamine bovine albumin counter stain had been added.

PHOTOGRAPHY

Kodak Super Ektochrome and Ansco Super Anscochrome film were used initially, but the Kodak Super Ektochrome did not give satisfactory color reproduction of the specimen.

Ansco Super Anscochrome produced transparencies which could be printed when an exposure time of 10 to 15 minutes was used. Transparencies for projection could be obtained using exposure times of 3 to 5 minutes.

The prolonged exposure time was necessary due to the low intensity of the fluorescence. It was necessary to have copies of the control slides made to obtain color prints.

RESULTS

Rabbit anti-chicken globulin conjugate was used for some tests by the indirect procedure (Figs. 1 to 16) and goat anti-chicken globulin conjugate was used for other tests (Figs. 17 to 22) in the indirect procedure. The use of goat anti-chicken globulin conjugate was necessitated when the supply of rabbit anti-chicken globulin conjugate was exhausted.

Unconjugated anti-IBV41 chicken serum added to erythrocytes agglutinated by trypsinized IBV41-7 produced a yellow-green fluorescence of high intensity on the surface of the erythrocytes (Fig. 1). The halo effect or widely diffused fluorescence around the group of agglutinated erythrocytes was not nearly as pronounced microscopically as it is in the photomicrograph. It was necessary to expose the film for 15 minutes to obtain acceptable photomicrographs; and this unusually long period probably distorted the intensity of the fluorescence present, since fluorescein isothiocyanate is photosensitive, and the intensity of the emitted fluorescence decreases with prolonged exposure to ultraviolet light.

Unlabeled, normal, chicken serum added to erythrocytes

agglutinated by trypsinized IBV41-7 and treated with rabbit anti-chicken globulin conjugate produced a yellow-green fluorescence of low intensity. This was considered to be non-specific staining due to the adsorption of the serum to the cell wall (Fig. 2). As determined by subsequent experiments, the non-specific fluorescence could be minimized if rhodamine bovine albumin counter stain was used to react with non-specific antigens present. The fluorescence produced by the fluorescein isothiocyanate when combined with the antigen-antibody complex is of greater intensity than that of the rhodamine isothiocyanate antigen-antibody combination.

A yellow-green fluorescence was present on the surface of erythrocytes agglutinated by trypsinized IBV41-7 when treated with unlabeled anti-IBV46 chicken serum, and rabbit anti-chicken globulin conjugate (Fig. 3). The fluorescence was less intense than that with homologous anti-IBV41 chicken serum (Fig. 1).

Unlabeled rabbit serum added to erythrocytes agglutinated by trypsinized IBV41-7 produced a natural red-blue fluorescence of low intensity (Fig. 4).

Natural fluorescence only was present when anti-IBV41 chicken serum (Fig. 5), anti-IBV46 chicken serum (Fig. 6), unlabeled rabbit serum (Fig. 7), or rabbit anti-chicken globulin conjugate (Fig. 8) were added to erythrocytes that had not been agglutinated with trypsinized IBV41-7.

Rhodamine bovine albumin was used as a counter stain in all studies of chicken embryo kidney cells. The counter stain imparts a rose color to uninfected cells and masks non-specific staining.

The titer of IBV42-91C used to infect the CEKC was EID_{50} 8.7 per 0.1 ml of infected tissue culture fluid. Extra cellular virus produced by the CEKC at 30, 60, 90, 120, 360, and 720 minutes post inoculation was EID_{50} 4.0, 7.5, 8.0, 8.5, 6.5, 6.0 and 6.0, respectively.

The titer of IBV41-7 used in trypsinization and agglutination of chicken erythrocytes was found to have an EID_{50} 6.63. The neutralization index of anti-IBV41 chicken serum used in all test procedures demonstrated an $i.d._{50}$ NI 5.0.

Unlabeled anti-IBV41 chicken serum added to CEKC infected with IBV42-91C for two hours, and treated with rabbit anti-chicken globulin conjugate produced a pale yellow fluorescence of individual intracytoplasmic particles (Fig. 9).

Fluorescence of high intensity was not produced with CEKC which has been infected with IBV42-91C for two hours and then treated with normal chicken serum (Fig. 10), or unlabeled rabbit serum (Fig. 12). When anti-IBV46 chicken serum was added to CEKC infected for two hours with IBV42-91C, and then treated with rabbit anti-chicken globulin conjugate, fluorescence of lower intensity (Fig. 11) than that of anti-IBV41 chicken serum (Fig. 9) was present.

Natural fluorescence only was present when anti-IBV41 chicken serum (Fig. 13), anti-IBV46 chicken serum (Fig. 14), unlabeled rabbit serum (Fig. 15), or rabbit anti-chicken globulin conjugate (Fig. 16) were added to uninfected CEKC controls.

Unlabeled anti-IBV41 chicken serum and goat anti-chicken globulin conjugate added to CEKC infected with IBV42-91C for twelve hours produced a pale yellow fluorescence throughout the cytoplasm (Fig. 17).

Fluorescence of high intensity was not produced with CEKC which had been infected with IBV42-91C for twelve hours, and then treated with unlabeled goat serum (Fig. 18) or normal chicken serum (Fig. 19). When anti-IBV46 chicken serum was added to CEKC infected with IBV42-91C for twelve

hours (Fig. 20) fluorescence of a lower intensity than that with anti-IBV41 chicken serum (Fig. 17) was present.

Natural fluorescence was present when anti-IBV41 chicken serum (Fig. 21) or goat anti-chicken globulin conjugate only (Fig. 22) were added to uninfected CEKC controls.

Extra-cellular fluorescent particles were observed in some of the specimens that had been infected with IBV42-91C, but not in the specimens that were uninoculated.

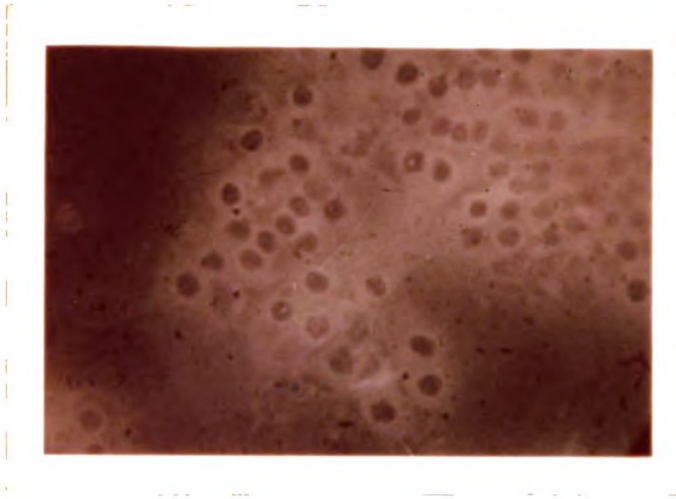

Figure 1. Chicken erythrocytes agglutinated by trypsinized IBV41-7 to which anti-IBV41 chicken serum and rabbit anti-chicken globulin conjugate have been added (485X).

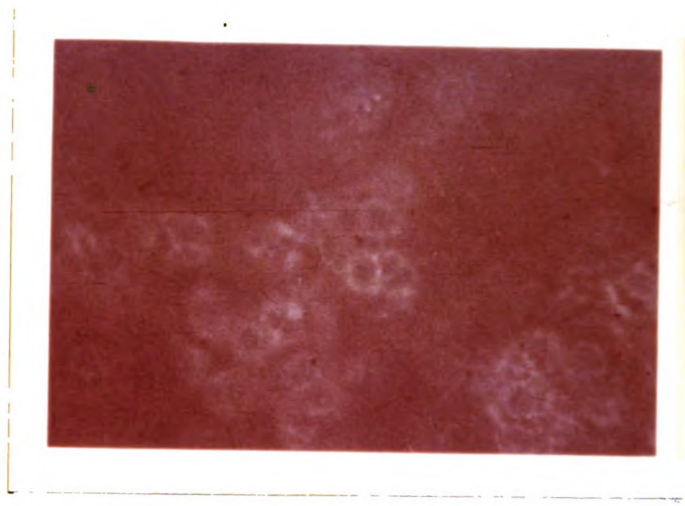

Figure 2. Chicken erythrocytes agglutinated by trypsinized IBV41-7 to which normal chicken serum and rabbit anti-chicken globulin conjugate have been added (485X).

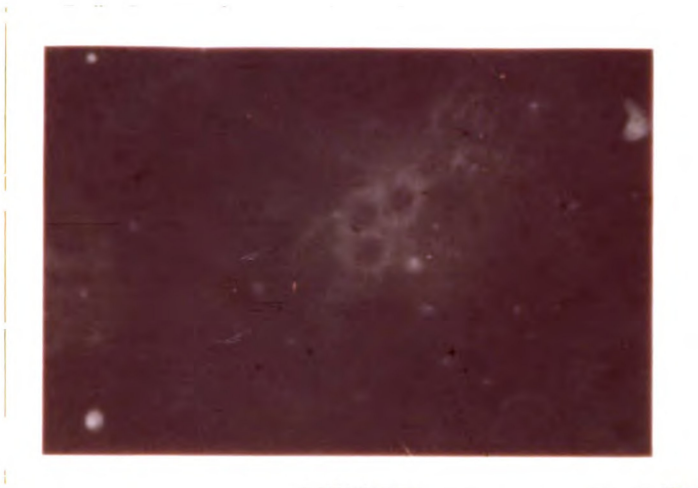

Figure 3. Chicken erythrocytes agglutinated by trypsinized IBV41-7 to which anti-IBV46 chicken serum and rabbit anti-chicken globulin conjugate have been added (485X).

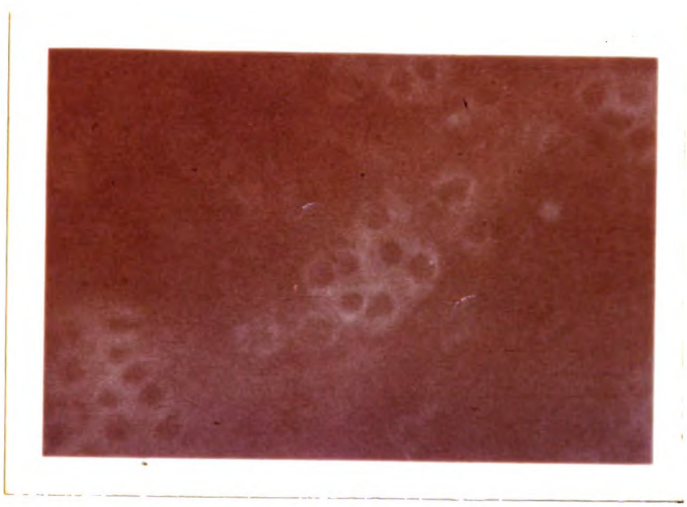

Figure 4. Chicken erythrocytes agglutinated by trypsinized IBV41-7 to which unlabeled rabbit serum and rabbit anti-chicken globulin conjugate have been added (485X).

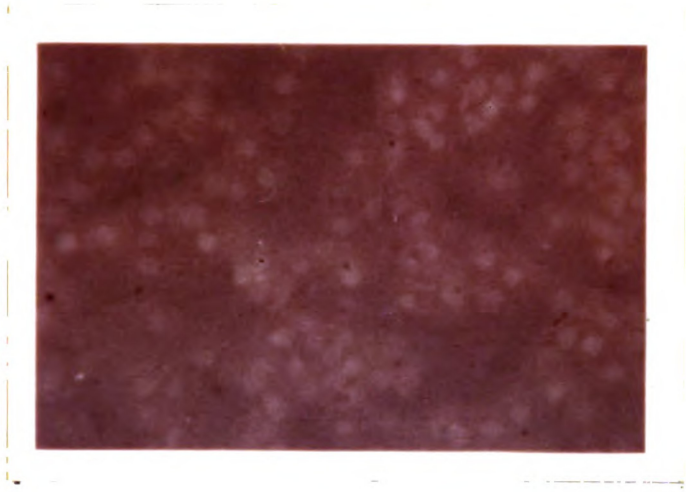

Figure 5. Normal chicken erythrocytes to which anti-IBV41 chicken serum and rabbit anti-chicken globulin conjugate have been added (485X).

Figure 6. Normal chicken erythrocytes to which anti-IBV41 chicken serum and rabbit anti-chicken globulin conjugate have been added (485X).

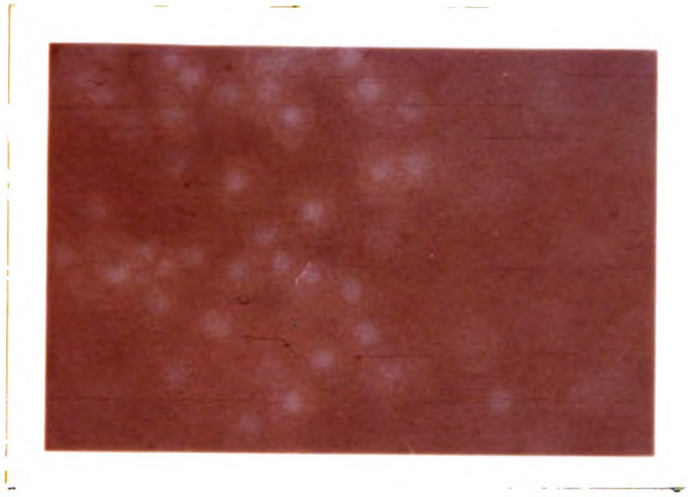

Figure 7. Normal chicken erythrocytes to which unlabeled rabbit serum and rabbit anti-chicken globulin conjugate have been added (485X).

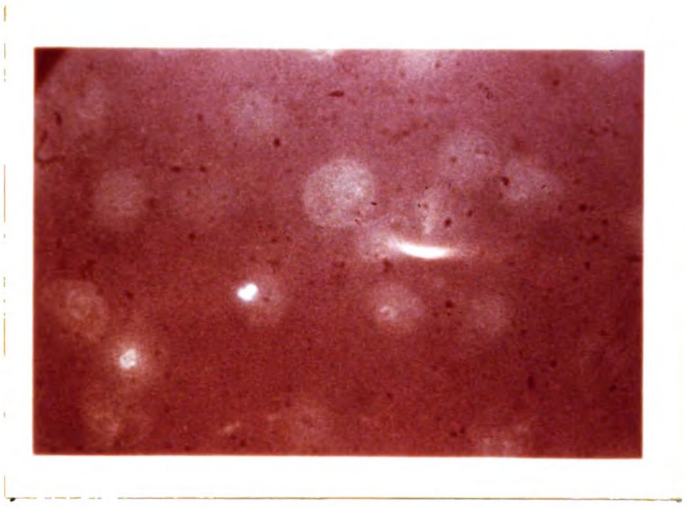

Figure 8. Normal chicken erythrocytes to which rabbit anti-chicken globulin conjugate has been added (485X).

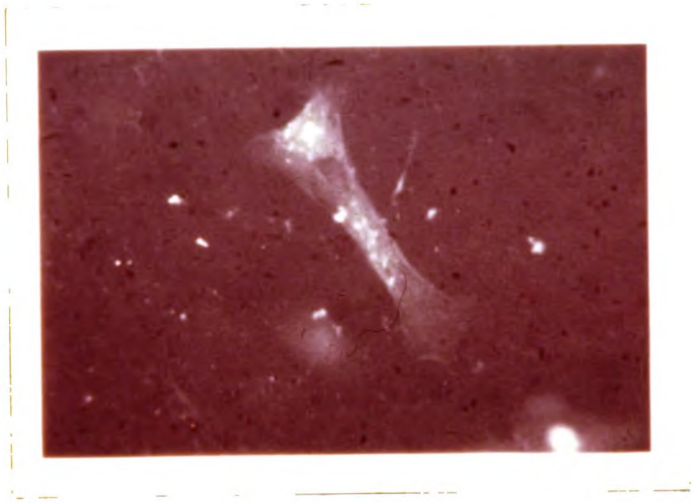

Figure 9. Chicken embryo kidney cells infected with IBV42-91C and treated with anti-IBV41 chicken serum and rabbit anti-chicken globulin conjugate two hours post inoculation (485X).

Figure 10. Chicken embryo kidney cells infected with IBV42-91C and treated with normal chicken serum and rabbit anti-chicken globulin conjugate two hours post inoculation (485X).

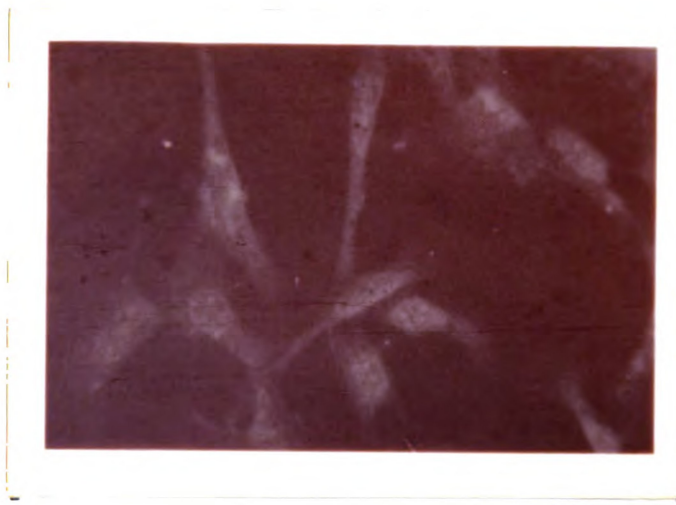

Figure 11. Chicken embryo kidney cells infected with IBV42-91C and treated with anti-IBV46 chicken serum and rabbit anti-chicken globulin conjugate two hours post inoculation (485X).

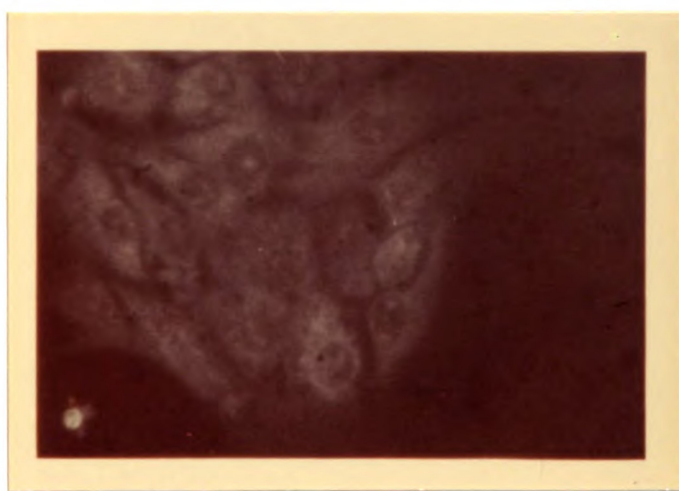

Figure 12. Chicken embryo kidney cells infected with IBV42-91C and treated with unlabeled rabbit serum and rabbit anti-chicken globulin conjugate two hours post inoculation (485X).

Figure 13. Normal chicken embryo kidney cells treated with anti-IBV41 chicken serum and rabbit anti-chicken globulin conjugate two hours post inoculation (485X).

Figure 14. Normal chicken embryo kidney cells treated with normal chicken serum and rabbit anti-chicken globulin conjugate two hours post inoculation (485X).

Figure 15. Normal chicken embryo kidney cells treated with unlabeled rabbit serum and rabbit anti-chicken globulin conjugate two hours post inoculation (485X).

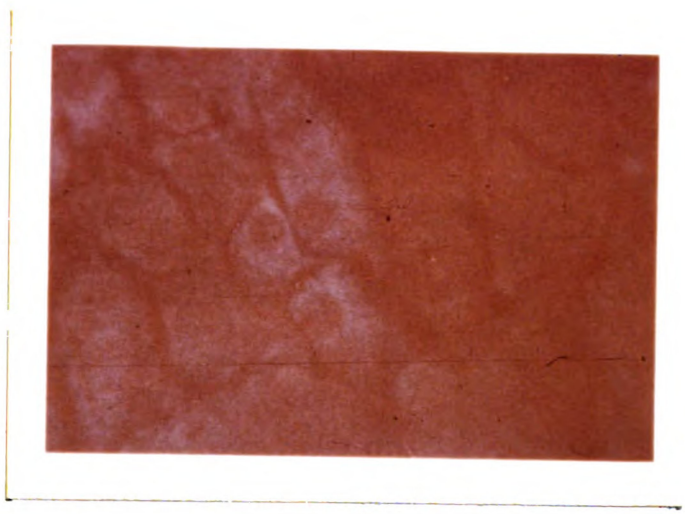

Figure 16. Normal chicken embryo kidney cells treated with rabbit anti-chicken globulin conjugate (485X).

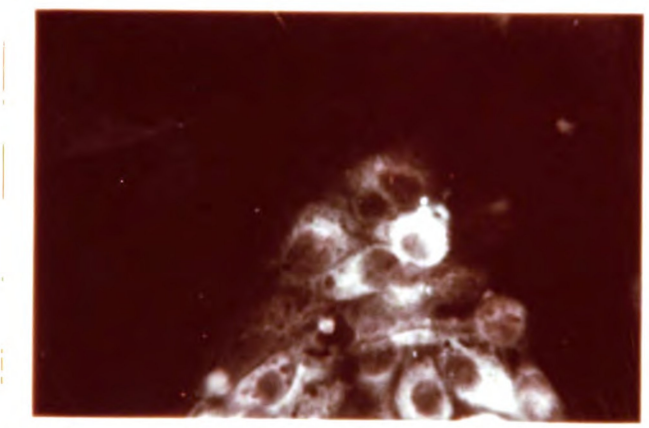

Figure 17. Chicken embryo kidney cells infected with IBV42-91C and treated with anti-IBV41 chicken serum and goat anti-chicken globulin conjugate twelve hours post inoculation (485X).

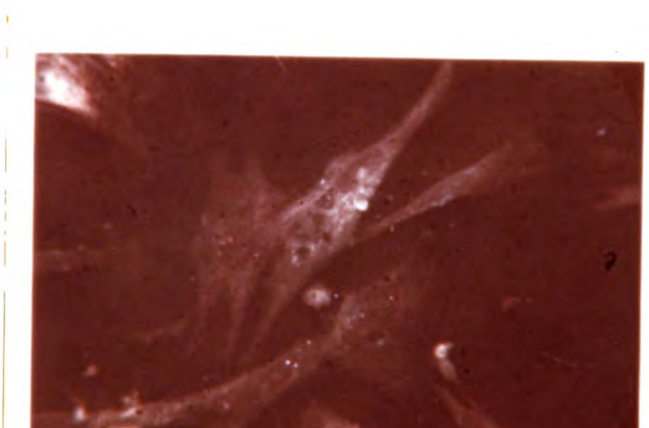

Figure 18. Chicken embryo kidney cells infected with IBV42-91C and treated with unlabeled goat serum and goat anti-chicken globulin conjugate twelve hours post inoculation (485X).

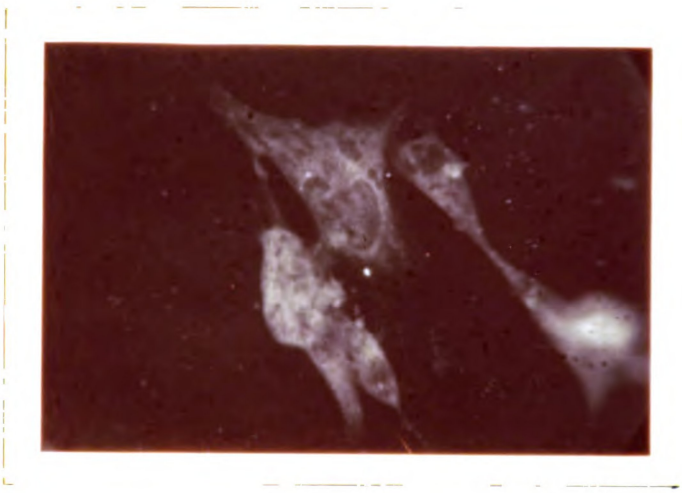

Figure 19. Chicken embryo kidney cells infected with IBV42-91C and treated with normal chicken serum and goat anti-chicken globulin conjugate twelve hours post inoculation (485X).

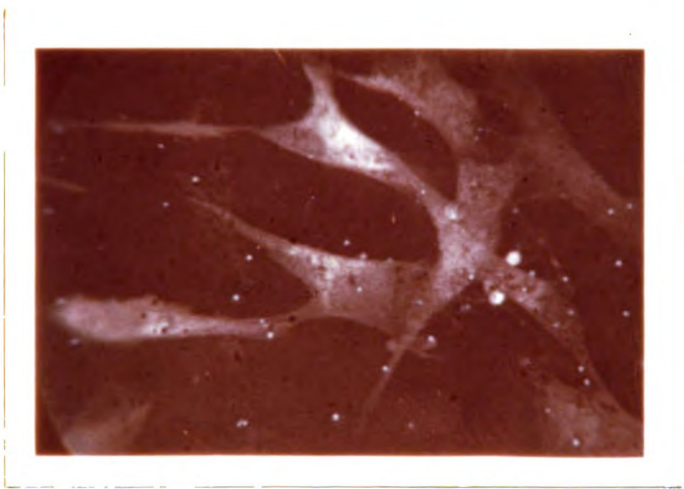

Figure 20. Chicken embryo kidney cells infected with IBV42-91C and treated with anti-IBV46 chicken serum and goat anti-chicken globulin conjugate twelve hours post inoculation (485X).

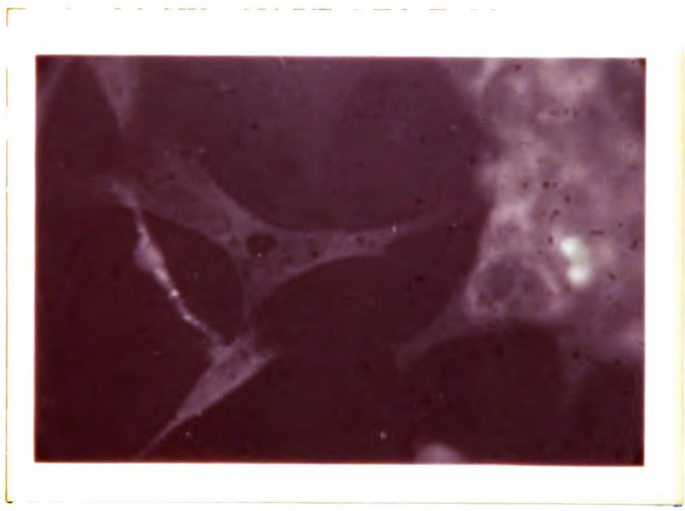

Figure 21. Normal chicken embryo kidney cells treated with anti-IBV41 chicken serum and goat anti-chicken globulin conjugate twelve hours post inoculation (485X).

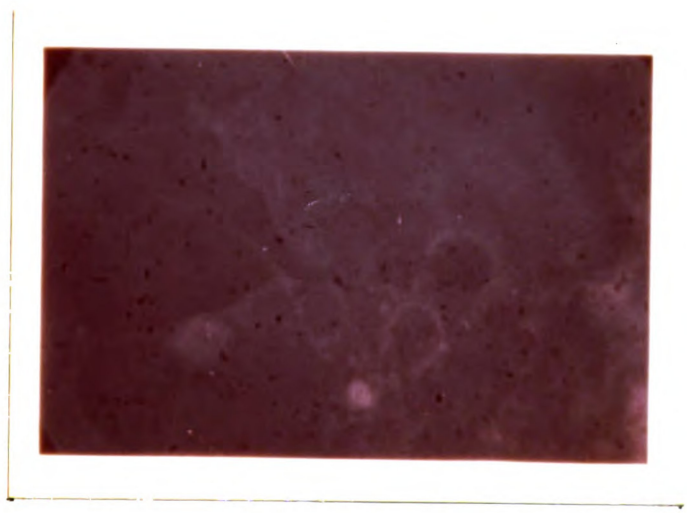

Figure 22. Normal chicken embryo kidney cells treated with goat anti-chicken globulin conjugate twelve hours post inoculation (485X).

DISCUSSION

A positive antigen-antibody reaction, as evidenced by fluorescence on the surface of chicken erythrocytes agglutinated by trypsinized IBV41-7 to which anti-IBV41 chicken serum and rabbit anti-chicken globulin conjugate had been added, confirms the findings of Nazerian (1960) that trypsinized IBV reacts with the surface of the erythrocyte. There was no fluorescence when non-trypsinized IBV41-7 was mixed with chicken erythrocytes, and treated with anti-IBV41 chicken serum and rabbit anti-chicken globulin conjugate. The absence of specific staining was probably due to the inability of the virus, in the non-trypsinized state, to adsorb to chicken erythrocytes to cause hemagglutination. Nazerian (1960) demonstrated by electron microscopy that non-trypsinized IBV41 does not react with the surface of chicken erythrocytes. Anti-IBV41 chicken serum neutralizes infectious non-trypsinized IBV41 as demonstrated by the neutralization test, but the antigen-antibody complex is not formed on the surface of the erythrocyte, and is thus easily removed by washing with phosphate buffered saline.

An antigen-antibody reaction between trypsinized IBV41-7

and anti-IBV46 chicken serum was evidenced by fluorescence on the surface of agglutinated chicken erythrocytes. This would appear to substantiate the report by Oshel (1961) who used cross neutralization tests and found that IBV41 could be neutralized by anti-IBV46 chicken serum. Thus there is the possibility that these two "serotypes" have at least one antigen in common.

Chicken embryo kidney cells infected with cell culture-adapted IBV42 had small fluorescent particles within the cytoplasm when treated with anti-IBV41 chicken serum and either rabbit or goat anti-chicken globulin conjugates. The particles, which could not be detected before two hours, were considered to be virus that had reacted with anti-IBV41 chicken serum. At twelve hours the entire cytoplasm was fluorescent. This would indicate that between the second and twelfth hour replication of the virus has occurred, and that the particles had increased in either size or number or both. The intensity of the fluorescence did not increase to any appreciable degree after twelve hours. At both periods, extra-cellular fluorescent particles were observed and were assumed to be viral particles. Whether or not these particles were active or inactive residual virus which had not

been completely washed from the cell cultures after inoculation, or represented newly formed virus released from the cells was not determined.

After the twenty minute adsorption period, and after the cell cultures had been thoroughly washed, chicken embryo-infective virus was not present in the last washing. This is in contrast to the report by Kisch et al. (1961) who demonstrated washing HeLa cells four times with four ml of BSS did not remove all of the respiratory syncytical virus inoculum. Thirty minutes after adsorbing IBV42-91C to the CEKC extra-cellular virus, EID_{50} 4.0, was present. Such a rapid multiplication of infective viral particles appears to be similar to that seen with influenza and Western equine encephalomyelitis viruses. These data support the results of Spring (1960) that cell culture-adapted IBV42 replicates with an EID_{50} 3.5 in the extra-cellular fluid thirty minutes post adsorption, and a maximum titer of EID_{50} 8.5, ninety to one-hundred-and-twenty minutes post adsorption.

Failure to demonstrate specific fluorescence using CEKC before two hours post adsorption may have been due to any one or all of the following factors: (1) reversibility of the antigen-antibody reaction, (2) antigen-antibody complex

may have been present, but not in sufficient quantity to be visualized with the procedure used, (3) the concentration of antibody, $i.d._{50}$ NI 5.0, may have destroyed the attachment sites on the virus particles present in the early stages of development; thereby negating the antigen-antibody complex, and (4) the concentration of the virus particles within the cytoplasm of the cell may have been so low that the specific antibody may not have been able to combine firmly to the particles, due to the overwhelming number of antibody molecules present, or the virus in low concentration may be soluble in an excess of antibody.

The reversible or irreversible reactions between a viral antigen and its specific antibody have been discussed by Burnet et al. (1937) who hypothesized that viral inactivation by specific antiserum was the result of a reversible combination of antibody with the surface of the virus. Dulbecco et al. (1956) theorized that neutralization was an irreversible reaction between a virus particle and an antibody molecule, was independent of the cell system, and was linearly dependent upon the antibody concentration. In the presence of excess antibody the neutralization kinetics were of a first order reaction which would indicate that one antibody

molecule would neutralize one infective unit of virus. Fazekas de St. Groth and Reid (1958) proposed that the antigen-antibody reaction was reversible and the antigen-antibody complex could be dissociated.

It is not known how animal viruses penetrate a cell wall or how many virus particles penetrate the cell wall. It has been assumed with plaque-forming viruses that one infective virus particle will form one plaque. This concept appears to hold for poliomyelitis virus, but a reliable plaque technique for infectious bronchitis virus has not been thoroughly demonstrated.

Spring (1960) reported data that would indicate a proportionality between the virus antigen and specific antibody in neutralization tests. This may account in part for the lack of fluorescence before two hours post adsorption. Fluorescence at two hours post adsorption may be due to the optimal concentration of antigen and antibody present to which the conjugated anti-chicken globulin combines to form the fluorescent particle that is observed.

Specific anti-IBV rabbit serum will precipitate its homologous antigen (Tevethia, 1962), but it is not known if the specific antibody causes agglutination of the virus, flocculation of the soluble antigen, or takes part in the

fluorescent antibody reaction in the absence of the other antibody types.

Inability of the rabbit or goat anti-chicken globulin conjugate to penetrate the cellular membrane of the host cell as demonstrated by Easton et al. (1962) using ferritin-antibody conjugate may be partially responsible for the lack of fluorescence in the early stages of infection. Detection of fluorescence at two hours post-adsorption with the accompanying increase in intensity up to twelve hours post-adsorption may have been due to the formation of aggregates of virus particles within the cytoplasm of the host cell as a result of metabolic impairment which prevents release of the virus from the cell. Non-release of the virus by the host cell may be the beginning of the observed cytopathic effect in cell cultures infected with viral agents.

The problems posed can not be solved from the present study since all of the work is qualitative and not quantitative in nature. The study does emphasize the need for quantitative study on the specific aspects of IBV multiplication with regard to the specific make-up of the virus itself, what component of the virus is responsible for infectivity, and what fraction of the specific antiserum participates in the fluorescent antibody reaction.

SUMMARY

1. The indirect fluorescent antibody technique was used with rabbit or goat anti-chicken globulin conjugates in combination with chicken erythrocytes agglutinated by trypsinized infectious bronchitis virus, and chicken embryo kidney cells infected with tissue adapted infectious bronchitis virus.
2. Anti-IBV41 and anti-IBV46 chicken serum reacted with trypsinized IBV41-7 on the surface of agglutinated chicken erythrocytes, but the fluorescence with anti-IBV41 chicken serum exhibited a greater intensity than that seen with anti-IBV46 chicken serum. From the evidence presented there would appear to be a common antigen shared by IBV41 and IBV46.
3. Intracytoplasmic particles appeared within chicken embryo kidney cells infected with tissue adapted infectious bronchitis virus when treated with both anti-IBV41 or anti-IBV46 chicken serum two hours post adsorption.
4. Intracytoplasmic particles appeared to increase in size and number within chicken embryo kidney cells to demonstrate confluent fluorescence at twelve hours post adsorption.

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