SOME STUDIES OF INFECTIOUS LARYNGOTRACHEITIS VIRUS CULTIVATED IN CHICKEN EMBRYO LIVER CELLS

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SOME STUDIES OF INFECTIOUS LARYNGOTRACHEITIS VIRUS CULTIVATED IN CHICKEN EMBRYO LIVER CELLS

By

DONALD L. CROGHAN

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Dedicated

to

my family

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INTRODUCTION

Previous studies of the process of adaptation of animal viruses to cell culture and some alterations in these viruses that might be attributed to the adaptation process, were primarily responsible for these studies.

The primary objective of this research was the adaptation of infectious laryngotracheitis virus to chicken embryo liver cells, and the use of the cytopathic effect of the virus on the cells as a method for the assay of the virus.

Other studies were made in an attempt to determine if some fundamental characteristics of the virus had been altered during the adaptive process. To ascertain in part the role of temperature in the stability of the virus, thermal inactivation at 56 C was performed. The antigenic properties of the virus were tested in the natural host and by serum neutralization tests. A time-rate multiplication experiment was performed to determine virus production in cell cultures. Susceptible chickens were inoculated with the virus to find whether adaptation had altered pathogenicity of the virus. Typical intranuclear inclusion bodies were demonstrated in cell culture, and their probable role in viral replication hypothesized.

REVIEW OF LITERATURE

Infectious laryngotracheitis is a highly contagious virus disease of chickens, which has been reported from Canada, Australia, New Zealand, Holland, Sweden, Poland, and the United States (Hofstad, 1959). Gwatkin (1924) first described the clinical signs of infection and the gross pathology. The etiological agent was determined to be a filterable virus (Beach, 1931a; and Graham et al., 1931).

Seifried (1931) described the histopathological manifestations which are limited to the upper respiratory tract, and include necrosis of the epithelium with edema, hemorrhage, and cellular infiltration of the submucosa. Characteristic intranuclear inclusion bodies were present in many epithelial cells.

Beach (1931b) isolated the virus from the livers of two of six chickens, and from the spleens of eighteen of thirty chickens. The virus was isolated from the tracheal exudate of all the chickens.

Infectious laryngotracheitis virus (ILV) was found to be host specific for the chicken with the exception that the pheasant could also be infected (Beach, 1931a; Hudson and Beaudette, 1932; Brandly and Bushnell, 1934; and Reagan et al., 1954).

Gibbs (1935) calculated the infectious laryngotracheitis virus particle to be 50 to 80 mμ in diameter by "Gradacol" filtration. Reagan et al. (1955), using centrifugally purified suspensions of the infected chorio-allantoic membrane (CAM) from the embryonating chicken egg, by electron microscopy determined the particle to be a 100 to 110 mμ diameter sphere. Electron microscopy of the infected ectodermal cells of the CAM, showed particles of 30 to 40 mμ and 80 to 100 mμ intranuclearly, and 180 to 240 mμ intracytoplasmically (Watrach et al., 1959).

Only the embryonating eggs of chickens and turkeys were found to be susceptible to infection with ILV (Brandly, 1936). Replication of ILV in the ectodermal cells of the CAM was identified by the formation of plaque-like lesions, consisting of proliferative and necrotic cellular changes (Burnet, 1934; and Brandly, 1935). Brandly (1936) found that the maximum growth of ILV in the CAM occurred at three days post-inoculation, and that the titer dropped markedly by the sixth and seventh days. With growth curve studies in cell culture, Atherton and Anderson (1957) found that the virus had an eclipse phase until 12 hours and the maximum replication was at about 48 hours when infectivity was measured on the CAM. Burnet and Foley (1941) demonstrated characteristic

lesions in the respiratory tract of chicken embryos by injecting ILV via the allantoic cavity.

Schalm and Beach (1935) showed that ILV was rendered non-infectious by exposure for one minute to 5 per cent phenol, 30 seconds to 1 per cent cresol, and 30 seconds to 1 per cent sodium hydroxide. Virus in tracheal exudate exposed to 55.5 C in a water bath for ten minutes was infective, but not infectious when exposed for fifteen minutes.

Burnet (1936), using the constant virus-decreasing serum method of serum neutralization with end-point determination by plaque counts on the CAM, showed that previous infection could be determined and that various strains of ILV showed no qualitative differences in their antigenicity. With the use of the decreasing virus-constant serum method, Hitchner et al. (1958) found that the neutralization index (NI) for ILV immune serum was generally in the range of 10^2 to 10^3, rarely above 10^4 and that with three standard deviations the NI for normal serum would not exceed $10^{1.28}$. Similar results were obtained by Benton et al. (1958) when comparing ILV strains that showed marked differences in pathogenicity.

Simmons et al. (1954), Satriano et al. (1957), and Cover and Benton (1957), isolated strains of ILV that produced

atypical clinical signs of infection, and could only be identified by cross-immunization or serum neutralization tests. Burnet (1936) and Brandly (1937) described the lesions produced by strains of low virulence as being smaller than those produced by more virulent strains. It was found that ILV strains of low virulence were not neutralized with immune serum produced using virulent virus as readily as were the strains of the high virulence (Burnet, 1936).

Infectious laryngotracheitis virus may be propagated in Maitland type tissue culture, using minced whole chicken embryo in Tyrode's solution (Beach, 1932). The virus was serially passed 22 times in this culture, and infectivity was demonstrated by inoculating susceptible chickens with 0.1 ml of the extra-cellular fluids. Atherton and Anderson (1957) successfully cultivated ILV in chicken embryo heart muscle and lung epithelium, both in explants and monolayers, with visible cytopathic effects (CPE). The virus was found to be cell associated, and not released into the extra-cellular fluid through the third cell passage. Webster (1959) found that ILV would multiply and was cytopathogenic for one-day-old chicken kidney and skeletal muscle cell cultures. The virus would multiply to a limited extent in HeLa cells, but was not cytopathogenic. Chang et al. (1960)

produced CPE with three strains of ILV in chicken embryo kidney cells and showed plaque production using the method of Hsiung and Melnick (1955).

Microscopic examination of ILV infected CAM (Burnet, 1934), showed proliferation of the ectodermal layer with infiltration of fibroblasts into the mesodermal layer. Final stages were evidenced by pyknosis of the nucleus and necrosis of the cytoplasm. In chicken embryo heart muscle fibroblast cultures, Atherton and Anderson (1957) noted a retraction of the cell processes and vacuolation of the cytoplasm. The nuclei became more compact and were intensely basophilic starting the fifth day after infection.

Inclusion bodies have been observed in cells contained in tracheal exudate (Seifried, 1931; and Thorp and Graham, 1934). Burnet (1934) found that many of the proliferated cells of the infected CAM contained intranuclear inclusions.

Cover and Benton (1958) were best able to demonstrate inclusion bodies in the infected CAM by using fixatives with a low pH and staining with hematoxylin and eosin. Armstrong (1959) used absolute methanol fixation with dilute Giemsa stain (1:10) for rapid inclusion body staining procedure with tracheal exudate smears. Water soluble "Carbowax 1000" as used by Pirozok et al. (1957) to embed infected tissue for

sectioning, greatly reduced the time for preparing sections. Atherton and Anderson (1957) found that Carnoy fixative and Giemsa stain gave good results for demonstration of inclusion bodies in cell culture.

MATERIALS AND METHODS

Virus

Infectious laryngotracheitis virus, strain 1323B, was received from Dr. M. S. Cover, University of Delaware, Newark, in the form of minced CAM from an embryonating chicken egg. The virus, originally isolated from a field outbreak, was in the fourth CAM passage, and produced signs of infection in susceptible chickens and pock lesions on the CAM.

Preparation of Chicken Embryo Liver Cells

Primary cultures of chicken embryo liver cells (CELC) were used throughout the study with certain exceptions. The livers from 16-day-old chicken embryos were removed aseptically and washed in Hanks balanced salt solution (BSS) in a petri dish. The livers were minced into pieces about 0.5 to 1.0 mm^3, and were again thoroughly washed in BSS. The pieces were transferred to a 250 ml fluted Erlenmyer flask containing a "Teflon" covered magnetic bar and 80 ml of Krueger-filtered 0.05 per cent trypsin, pH 8.0, at 4 C. The flask was held from 18 to 24 hours at 4 C. The trypsin was then decanted and replaced with an equal volume of fresh trypsin. The

flask was then placed over a "Magnestir" and trypsinization with agitation by the magnetic bar was performed at 37 C until pieces were no longer visible macroscopically. This process was usually completed in forty to sixty minutes.

The cell suspension was filtered through four layers of cheese cloth and centrifuged at 1000 rpm (195 X g) for ten minutes in an International PR-1 refrigerated centrifuge at 4 C. The supernatant fluid was discarded, and the packed cells were resuspended in Medium 199 (Microbiological Associates, Inc.). The same procedure of centrifugation was repeated. The packed cells from this washing were diluted 1:400 in a growth medium consisting of Medium 199, 10 per cent bovine serum, 100 units of penicillin G per ml, and 100 μg of dihydrostreptomycin per ml.

Leighton tubes, 16 x 125 mm, were seeded with 1.0 ml of the cell suspension, and 8 ounce prescription bottles were seeded with 10 ml of the cell suspension. The Leighton tubes were sealed with white rubber stoppers, and the prescription bottles with the original plastic caps. The tubes and bottles were incubated horizontally at 37 C with the flat surface down.

A complete monolayer was formed in from 48 to 54 hours in the Leighton tubes, at which time the growth medium was

replaced with 0.9 ml of Medium 199 as a maintenance medium. The monolayer was then inoculated with 0.1 ml of ILV strain 1323B. The prescription bottles were used to produce a large pool of virus, and the same ratio of virus to medium was used as in the Leighton tubes.

Adaptation of ILV to CELC

The first passage of ILV in CELC was made by inoculating each of five tubes with 0.1 ml of virus infected CAM ground in a Tenbroeck tissue grinder. Microscopic examinations were made daily, and at 72 hours there was a clumping of the epithelial cells. The cells and extracellular fluids were harvested 48 hours later when the majority of the cells had sloughed off the glass surface.

The cells adhering to the glass surface were removed either by freezing and thawing or by mechanically scraping the monolayer with a "rubber policeman." Both fluid and cells were collected in the mortar portion of a 7 ml capacity Tenbroeck tissue grinder. The cells were sedimented by centrifugation at 1500 rpm (437 x g) at 4 C, and the supernatant fluid was removed. The cells were ground and resuspended in the supernatant fluid and stored at -60 C until used as

inoculum. This procedure was used for the first 14 passages of the virus. At the 15th passage, the extra-cellular fluid and cells were collected separately and assayed for viral infectivity. It was found that the virus was being released into the extra-cellular fluid, and that the cells contained practically no infectious virus. For subsequent passages, the extra-cellular fluid alone was used for the inoculum.

As a standard procedure, all microscopic observations of the infected monolayers were recorded 72 hours post-inoculation.

Titration of ILV in CELC

Serial ten-fold dilutions of the 10th cell passage of virus were prepared using as a diluent 4.5 ml of Medium 199. The virus was thawed and centrifuged at 1500 rpm (437 X g) for ten minutes at 4 C. To the first tube was added 0.5 ml of virus with a 1 ml serological pipette. Using a 2 ml serological pipette, the contents of the tube were mixed by aspirating and expelling the mixture 20 times, and then transferring 0.5 ml to the next tube. This was continued until all the dilutions were made. All ingredients were kept in an ice bath to prevent inactivation of the virus at room temperature. Each dilution of virus was used to inoculate

five tubes of cells, 0.1 ml per tube, and to inoculate five 10-day-old chicken embryos, 0.1 ml per embryo, by the CAM route according to the procedure of Gorham (1957). The tubes were incubated at 37 C for 72 hours before microscopic examinations were made. The eggs were incubated at 37 C for 120 hours when macroscopic examination was made.

The titer of the virus was calculated by the method of Reed and Muench (1938), and expressed as cell culture $dose_{50}$ ($c.c.d._{50}$) and egg infective $dose_{50}$ ($e.i.d._{50}$).

Thermal Inactivation of ILV

The 10th cell passage of the virus was thawed, centrifuged at 1500 rpm (437 X g) for ten minutes at 4 C, and dispensed in 2 ml amounts into sterile 13 x 100 mm tubes. The tubes were placed in a water bath at 56 C. At the end of two minutes, the time required for thermal equilibration, one tube was removed to a beaker of crushed ice. At the end of each three succeeding minutes, another tube was removed and placed in the beaker of crushed ice. After one minute in the crushed ice, the tubes were removed and serial ten-fold dilutions of the virus were made, and cell cultures and eggs were inoculated as previously described.

Neutralization Tests

Decreasing virus-constant serum method

Anti-1323B serum was produced by inoculating chickens with 0.1 ml of the 9th cell culture passage of virus into the frontal sinus. The chickens had been vaccinated fourteen days previously with a commercial ILV vaccine. A second inoculation of 9th cell passage virus was given after fourteen days, and serum was collected seven days later.

Serial ten-fold dilutions of the 10th cell culture of virus were prepared. Anti-1323B serum and normal chicken serum were passed through Krueger filters and inactivated at 56 C for thirty minutes. For the first neutralization test, serum diluted 1:10 with Medium 199 was used. A previous report (Spring, 1960) indicated that undiluted chicken serum used with chicken embryo kidney cells produced a precipitate which partially obscured CPE as observed by microscopic examination. This was not found to be the case with the present cell culture system and in subsequent experiments undiluted serum was used.

The undiluted serums and virus dilutions were mixed, 1.0 ml each, and incubated for thirty minutes at room temperature. Each of five cell cultures and five eggs were

inoculated with 0.1 ml of the mixtures. The tubes and eggs were incubated and examined as previously described.

The NI (Cunningham, 1960), for the serums was the reciprocal of the differences between the infectious dose$_{50}$ (i.d.$_{50}$) end-points of the virus alone and the virus-serum mixtures. Hereafter, the NI will be expressed as a number and not as a logarithm.

Constant virus-decreasing serum method

To study the effect of the ratio of antigen to antibody as a possible variable in the neutralization test, two-fold serial dilutions of serum were tested against constant amounts of virus which differed by decimal solution.

Serial ten-fold dilutions of the 20th CELC passage virus were prepared using the procedure previously described. Serial two-fold dilutions of the filtered, heat inactivated, immune chicken serum were prepared using as a diluent 5.0 ml of Medium 199, and an equal quantity of serum. Using a 5 ml serological pipette, 5 ml of serum was transferred to the first tube. With another 5 ml pipette, the content of the tube was mixed by aspirating and expelling the mixture 20 times, and then 5 ml was transferred to the next tube. This was continued until all the dilutions had been made.

The serum dilutions and virus dilutions were mixed, 1.0 ml each, and incubated for thirty minutes at room temperature. Each of five cell cultures and five eggs were inoculated with 0.1 ml of the mixtures. The tubes and eggs were incubated and examined as before.

Cross-Immunization Test in Chickens

Chickens, 4-weeks-old, were divided into four groups of three each. The chickens in group one were injected with 0.1 ml of 15th cell passage virus into the frontal sinus. For the second group, the virus was applied to the cloaca with a brush. Groups three and four received commercial ILV vaccine by the same routes as for groups one and two respectively. All chickens were examined daily for fourteen days for signs of response to the virus such as swelling around the eye and edema or hyperemia of the cloaca.

On the fourteenth day, the chickens in groups one and two received via the trachea 0.1 ml of the commercial ILV vaccine, and the chickens in groups three and four received 0.1 of the 15th cell passage virus via the same route. These chickens were observed daily for an additional fourteen days.

Chickens of the same lot as those in the above groups were used for controls. Two chickens received the commercial vaccine virus and two the 15th cell passage virus via the trachea at the same time that the chickens in the above groups were challenged. These control birds were to be observed daily for fourteen days, or until severe symptoms were manifested.

Multiplication Cycle of ILV in CELC

Cell cultures were prepared in 54 tubes and after 48 hours the growth medium was replaced with maintenance medium. Each tube was inoculated with 0.1 ml of a 10^{-1} dilution of the 15th cell passage virus. At 5, 15, 30, 45, 60, and 90 minutes and 2, 4, 6, 8, 12, 24, 30, 36, 48, and 96 hours, three tubes were removed and the monolayers were scraped from the wall of the tube with a "rubber policeman." The extra-cellular fluid and cells were transferred to the mortar portion of a 7 ml Tenbroek tissue grinder and centrifuged at 1500 rpm (437 X g) for ten minutes at 4 C. The supernatant fluid was transferred to a 2 dram screw cap vial and immediately stored at -60 C. The cells in the tissue were resuspended in BSS and the process of centrifugation was repeated. The BSS was discarded and the cells were

ground rapidly for one minute in the Tenbroek grinder, then 3 ml of Medium 199 was added to resuspend the cell material. The resuspended cell material was transferred to a 2 dram screw cap vial and immediately stored at -60 C.

For titration of the virus in the extra-cellular fluids and ground cell material of the same time interval, the stored samples were thawed rapidly in warm tap water and centrifuged at 1500 rpm (437 X g) for ten minutes at 4 C, to sediment the cellular debris. Serial ten-fold dilutions of the samples were made in Medium 199, and five cell cultures and five eggs were inoculated with 0.1 ml of each dilution.

Intranuclear ILV Inclusion Bodies in CELC

Leighton tubes containing 11 x 35 mm No. 1 coverslips were seeded with CELC, and after 48 hours the growth medium was replaced with maintenance medium. The cultures were inoculated with approximately 10^3 $c.c.d._{50}$ of the 20th cell passage of the virus.

At approximately 18 hours after inoculation with ILV there was a swelling of the nucleus and an apparent diminution of the cytoplasm, so that only the massed nuclear membranes were visible. At this time the coverslips were removed from the tube, and washed for twenty minutes in BSS

at room temperature. The coverslips were placed in Carnoy fixative for thirty minutes, and then placed in Giemsa stain overnight (Atherton and Anderson, 1957).

The staining reaction was quite variable. If the stained preparation was darker than magenta, it was slightly decolorized in absolute methyl alcohol. The specimen was dehydrated and cleared for five minute intervals, respectively, through acetone, acetone-xylol (equal parts), and xylol. The coverslip was then mounted on a slide with "Damar."

For comparison, 10-day-old embryos were inoculated via the CAM with undiluted 20th cell passage virus. Both infected and normal CAMs were harvested six days later, and were placed overnight in Bouin fixative containing 5 per cent glacial acetic acid. The CAM was then imbedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections were then mounted on slides using "Damar" as the mounting medium.

Cell Spectrum for CELC ILV

To determine in part the spectrum of cells susceptible to CELC ILV, a minimum of three cell culture tubes of Chang's liver cells (Microbiological Associates, Inc.), bovine embryo kidney cells, monkey kidney cells, chicken embryo kidney cells,

and chicken embryo lung cells were used. The virus from the 15th chicken embryo liver cell passage was used as inoculum, employing 0.1 ml per tube. With Chang's liver, bovine embryo kidney and monkey kidney cells, extra-cellular fluid was collected on the fifth day and used for subsequent passages. The virus was passaged three times in Chang's liver cells and twice in bovine and monkey kidney cells.

RESULTS

Adaptation of ILV to CELC

The CPE, at 72 hours in the first and subsequent passages of ILV in CELC, consisted of a clumping of the epithelial cells which were spheroid with a refractile, hyaline material encapsulating the clumps of cells. This clumping of cells became generalized throughout the monolayer, and by 48 hours the majority of the epithelial cells had sloughed off the glass surface, but numerous fibroblasts remained (figures 1 and 2). In subsequent experiments where viral infectivity was tested, it was noted that in the tubes near the end-point of infection, the virus appeared to have spread centrifugally from the foci of infection.

The interval between inoculation of the virus and the appearance of CPE decreased with successive passage of the virus. Using undiluted 10th cell passage virus, CPE was evident at 24 hours, however with dilutions of virus near the end-point of viral activity, CPE was not evident until the 72nd hour.

Titration of ILV in CELC

Titration for viral activity was first done with

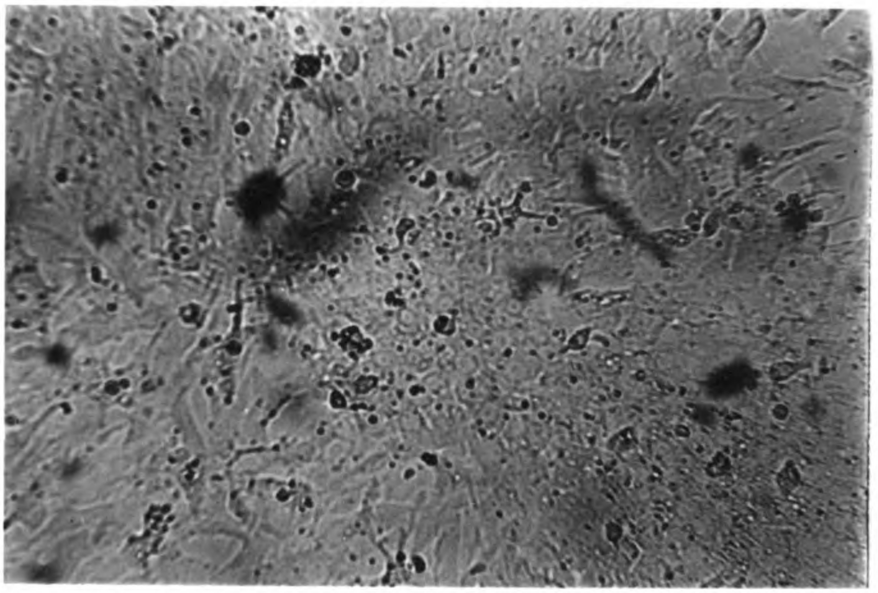

Figure 1. Normal chicken embryo liver cells at 96 hours (200X).

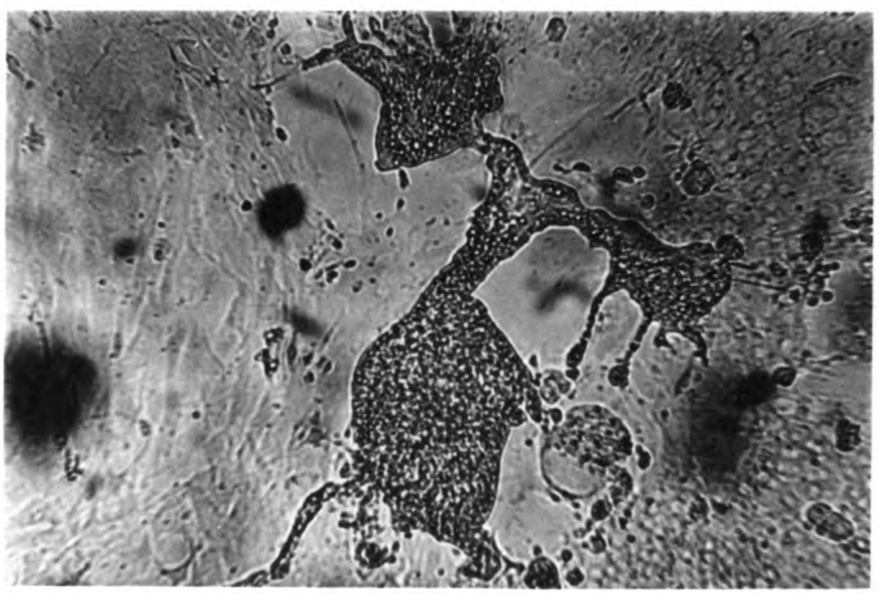

Figure 2. Chicken embryo liver cells at 96 hours infected with 17th chicken embryo liver cell passage of infectious laryngotracheitis virus at 48 hours (200X).

the 10th cell passage of virus using CPE on cell cultures and pock formation on the CAM as the criteria of a positive response. The titer was $10^{3.5}$ c.c.d.$_{50}$/0.1 ml and $10^{4.5}$ e.i.d.$_{50}$/0.1 ml. While titrations for viral activity were not performed at each cell culture passage, titrations were conducted routinely with other experiments, and at the 15th cell passage, the titer was $10^{4.3}$ c.c.d.$_{50}$/0.1 ml and $10^{3.7}$ e.i.d.$_{50}$/0.1 ml, while at the 20th cell passage the titer was $10^{6.2}$ c.c.d.$_{50}$/0.1 ml and $10^{2.6}$ e.i.d.$_{50}$/0.1 ml.

Thermal Inactivation of ILV

With inactivation of 10th cell passage ILV at 56 C, the initial titer in both cell culture and on the CAM was $10^{3.8}$ i.d.$_{50}$/0.1 ml at zero time, whereas in the original titration of 10th cell passage ILV on the CAM the titer was $10^{4.5}$ e.i.d.$_{50}$/0.1 ml. The most likely explanation for this decrease in titer would be the three weeks storage period between experiments.

After the zero time the titer decreased rapidly and at fifteen minutes viral infectivity could be detected but not quantitatively measured (figure 3). The results obtained by titration in CELC during the first six minutes fit an

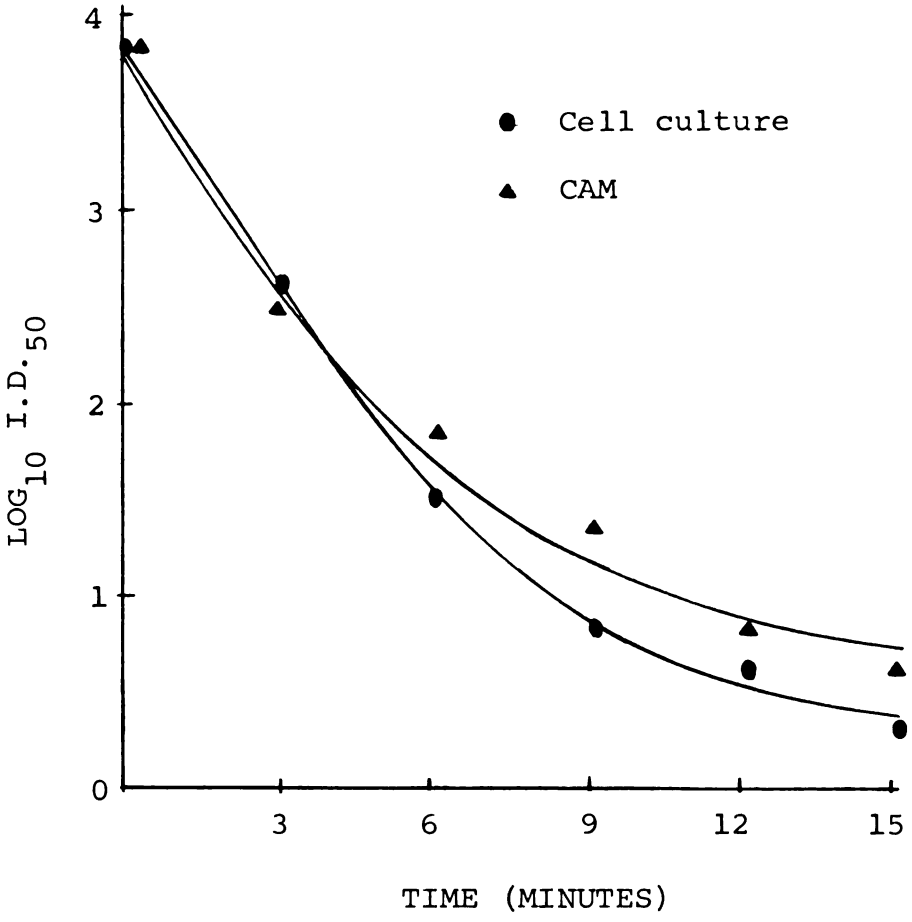

Figure 3. Inactivation at 56 C of 10th passage chicken embryo liver cell infectious laryngotracheitis virus tested in cell culture and on the chorio-allantoic membrane.

approximate straight line, thereafter, the rate of inactivation decreased and the line skewed. Measurement of viral infectivity on the CAM closely paralleled that of cell culture.

Neutralization Tests

Decreasing virus-constant serum method

Cell cultures and the CAM were used to obtain a comparison as to their relative sensitivity as indicator systems in the serum neutralization tests. Using the 10th cell passage of virus, the titer on the CAM was found to be $10^{3.3}$ e.i.d.$_{50}$/0.1 ml, whereas in the original titration it was $10^{4.5}$ e.i.d.$_{50}$/0.1 ml. The only explanation of this difference in titer of the virus was that the virus had been stored for four weeks between experiments.

The NI for the immune serum was 3.0 in cell culture and 2.7 on the CAM (table 1). For the normal serum, the NI was 2.3 in cell cultures and 2.0 on the CAM. With the 20th cell passage of the virus, the NI for the same immune serum was 4.5 in cell culture and 2.6 on the CAM. For the normal serum, the NI was 2.0 in cell culture and 0.4 on the CAM.

Constant virus-decreasing serum method

With the data obtained from the constant virus-

Table 1.

Neutralization Tests

Decreasing virus-constant serum method

10th CELC passage virus

Indicator	serum i.d.$_{50}$	virus i.d.$_{50}$	NI i.d.$_{50}$
cell culture (immune serum)	1.6*	3.6	3.0
cell culture (normal serum)	2.3*	3.6	2.3
CAM (immune serum)	1.6*	3.3	2.7
CAM (normal serum)	2.3*	3.3	2.0

20th CELC passage virus

Indicator	serum i.d.$_{50}$	virus i.d.$_{50}$	NI i.d.$_{50}$
cell culture (immune serum)	1.7#	6.2	4.5
cell culture (normal serum)	4.2#	6.2	2.0
CAM (immune serum)	0.0#	2.6	2.6
CAM (normal serum)	2.2#	2.6	0.4

*serum diluted 1:10. NI adjusted to compensate for dilution.

#serum undiluted.

decreasing serum experiment of neutralization with ten-fold dilutions of the virus and two-fold dilutions of serum (table 2), the logarithm of the dilution of serum was plotted against the logarithm of the amount of virus neutralized (figure 4). This gave a line of neutralization, the slope of which was obtained by the formula,

$$\text{slope} = \frac{y_1 - y_2}{x_1 - x_2}$$

The slopes of the lines for both the cell culture and the CAM were 2.0.

Cross-Immunization Test in Chickens

In the cross-immunization test, only one of the chickens in group three which received commercial ILV failed to show a response to the vaccination. All other chickens showed signs of infection of varying degrees. Following challenge fourteen days later, none of the vaccinated chickens in groups one to four respectively showed any response to the virus.

All the control chickens showed severe signs of respiratory disturbance on the third day. Those receiving the commercial vaccine virus died on the fourth and fifth days. The two control chickens inoculated with 15th cell passage virus were comatose on the fifth and seventh days respectively,

TABLE 2.

Neutralization Tests.

Constant virus-decreasing serum method

20th CELC passage virus in cell culture

$\log_{10}$ virus dil.	serum dilution 1:2	1:4	1:8	1:16	1:32	virus neutralized
-1	5/5	5/5	5/5	5/5	5/5	$10^{4.03}$
-2	3/5	5/5	5/5	5/5	5/5	$10^{3.57}$
-3	0/5	1/5	4/5	5/5	5/5	$10^{2.52}$
-4	0/5	0/5	2/5	3/5	5/5	$10^{2.03}$
-5	0/5	0/5	0/5	0/5	1/5	$10^{1.57}$

$c.c.d._{50}$ of virus 6.2

20th CELC passage virus on the CAM

$\log_{10}$ virus dil.	serum dilution 1:2	1:4	1:8	1:16	1:32	virus neutralized
-1	1/5	3/5	5/5	5/5	5/5	$10^{2.97}$
-2	0/5	1/5	2/5	4/5	5/5	$10^{2.10}$
-3	0/5	0/5	1/5	2/5	3/5	$10^{1.60}$
-4	0/5	0/5	0/5	0/5	0/5	$10^{0.92}$
-5	0/5	0/5	0/5	0/5	0/5	$10^{0.0}$

$e.i.d._{50}$ of virus 3.6

The denominator is the number of cell cultures or eggs used per dilution. The numerator is the number of infected cell cultures or CAMs per dilution.

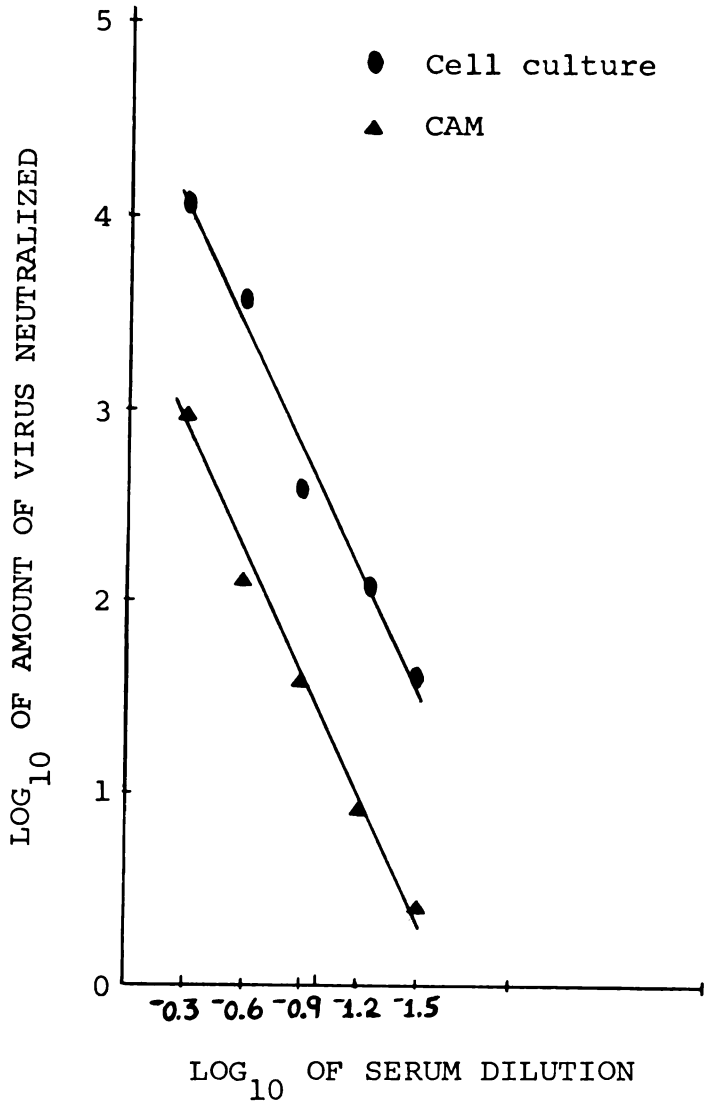

Figure 4. Slope of the neutralization obtained by plotting $\log_{10}$ of the serum dilution against the $\log_{10}$ of the amount of virus neutralized.

and were killed. At necropsy, all control chickens showed gross evidence of infection characteristic of that produced by ILV.

Multiplication Cycle of ILV in CELC

During the first fifteen minutes of the multiplication cycle, the virus in the supernatant fluid as assayed in cell culture, decreased from $10^{3.3}$ c.c.d.$_{50}$/0.1 ml to $10^{1.5}$c.c.d.$_{50}$ /0.1 ml (figure 5). This indicated that 98.5 per cent of the virus had been adsorbed during this time. Following this lag phase, there was a marked increase or log phase in detectable extra-cellular virus to $10^{2.83}$ c.c.d.$_{50}$/0.1 ml at ninety minutes. The titer remained about on a plateau to the 4th hour and then started to gradually rise to $10^{3.17}$ c.c.d.$_{50}$/0.1 ml at the 12th hour.

Only a few samples could be tested at one time, due to the numbers of cell culture tubes and eggs required to test a sample. Between the titration of the 12th hour sample and samples collected at the 24th and subsequent hours, mechanical failure of the -60 C freezer occurred, with the result that the samples were transferred to a freezer at -20 C for one week. With the 24 hour sample, it was noted that the titer was $10^{1.83}$ c.c.d.$_{50}$/0.1 ml or a decrease of

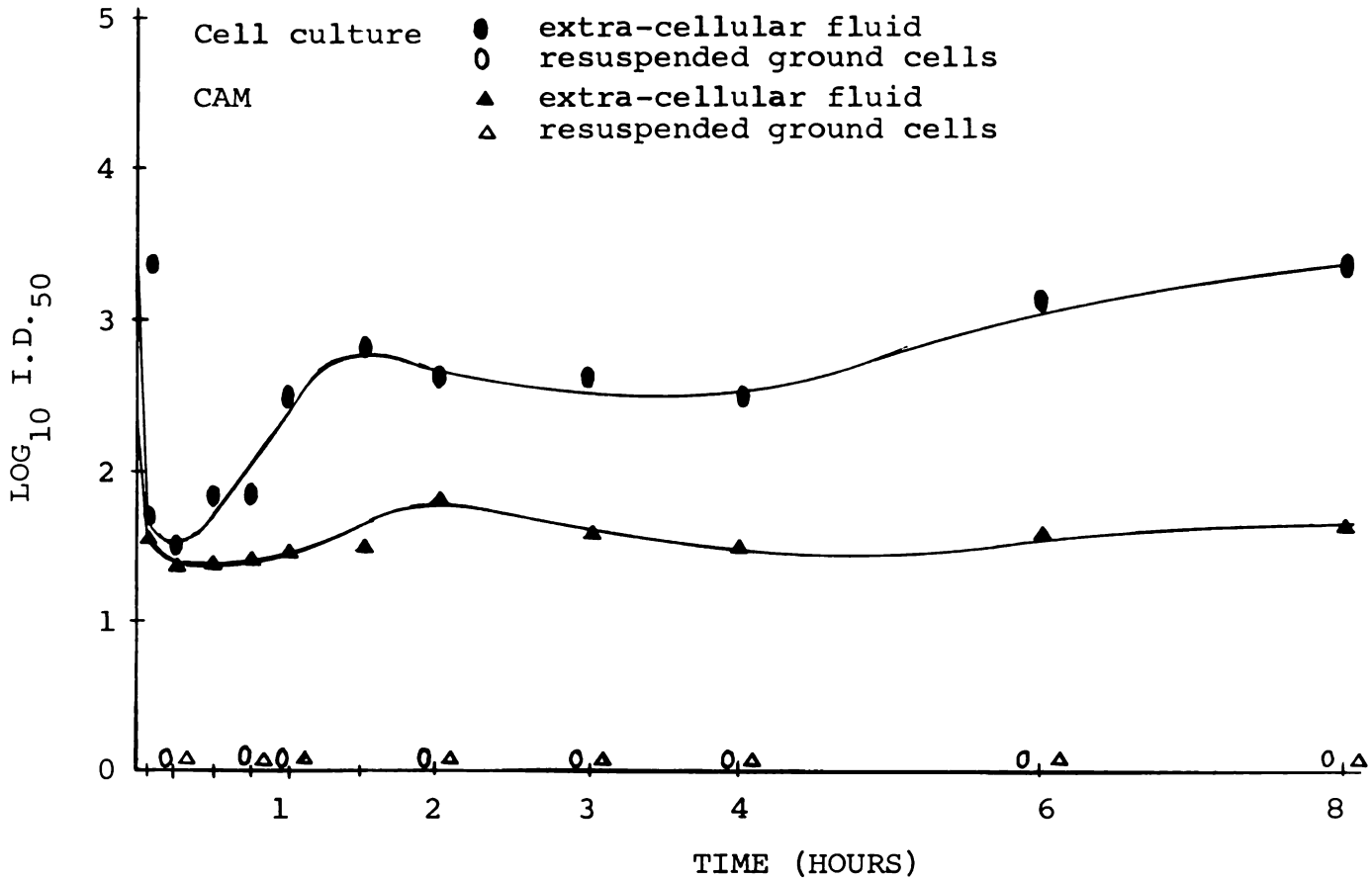

Figure 5. Multiplication cycle of 15th cell culture passage infectious laryngotracheitis virus in chicken embryo liver cells and on the CAM.

$10^{1.24}$ c.c.d.$_{50}$/0.1 ml from the 12th hour sample as assayed on cell culture (figure 6). It is assumed that all subsequent samples were affected to the same degree.

Regardless of this possible inactivation due to temperature fluctuations, the titer increased rapidly from $10^{1.83}$ c.c.d.$_{50}$/0.1 ml at the 24th hour and reached a maximum of $10^{3.83}$ c.c.d.$_{50}$/0.1 ml at the 48th hour. From the 48th to the 96th hour, the titer decreased.

Similar results were obtained when the infectivity of the virus was measured on the CAM. At fifteen minutes the extra-cellular virus was $10^{1.25}$ e.i.d.$_{50}$/0.1 ml and gradually increased to $10^{1.83}$ e.i.d.$_{50}$/0.1 ml at 2 hours. The titer remained about on a plateau through the 12th hour. With titration of the 24th hour sample, the titer decreased to an estimated $10^{0.83}$ e.i.d.$_{50}$/0.1 ml, but thereafter increased rapidly to a maximum of $10^{2.5}$ e.i.d.$_{50}$/0.1 ml at 48 hours. From the 48th to the 96th hour, the titer decreased.

When the resuspended ground cell material was assayed in cell culture and on the CAM, slight evidence of infection was obtained with the undiluted inoculum only, and quantitative assay was not possible.

Intranuclear ILV Includion Bodies in CELC

For demonstration of includion bodies, the inoculum

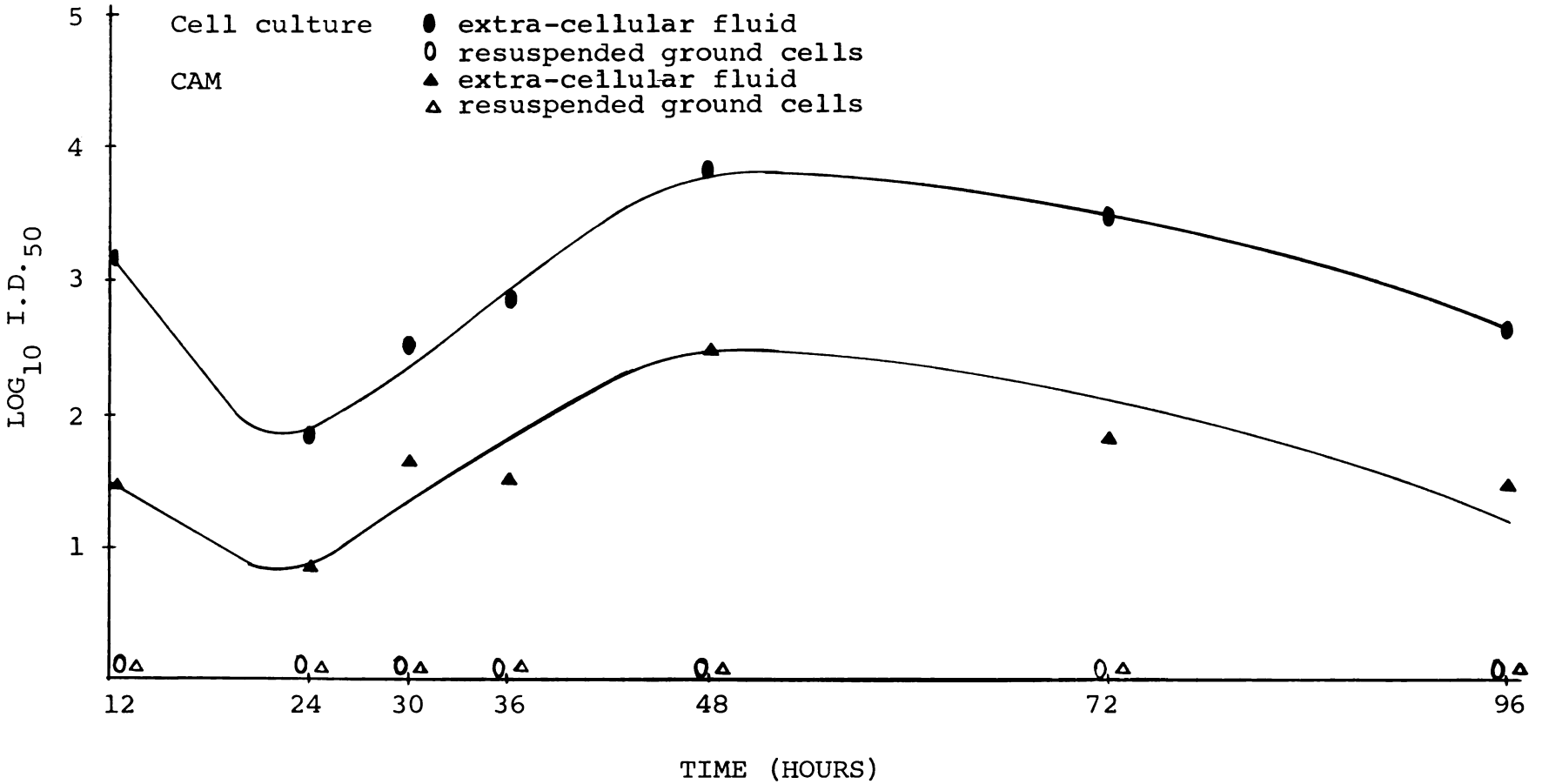

Figure 6. Multiplication cycle of 15th cell culture passage infectious laryngotracheitis virus in chicken embryo liver cells and on the CAM.

was diluted so that on the same slide there would be cells showing different stages of infection (figures 7 and 8). When stained at 18 hours after infection two types of inclusion bodies could be observed. At the edge of a group of infected cells, the inclusion bodies were smaller, irregular in shape, and stained darker, whereas the inclusion bodies observed that were closer to the area containing normal appearing cells, were stained lighter and filled a larger portion of the nucleus.

The ectodermal layer of the infected CAM showed extensive proliferation of cells, and many of the cells in the proliferated area showed intranuclear inclusion bodies (figures 9 and 10). These inclusion bodies were darker stained than those in CELC, and none were observed that filled the nucleus to the extent that some of the inclusion bodies did in the CELC.

Cell Spectrum for CELC ILV

Chicken embryo lung and kidney cells were susceptible to CELC ILV as evidenced by CPE similar to that exhibited by infected chicken embryo liver cells.

Cytopathic effect was not observed through three serial passages in Chang's liver cells, or two serial passages, respectively, in bovine embryo kidney cells and monkey kidney cells.

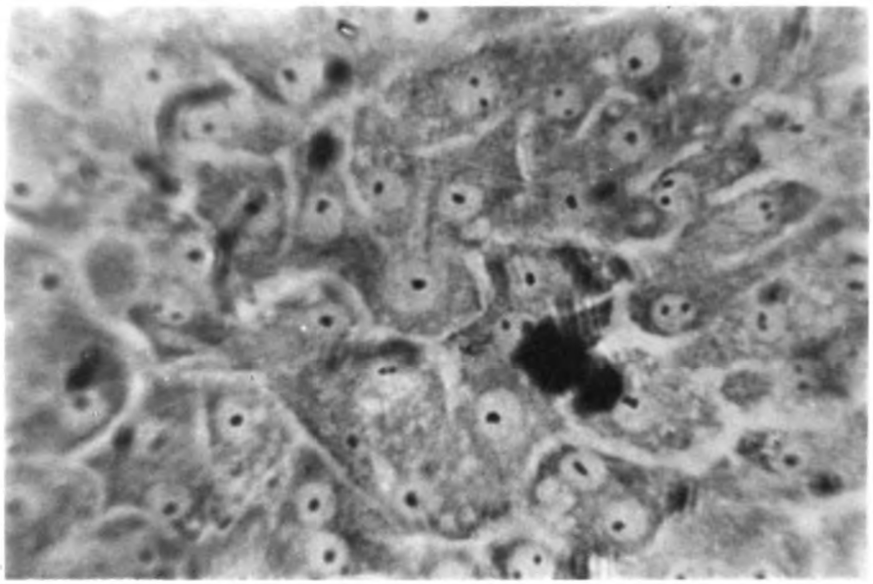

Figure 7. Normal chicken embryo liver cells at 72 hours. Carnoy fixative and Giemsa stain (970X).

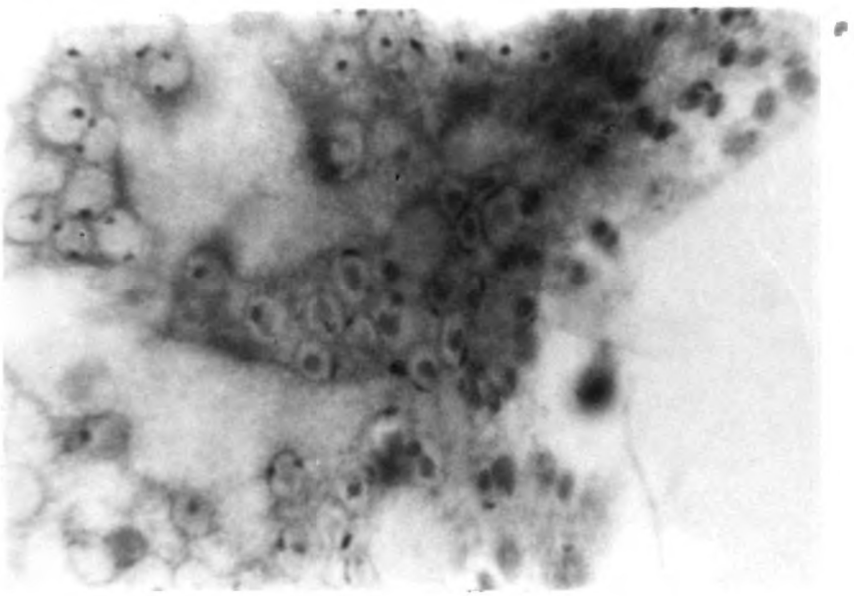

Figure 8. Infected chicken embryo liver cells showing inclusion bodies at 72 hours, inoculated at 54 hours. Carnoy fixative and Giemsa stain (970X).

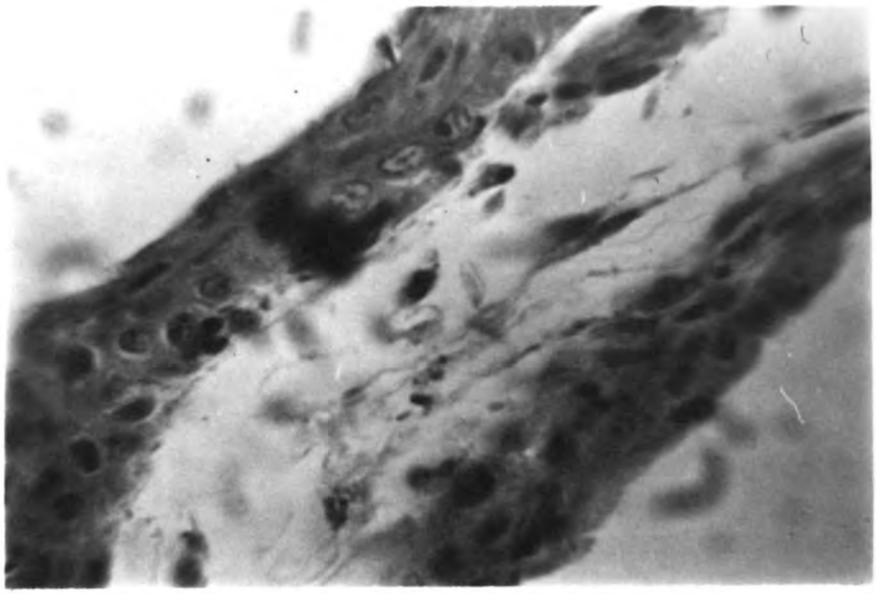

Figure 9. Normal CAM from a 16-day-old chicken embryo. Bouin fixative and hematoxylin and eosin stain (970X).

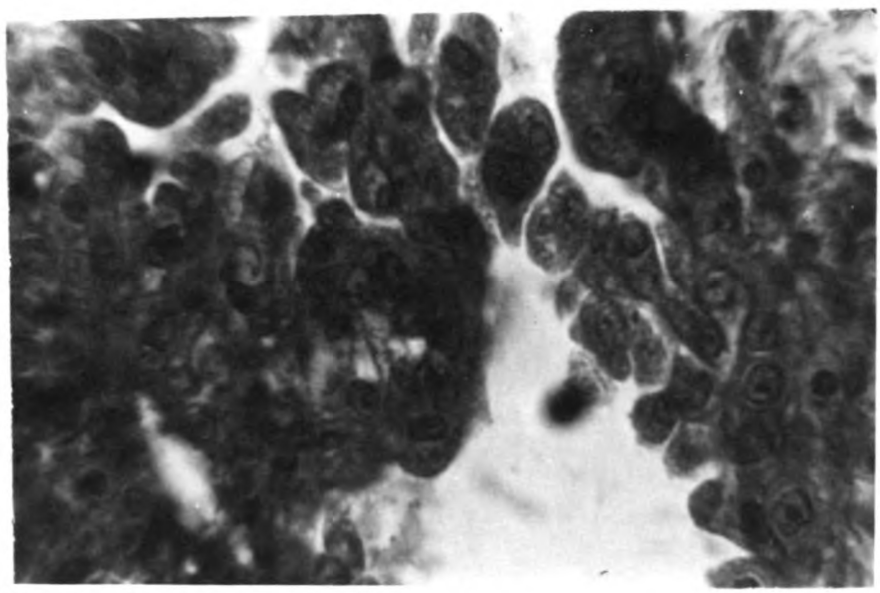

Figure 10. Infected CAM showing intranuclear inclusions from a 16-day-old chicken embryo inoculated on the 10th day. Bouin fixative and hematoxylin and eosin stain (970X).

DISCUSSION

The essential prerequisite for experimental investigations of a virus is the production in some host of recognizable signs of infection, specifically associated with the virus in question. Many viruses exhibit an affinity for certain tissues and must undergo a process of adaptation to become infectious for different tissues.

With ILV isolated initially from the natural host the chicken, adaptation to cultivation in the CAM was not necessary (Burnet, 1934), neither was adaptation required to cultivate CAM passaged 1323B strain ILV in chicken embryo liver cells in the present study. The virus became better adapted after continued serial passage in cell culture. The time required to demonstrate CPE, the criterion of infection, became shorter in subsequent passages, and the $c.c.d._{50}$ of the virus increased when assayed in cell culture. During this period of adaptation, the cell culture virus became less infective for the CAM.

Although the results of thermal inactivation of ILV at 56 C, as measured by CPE in cell culture, do not agree with those of Schalm and Beach (1935), the two systems are hardly comparable. Schalm and Beach (1935) used virus from

tracheal exudate and chickens as the indicator system. In the present study, cell culture virus was used and either cell cultures or the CAM was employed as the indicator system.

Another variable that must be considered is the fact that Schalm and Beach (1935) used the "thermal death point" of the virus which under their conditions gave purely qualitative results. Whereas, with the present system of measurements and conditions, using either cell cultures or the CAM, quantitative results were obtained.

In the present study, thermal inactivation was not found to be a linear function throughout the range of the reaction. A possible explanation for this might be that the virus had formed aggregates which protected a small portion of particles that remained viable and infective. In addition, the skewing of both of the curves, when measured in cell culture and on the CAM, might indicate that a possible change in the fundamental character of the virus had occurred. Singh (1957) found in studies of chicken embryo cultivated infectious bronchitis virus at 56 C, that the inactivation rates were not linear throughout the reaction, but were best fitted by a curve from which he could extrapolate two independent linear reactions. He hypothesized that this was due to a heterologous population of virus particles that had two

different rates of inactivation based on different thermostability. It is possible that the adaptation of ILV to cell culture has resulted in a heterologous population with two different rates of inactivation.

Since the NI is determined from the difference between the $i.d._{50}$ of the virus alone and the virus-serum mixture, the titer of the virus is a major variable in the results obtained with a decreasing virus-constant serum neutralization test. This is demonstrated by the comparison of NIs obtained using the same immune serum and the 10th and 20th cell passage virus. With the virus titer of $10^{3.6}$ $c.c.d._{50}$/0.1 ml at the 10th cell passage and $10^{6.2}$ $c.c.d._{50}$/ 0.1 ml at the 20th cell passage, the respective NIs were 3.0 and 4.5. When the CAM was used to assay the virus with $10^{3.3}$ $e.i.d._{50}$/0.1 ml at the 10th cell passage and $10^{2.6}$ $e.i.d._{50}$/ 0.1 ml at the 20th cell passage, the NIs were 2.7 and 2.6, respectively.

With the constant virus-decreasing serum neutralization tests, the slope of the lines of neutralization indicates that the ratio between the virus and antibody was not constant but changed progressively as the two values were altered (Horsfall, 1957). The apparent greater neutralization observed as measured on the CAM is only relative, as parallel titrations

of the virus in CELC and on the CAM indicate that the virus was less infective for the CAM.

The multiplication cycle of western equine encephalomyelitis virus in whole chicken embryo cell culture was shown to be a one-step exponential rise that reached a maximum in 6 to 8 hours (Dulbecco and Vogt, 1954). Dunnebacke (1956) described the same exponential rise with poliomyelitis virus in HeLa cell cultures with a maximum at 18 hours. In these experiments, the titer of the virus in the inoculum was high enough to insure that all the cells in the monolayer were initially infected. Spring (1960) using cell culture adapted infections bronchitis virus, found that when monolayers were infected with a low titer of virus, that after a five minute latent period the replication was so rapid that the rise in titer was exponential.

However, all viruses do not display the one-step exponential rise in titer, as Scott et al. (1953) described the multiplication cycle as wave-like when using herpes simplex virus on the CAM. They found that after a variable latent period, the length of which was dependent upon the initial titer of the inoculum, that the virus then entered an exponential increase period which lasted for approximately 10 hours, remained on a plateau for 6 hours, and gradually

increased to a maximum at 48 hours. The wave-like curve was a constant feature regardless of the titer of the virus in the inoculum.

The curve in the multiplication cycle of ILV in CELC showed similarity to that described by Scott et al. (1953), however, the latent period for ILV in CELC was a matter of minutes rather than hours. It is also possible that had the titer of the ILV in the initial inoculum been sufficient to initially infect all susceptible cells, that the results might have been a one-step exponential rise.

A possible explanation for the low titer of intracellular ILV in the ground cell material, could be that the infective particles were released as soon as they were formed, and that the virus remaining in the cells was in an incomplete or non-infective stage. When the cellular material was ground, it is possible that the majority of the particles released were of the non-infective form.

Cellular inclusion bodies have been accepted as evidence of viral infection, and the presence of cellular inclusion bodies as a diagnostic aid in many viral diseases. Intracytoplasmic inclusion bodies in certain viral diseases such as fowl pox, vaccinia, and others, have been found to be aggregations of virus particles. Intranuclear inclusion

bodies have been recognized as a stage in viral infection, but the exact significance has not been conclusively determined. Crouse et al. (1950) found that with herpes simplex virus in monkey kidney cells, the virus was being released into the extra-cellular fluid at about the same time that typical intranuclear inclusion bodies could be demonstrated. Sherer (1953) growing pseudorabies and herpes simplex viruses in Earle cells, stated that there was a relatively large yield of virus when only a few cells showed intranuclear inclusion bodies. Lebrun (1956) working with the fluorescent antibody technique with herpes simplex virus in human epidermoid carcinoma cells, found that some of the infected nuclei contained antigen as early as 24 hours post-infection. At 66 to 72 hours, typical intranuclear inclusion bodies were demonstrable, but the same nuclei contained little or no antigen.

Results similar to the above were obtained when the results of several of the present experiments were correlated. With ILV in the multiplication cycle as assayed in CELC, virus was being released from the cells at ninety minutes post-inoculation, but the typical intranuclear inclusion bodies were not demonstrable until the 18th hour. From this difference in time it may be hypothesized that the intranuclear inclusion bodies per se, were not involved in the actual replication of the virus.

To further confirm this observation, the forming of aggregates of nuclei filled with intranuclear inclusion bodies occurred before these same infected cells formed clumps which then sloughed off the surface of the glass. When these sloughed cells were assayed as was done throughout the entire multiplication cycle study, infective virus could not be quantitatively measured.

It may be hypothesized from these observations that the intranuclear inclusion bodies found in CELC infected with cell passaged virus probably represent a visible residue remaining after the interaction of the virus with the host cell.

Although no CPE was evidenced in the cell cultures from certain animal sources other than those from the chicken embryo, it is possible that after a greater number of serial passages that the CELC ILV would adapt and produce CPE. It is possible that there are untested cell types in which CELC ILV would produce CPE.

SUMMARY

1. Infectious laryngotracheitis virus strain 1323B was propagated for 20 serial passages in primary chicken embryo liver cells and produced a cytopathic effect.
2. The titer of the virus increased from $10^{3.5}$ c.c.d.$_{50}$/0.1 ml to $10^{6.2}$ c.c.d.$_{50}$/0.1 ml from the 10th to the 20th passage as titrated in cell culture.
3. After fifteen minutes at 56 C, the titer of the virus had decreased from $10^{3.8}$ c.c.d.$_{50}$/0.1 ml to an estimated $10^{0.3}$ c.c.d.$_{50}$/0.1 ml when assayed in cell culture.
4. The virus, as determined by CPE, was neutralized by anti-ILV serum.
5. The relationship between the amounts of virus used and the degree of neutralization indicated a variable proportionality between antigen and antibody.
6. Within fifteen minutes 98.5 per cent of the virus was adsorbed to the cells. The extracellular virus titer rose rapidly during the next ninety minutes, and then gradually increased to a maximum at 48 hours. Following this, the titer gradually decreased during the next 48 hours.
7. Infective intra-cellular virus was present in the ground

cellular material in amounts too small to be quantitatively measured.

8. Intranuclear inclusion bodies were demonstrable 18 hours post-inoculation. It was hypothesized that the intranuclear inclusion body is a visible residue of infection with CELC ILV, because the extra-cellular virus was being released in ninety minutes, whereas the inclusion bodies were not demonstrable until the 18th hour. In addition, when cells that contained intranuclear inclusion bodies were ground, the infective virus released was of such a low titer that it could not be quantitatively measured.
9. Cells from the chicken embryo lung and kidney could be infected with CELC ILV with CPE being evident in the first passage.
10. Three passages in Chang's liver cells, and two passages in bovine and monkey kidney cells failed to show infection as evidenced by CPE.

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