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MULTIPLICATION OF INFECTIOUS CANINE HEPATITIS VIRUS IN HELA CELLS

Toshio KINJO and Ryo YANAGAWA

Department of Hygiene and Microbiology
Faculty of Veterinary Medicine
Hokkaido University, Sapporo, Japan

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INTRODUCTION

The use of a susceptible cell line for virus assay is rather favorable in order to keep cells under appropriate conditions in each experiment.

At present, we have no cell line susceptible to infectious canine hepatitis (ICH) virus. Primary or secondary cultures of dog kidney cells have been used to propagate ICH virus.

The use of such cultures always involves difficulty in regulating cellular susceptibility uniformly and also involves the risk of contamination with unknown viral agents from the host animal. This experiment was initiated to establish or to search for cell lines which support the growth of ICH virus.

Since several trials to establish cell lines from dog kidney cell cultures were unsuccessful, attempts were made by the authors to investigate whether already established cell lines, even if derived from non-canine origin, were available for the above purpose.

In the course of such experiments, we found HeLa cells are capable of supporting partial multiplication of ICH virus. This report describes the multiplication of ICH virus in HeLa cells in comparison with that in dog kidney cell cultures.

MATERIALS AND METHODS

Virus strains Four strains of ICH virus were used. The strains Woc-4, Lewis and D-43 were provided by the Department of Veterinary Bacteriology, University of Tokyo, and the strain Matsuda originally isolated in this department by OSAMURA et al. (1957).

These viruses were grown in dog kidney cell cultures. All virus stocks were stored at $-20°C$.

Cell cultures and media Serially propagated HeLa cells were used. The cells were, after trypsinization, counted and diluted in the growth medium in a concentration of 1×10^5 cells per ml.

Primary dog kidney cell cultures (DKC) were prepared as described by YOUNGNER (1954). After incubating 3 to 4 days at 37°C, the cells were dispersed by trypsinization and diluted in

the growth medium as described above. The secondary cultures of DKC were usually used.

The cell suspension thus prepared was then dispensed into tubes (1×11 cm) or small bottles (3×5×3 cm), 0.5 ml to a tube and 5 ml to a bottle. Each bottle contained 4 coverslips. The cell monolayer was ready for use after 3 days of incubation.

For the growth medium, Hanks balanced salt solution containing 0.5% lactalbumin hydrolysate and 10% calf serum was used. For the maintenance medium, EARLE's balanced salt solution containing 0.5% lactalbumin hydrolysate, 0.1% yeast extract and 1% calf serum was used. Both media contained 200 units of penicillin and 200 μg streptmycin per ml.

Virus inoculation and titration After removal of the culture medium, virus material of 1 ml volume was inoculated onto cell monolayers in the bottles. Following 2 hr incubation at 37°C, the cells were washed twice with phosphate buffered saline (PBS), and then the maintenance medium was added.

For infectivity titration, 0.1 ml volume of tenfold dilution of virus material was inoculated into a tube. Four tubes of DKC were used for each virus dilution. Readings were made 6 or 7 days after inoculation. The infective titer was calculated statistically and expressed as the number $\log_{10} TCID_{50}$ per ml.

Cytological examinations At various intervals, 3 coverslips were withdrawn and placed in appropriate fixatives depending on the staining techniques to follow. At the same time, an aliquot of tissue culture fluid was taken and stored for both virus titration and serological tests.

Of the 3 coverslips one was stained with the Giemsa solution following methanol fixation, one was stained with 0.01% acridine orange solution using the method of TANAMI et al. (1961) and the remaining one was used for direct fluorescent antibody staining using the procedure described by GOLDSTEIN et al. (1961). The coverslips stained with acridine orange or fluorescent antibody were examined in a fluorescent microscope.

Serological experiments Hyper immune dog serum against strain Matsuda was used for both complement fixation and fluorescent antibody tests. The complement fixation (CF) test was performed using the method followed by the U.S. Army Medical School. The hemagglutination (HA) test was performed following the procedure described by SHIMIZU et al. (1960) with a slight modification in that physiological saline was used as diluent.

Electron microscopy Harvested culture fluids were first centrifuged at 1,800 g for 30 min and the supernatant fluids were recentrifuged at 4,500 g for 30 min to remove cellular debris from the materials. Finally, the supernatants were spun at 105,000 g for 1 hr in order to sediment the ICH virus. The resultant pellets were resuspended in distilled water 1/300 volume of the original culture fluids.

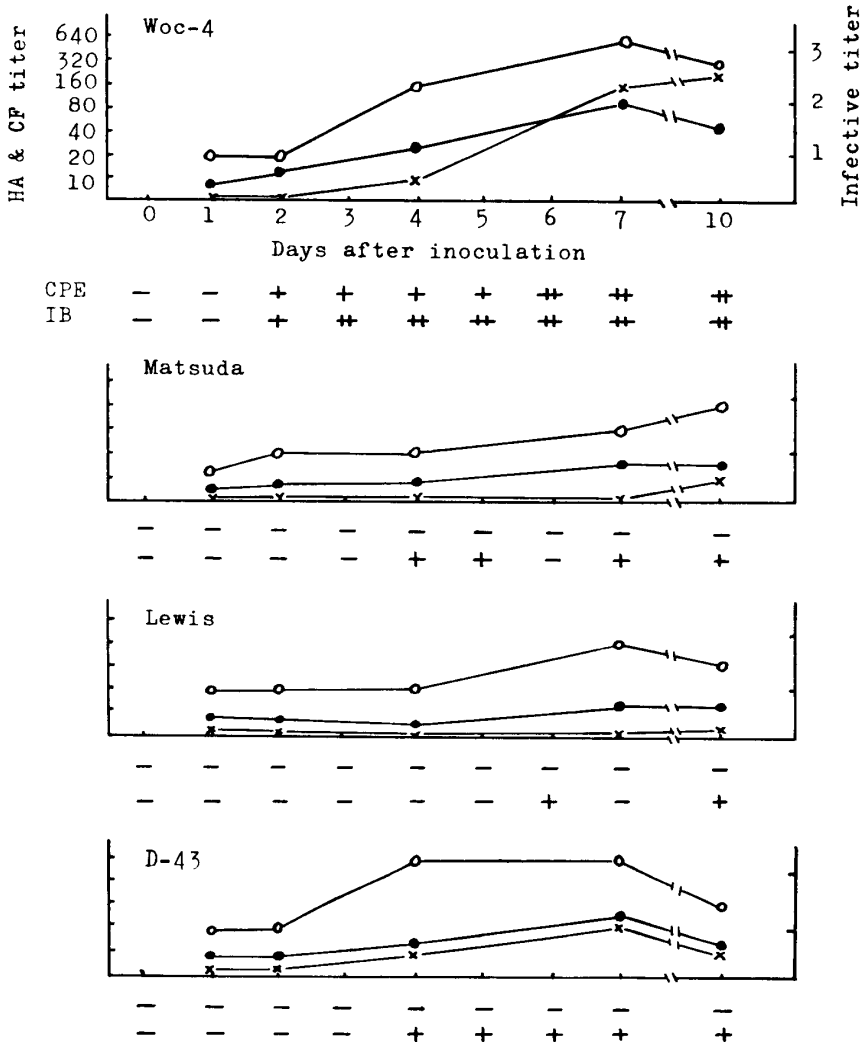
A microdrop of the virus suspensions was placed on carbon-coated grids. One minute later, the excessive fluids were drained, and specimens were stained with 2% phosphotungstic acid (pH 7.0) for 1 min.

Preparations were examined in the JEM-7 type electron microscope at an instrumental magnification of 40,000 ×.

RESULTS

1 Multiplication of 4 strains of ICH virus in HeLa cells Multiplication of ICH virus

FIGURE 1 *Multiplication of 4 strains of infectious canine hepatitis virus in HeLa cells*



Notes HA: Hemagglutinin ○—○, CF: Complement fixing antigen ●—●
HA and CF titers were expressed as the reciprocals of the highest antigen dilutions showing positive reactions.
Inf: Infectivity ×—×. Infective titers were expressed as $\log_{10}$ $TCID_{50}$/ml.
CPE: Cytopathic effect, IB: Inclusion body formation
Criterion of CPE and IB was defined as follows.
− : negative. Positive in less than 25% of cells was taken as +, 25–50% as ++, 50–75% as +++, and 75–100% as ++++.

in HeLa cells was studied using 4 virus strains. In all experiments, inoculum size calculated with DKC was standardized to 5.0 $\log_{10}$ $TCID_{50}$. Cytological findings, HA–, CF–, and infective-titers in culture fluids at indicated days after inoculation are illustrated in figure 1.

A cytopathic effect (CPE) in HeLa cells was produced by strain Woc-4, but not the other

3 strains. Strain Woc-4 produced generally CPE 48 hr after inoculation. As shown in figures 3 and 4, CPE produced by strain Woc-4 in HeLa cells was apparently similar to that produced in DKC.

Intranuclear inclusion bodies were observed in Woc-4 infected HeLa cells a day prior to the appearance of CPE as shown in figure 5. Similar nuclear changes were also detected in all cultures inoculated with the other 3 strains, despite the fact that they did not produce CPE. However, in the case of infection with these 3 strains, the appearance of the inclusion bodies was delayed and the proportion of the cells showing such nuclear changes was low.

An infective titer was detectable in the fluids of HeLa cell cultures inoculated with strain Woc-4 and D-43. The titer was low (fig. 1).

HA titer was, on the other hand, demonstrable in HeLa cell cultures inoculated with each of the 4 strains. The titer was only slightly reduced when compared to that obtained in DKC.

CF titer was also detectable in all HeLa cell cultures but the titer was low except in the culture inoculated with strain Woc-4. In the latter case, the CF titer detected was almost same as that of DKC.

It was suggested from the above experiments that the multiplication of ICH virus in HeLa cells was not same among the strains used and that multiplication was obviously found only when strain Woc-4 was used. Therefore, our efforts were concentrated mostly on the multiplication of strain Woc-4.

2 Characteristics of multiplication of strain Woc-4 in HeLa and dog kidney cells

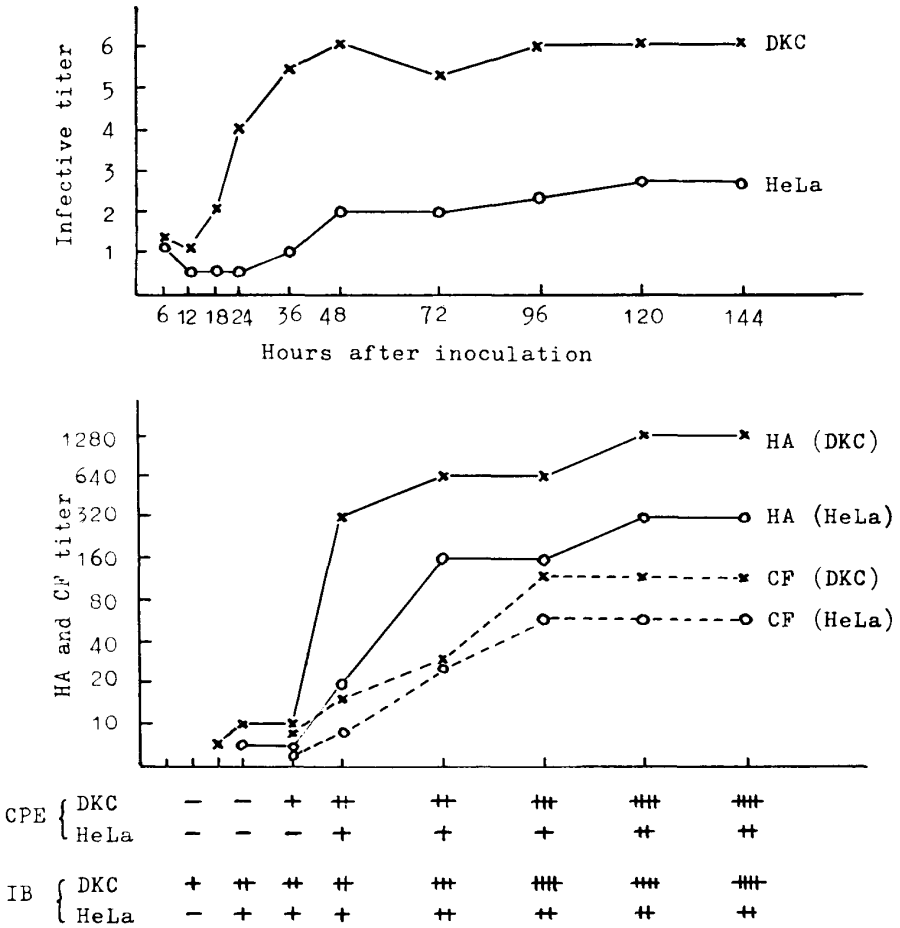
Although the CPE produced by strain Woc-4 was apparently similar in HeLa cells and DKC as mentioned before (figs. 3 & 4), it was found that the rate of degenerated cells examined in stained preparation was quite different between the two: almost 100% in DKC but only 30% in HeLa cells. Furthermore, the appearance of the inclusion bodies was about 12 hr later in HeLa cells than in DKC. Inclusion bodies produced by strain Woc-4 in HeLa cells and DKC are shown in figures 5 and 6 respectively.

As shown in figure 2, the infective titer in culture fluids was clearly different between DKC and HeLa cells. In DKC, the maximum infective titer in culture fluids was 6.0 $\log_{10} TCID_{50}$, while in HeLa cells it was only 2.7. On the other hand, viral antigens such as hemagglutinin and complement fixing antigens were demonstrated in both culture fluids without much differences in titers.

Since the viral antigenic proteins which were detectable by HA and CF tests were produced in HeLa cells, the reason for the small quantity of infective particles produced in HeLa cells was thought to be attributed to less active DNA synthesis.

Acridine orange staining was, therefore, applied in order to see whether nucleic acid synthesis progressed after infection in HeLa cells. The nuclei of uninfected HeLa cells showed a uniform light green appearance lightly flecked with DNA, and the cytoplasms were a uniform flame red (fig. 7). Sixteen hours after inoculation, there was a marked increase in the brilliance of nuclear DNA in the HeLa cells and a clumping of DNA in the central regions of nuclei and the periphery of the nuclear membrane. There was no detectable increase in the intensity of staining in cytoplasmic RNA. As shown in figures 8~10, DNA staining appeared bright in the central parts, which corresponded to the inclusion bodies

FIGURE 2 *Multiplication of infectious canine hepatitis virus (strain Woc-4) in HeLa and dog kidney cells*



Note: Signs and abbreviations are same with those of figure 1.

seen in the Giemsa stained preparations, and also in the margins of nuclei as described above. As the infection progressed, the infected cells began to round up, the nuclei becoming eccentric and pyknotic with brilliant DNA staining. No detectable increasing of nucleic DNA staining was found in uninoculated cells except the cells undergoing mitosis which stained brilliantly.

In DKC, DNA staining after infection showed a process apparently similar to that in HeLa cells, but the virus-induced nuclear DNA as shown by acridine orange staining, appeared about 12 hr earlier than in HeLa cells.

Thus it was demonstrated with this staining method that an increase in DNA synthesis due to infection progressed in HeLa cells too, but the rate was slower than in DKC.

Viral antigen staining with fluorescent antibody was also conducted at the same time. In HeLa cells, brightly fluorescent nuclear granules were first observed 24 hr after inoculation. Thereafter, these granules increased in number, aggregated, and then immigrated

toward the inner zone of the nuclear margins (figs. 11, 13 & 14). At a later stage of infection, the cells exhibited decreased fluorescent staining in the inclusion bodies, and in a few cases, diffuse fluorescent staining was seen in the cytoplasmic region around the nucleus. In DKC, fluorescent granules were already detectable at the 12th hr of infection, but subsequently, the process of increased and decreased fluorescent staining was almost similar to that in HeLa cells (figs. 12 & 15).

3 Relation between titers of infectivity and viral antigens Infective-hemagglutinin titer (I-H) ratio which is used in influenza virus preparation as indicator of the presence of incomplete virus (VON MAGNUS, 1954) was obtained in both DKC and HeLa cells inoculated with Woc-4. Three experiments were performed, each titer at the 6th day of infection was calculated and expressed as $\log_{10}$ titer in table 1. I-H ratio of HeLa cells was always smaller than that of DKC. Thus, the reason HeLa cells did not produce many infectious particles was explained by the production of noninfectious virus particles, which possessed viral antigens.

TABLE 1 *Relationship between titers of infectivity and viral antigens in culture fluids of HeLa and dog kidney cells 6 days after inoculation with infectious canine hepatitis virus (strain Woc-4)*

EXP. NO.	HELA				DKC			
	Inf	CF	HA	I/H	Inf	CF	HA	I/H
1	2.7	1.8	2.5	0.2	6.3	2.4	3.3	3.0
2	2.5	1.5	2.2	0.3	5.5	2.4	2.8	2.7
3	2.5	1.8	2.2	0.3	6.0	2.1	3.3	2.7

Notes: Inf: Infective titer was expressed as $\log_{10}$ $TCID_{50}$/ml.
CF, HA: Complement fixing antigen and hemagglutinin titers were expressed as the reciprocals of the highest antigen dilutions showing positive reactions.
I/H: Infective-hemagglutinin titer ratio

4 Effects of inoculum size on the multiplication of ICH virus in HeLa cells To determine the inoculum size required by HeLa cells to support ICH virus multiplication, the virus material was serially diluted and inoculated. The results are shown in table 2. It was found that a virus dose of 4.5 $\log_{10}$ $TCID_{50}$/ml was necessary to produce CPE and infectious particles in HeLa cells. On the contrary, a virus dose of 2.5 $\log_{10}$ $TCID_{50}$, which was the smallest employed, was sufficient to produce CPE and infectious particles in DKC. The viral antigens produced in HeLa cells inoculated with the diluted virus materials were low in titers, throughout the period of the experiments (8 days). In contrast, infective virus and viral antigens produced in DKC were high in titers on the 6th day of infection and not markedly affected by the serially diminished inoculum size.

Absorption efficiency of strain Woc-4 to both cells was compared. After a 2 hr

TABLE 2 *Effects of inoculum size on the multiplication of infectious canine hepatitis virus (strain Woc-4) in HeLa and dog kidney cells*

SIGNS OF VIRAL MULTIPLICATION	INOCULUM SIZE	HELA								DKC					
		Days after Inoculation								Days after Inoculation					
		1	2	3	4	5	6	7	8	1	2	3	4	5	6
CPE	5.5	−	+	+	+	++	++	++	++	+	++	++	+++	++++	++++
	4.5	−	−	−	−	+	+	+	+	−	+	++	++	+++	++++
	3.5	−	−	−	−	−	−	−	−	−	−	+	++	+++	++++
	2.5	−	−	−	−	−	−	−	−	−	−	+	++	++	+++
Inf	5.5	0.5*1	0.5		2.0		2.0		2.5	2.0	5.0		5.5		6.3
	4.5	<0.5	<0.5		<0.5		1.0		0.5	<0.5	4.5		6.0		6.0
	3.5	<0.5	<0.5		<0.5		<0.5		<0.5	<0.5	3.0		5.3		6.0
	2.5	<0.5	<0.5		<0.5		<0.5		<0.5	<0.5	1.5		4.5		5.5
HA	5.5	10*2	10		40		80		160	20	320		640		640
	4.5	5	5		5		10		10	5	160		640		320
	3.5	< 5	< 5		< 5		5		5	< 5	20		160		640
	2.5	< 5	< 5		< 5		< 5		5	< 5	20		320		320
CF	5.5	4*2	3		32		64		64	16	16		64		128
	4.5	2	2		4		8		8	< 2	16		64		64
	3.5	< 2	< 2		2		2		2	< 2	< 2		32		32
	2.5	< 2	< 2		< 2		< 2		< 2	< 2	8		32		64

Notes: *1 $\log_{10}$ TCID$_{50}$/ml *2 Reciprocals of the highest antigen dilutions showing positive reactions
Other signs and abbreviations are same with those of figure 1.

absorption period, the unabsorbed virus material was taken from the cultures, and its infective titer obtained. When a maximum dose (5.5 $\log_{10}$ $TCID_{50}$) was employed as the inoculum, the fluid remorved after the 2 hr adsorption period showed infective titers of 3.5 in the HeLa cells and 2.0 in DKC respectively. Therefore it could be said, that more than 10 times the number of virus particles were absorbed in DKC than in HeLa cells.

5 Confirmation of the Woc-4 strain as ICH virus Woc-4 was the only strain, out of the 4 strains used, which showed CPE accompanying viral multiplications in HeLa cells.

To solve the question of possible contamination of human adenoviruses in our stock of strain Woc-4, the following experiments were carried out. PEREIRA (1958) reported the early adenovirus CPE, known to be caused by a toxin, could be abolished by trypsin digestion of viral materials without loss of infectivity. To demonstrate CPE in HeLa cells produced by the infection of strain Woc-4 was not caused by such a toxin, PEREIRA's method was employed. One-tenth ml of a 0.25% solution of trypsin in Hanks solution was added to 0.4 ml of the virus material and incubated for 1 hr at 37°C, following which, 0.1 ml of a 0.25% solution of soybean trypsin inhibitor was added to the mixture. The trypsin-treated and untreated virus materials were inoculated into HeLa cells respectively.

The both HeLa cell cultures showed completely the same CPE and no effect of trypsin-treatment on CPE production was observed.

Heat treatment of virus materials was also performed since the toxin was found to be relatively more heat stable than virus infectivity (EVERETT & GINSBERG 1958). When infected-culture fluids of strain Woc-4 was heated for 30 min at 56°C or for 10 min at 60°C, under these conditions early adenovirus CPE was thought to be unaffected, the CPE in HeLa cells produced by this strain was abolished the parallels with infectivity by such treatments.

For more direct demonstration of the strain Woc-4 as a ICH virus, cross neutralization tests were conducted using antisera against the 4 strains of ICH virus used and also commercial antisera against human adenovirus types 1~14. As expected, the results of the experiments showed that the strain Woc-4 was completely identical with the other 3 strains of ICH virus and no evidence of contamination with human adenoviruses was obtained.

6 Electron microscopic observation Culture fluids harvested from HeLa cells, which were inoculated 6 days before with strain Woc-4, were concentrated by centrifugation, as described above, and examined electron microscopically. No other procedures for virus purification were employed.

As illustrated in figure 16, both full and empty particles were observed. Although the size and structure of these particles are identical to that of typical ICH virus which originated from the infected DKC, empty or irregularly structured particles (shown in fig. 17) were much more frequently found in the virus material collected from HeLa cells than those from DKC.

Small virus-like particles about 20~30 mμ in diameter were found in the preparations. Since the size is much smaller than that of ICH virus the virus-like particles might not be ICH virus. The nature of the particle was not examined.

DISCUSSION

The ICH virus has been hitherto considered as a virus with considerably high

host specificity.

Tissue culture cells susceptible to ICH virus, primary kidney cell cultures of ferret (FIELDSTEEL & YOSHIHARA, 1957), pig (EMERY & YORK, 1958), racoon (BOLIN et al., 1958) and hamster (MOTOHASHI, 1961), and cell-lines of pig kidney (FIELDSTEEL & YOSHIHARA, 1957) have been reported. Susceptibility of 2 cell-lines of pig kidney, established in this department, have been investigated by the authors, so far with negative results. In the course of such experiments, however, the authors found HeLa cells were capable of supporting a limited multiplication of ICH virus.

From the results obtained, HeLa cell may not be suitable as a tool for ICH virus assay because the susceptibility is not high. But the ICH virus and HeLa cells give us an interesting system.

As described in the text, out of the 4 virus strains used only Woc-4 showed specific CPE in HeLa cells accompanied by the production of the infectious virus which was low in titer. Inoculum of 4.0 $\log_{10}$ $TCID_{50}$ was requried to produce such CPE.

However, the nuclear inclusion bodies resulting from the virus infection were detected in HeLa cells inoculated with any ICH virus strain used. Similar results were obtained in other experiments which were not mentioned in this paper. In those experiments, 4 of 12 strains showed CPE. The frequency of the appearance of inclusion bodies varied among the strains used.

As to the mechanism of cytopathic changes, MOULTON & FRAZIER (1964) reported, from their cytochemical experiments using DNA inhibitor, that the formation of typical CPE is dependent to a great extent on over-production of DNA in the cells and also that the formation of the inclusion body is associated with DNA synthesis rather than with any destructive effects of the virus. Therefore, strain differences in CPE production shown in our experiment might depend on the differences in the ability of viral DNA synthesis of the strains used. At present, however, we have no data to support such an explanation of strain differences. Serologically, no difference was found among the strains used.

It is of interest that in spite of the lower yields of infective virus, viral antigens are produced in HeLa cells nearly to the extent that they are produced in DKC.

A similar phenomenon has been reported in influenza virus-chick embryo system by VON MAGNUS (1954), and also recently in SV_{40}-African green monkey kidney cell system by UCHIDA et al. (1966). It is caused by serial passages using undiluted virus. The phenomenon is supposedly due to incomplete virus particles contained in the virus materials. The incomplete virus interfered the conversion of the newly formed particles into fully active virus. And it is proposed that

the proportion of fully active virus to incomplete virus in a given preparation can be estimated fairly easy by means of the infective-hemagglutinin titer (I–H) ratio.

The authors calculated the I–H ratio for the purpose of estimation of the proportion of incomplete to complete virus in virus materials grown in HeLa cells. The I–H ratio was always smaller in HeLa cells than in DKC (table 1). Thus, it appeared that the virus grown in HeLa cells contained many incomplete virus particles.

Electron microscopic observation roughly confirmed the above estimation. There are, of course, several reports showing such incomplete, "empty" ICH virus particles in infected DKC preparations (KUNISHIGE et al., 1961; HORNE, 1962). However the proportion of incomplete to complete virus particles seemed to be higher in the viruses grown in HeLa cells than those grown in DCK.

To clarify the process of ICH virus multiplication in HeLa cells, the fluorescent antibody- and acridine orange- stainings were applied. The former method almost resembled the process of antigen formation in both cell systems, though the appearance of fluorescent nuclear granules was about 12 hr earlier in DKC. By acridine orange staining, increased synthesis of DNA was noted, after ICH virus infection, in the nuclei of both HeLa cells and DKC.

Thus, it is supposed that the virus induced DNA production and viral antigens were produced in HeLa cells as well as in DKC.

In spite of the production of viral components such as complement fixing, hemagglutination antigens and DNA, yields of infective virus particles were decreased in HeLa cells. In addition, HeLa cells produced more empty particles.

A simple explanation of such incomplete, "empty" virus formation in HeLa cells would be that the cells are able to support the synthesis of viral components but not the stage of assembly, when infective virus particles are completely constructed from the viral components.

It has been well known that the active virus production in infected cells is interfered and inhibited by incomplete particles and also by antiviral factors such as interferons and inhibitors. To clarify whether lower yields of infective virus in HeLa cells are affected by such factors, investigations by the authors are in progress.

CARMICHAEL (1965) reported an incomplete cycle of adenovirus type 4 multiplication in DKC. He found by autoradiography in combination with immunofluorescent techniques that both DNA and viral antigen synthesis occurred in DKC. But he could not detect any infective virus. He considered that, by unknown mechanisms, these virus components might fail to combine to produce the infective virus or are structurally deficient.

We could detect a few of the infective virus particles in HeLa cells, in this respect CARMICHAEL's report differes with us, the mechanism of incomplete virus growth in cultured cells derived from unsusceptible hosts appears to be in a similar category.

Other important points that should be clarified are (1) why more than 70 per cent of the HeLa cells were incapable of supporting ICH virus production, even when the multiplicity of infection was high, and (2) also why large doses of virus such as 4.5 $\log_{10} TCID_{50}$ were necessary to induce viral multiplication. Experiments relating to these problems are also in progress.

Small virus-like particles (20~30 mμ) were found, during electron microscopic observation, in the preparation of ICH virus grown in HeLa cells. Similar particles have been observed in preparations of human and simian adenoviruses, and have been known as adenovirus associated virus (AAV) (ATCHISON et al., 1965) or as adeno-satellite virus (ASV) (PARKS et al., 1967). It is supposed that the particle is a distinct viral entity but not a structural component of adenovirion and is a defective virus which requires adenovirus for its replication. SUGIMURA & YANAGAWA (1968) also observed such small particles in a strain of ICH virus (Matsuda) grown in DKC. It will be an important future problem to study possibility of interaction of these small virus-like particles on the multiplication of ICH virus.

SUMMARY

HeLa cells were capable of supporting partial multiplication of infectious canine hepatitis (ICH) virus, the strain Woc-4.

Infective virus grown in HeLa cells was low in titer despite the fact that the virus antigens were detected serologically and by the fluorescent antibody technique. Also deoxyribonucleic acid synthesis was demonstrable by acridine orange staining nearly as much as those detected in dog kidney cells. Thus it was considered that HeLa cells were able to support the synthesis of viral components but could not properly support the stage of assembly.

By electron microscopic observation, the virus materials grown in HeLa cells were found to contain many empty, incomplete virus particles.

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EXPLANATION OF PLATES

PLATE I

Fig. 3 Cytopathic effect in HeLa cells 72 hr after inoculation with infectious canine hepatitis virus (strain Woc-4).
unstained preparation
The virus strain used for inoculation in the following figures is same with the strain employed in this figure.

Fig. 4 Cytopathic effect in dog kidney cells 36 hr after inoculation
unstained preparation

Fig. 5 Intranuclear inclusion bodies in HeLa cells 48 hr after inoculation
Giemsa stained preparation

Fig. 6 Intranuclear inclusion bodies in dog kidney cells 36 hr after inoculation
Giemsa stained preparation

Fig. 7 Uninoculated HeLa cells
acriding orange staining

Figs. 8~10 Acridine orange stained HeLa cells 36 hr (fig. 8) and 48 hr (figs. 9 & 10) after inoculation.
Notice intranuclear inoclusion bodies and margins of nuclei that stain lightly for deoxyribonucleic acid

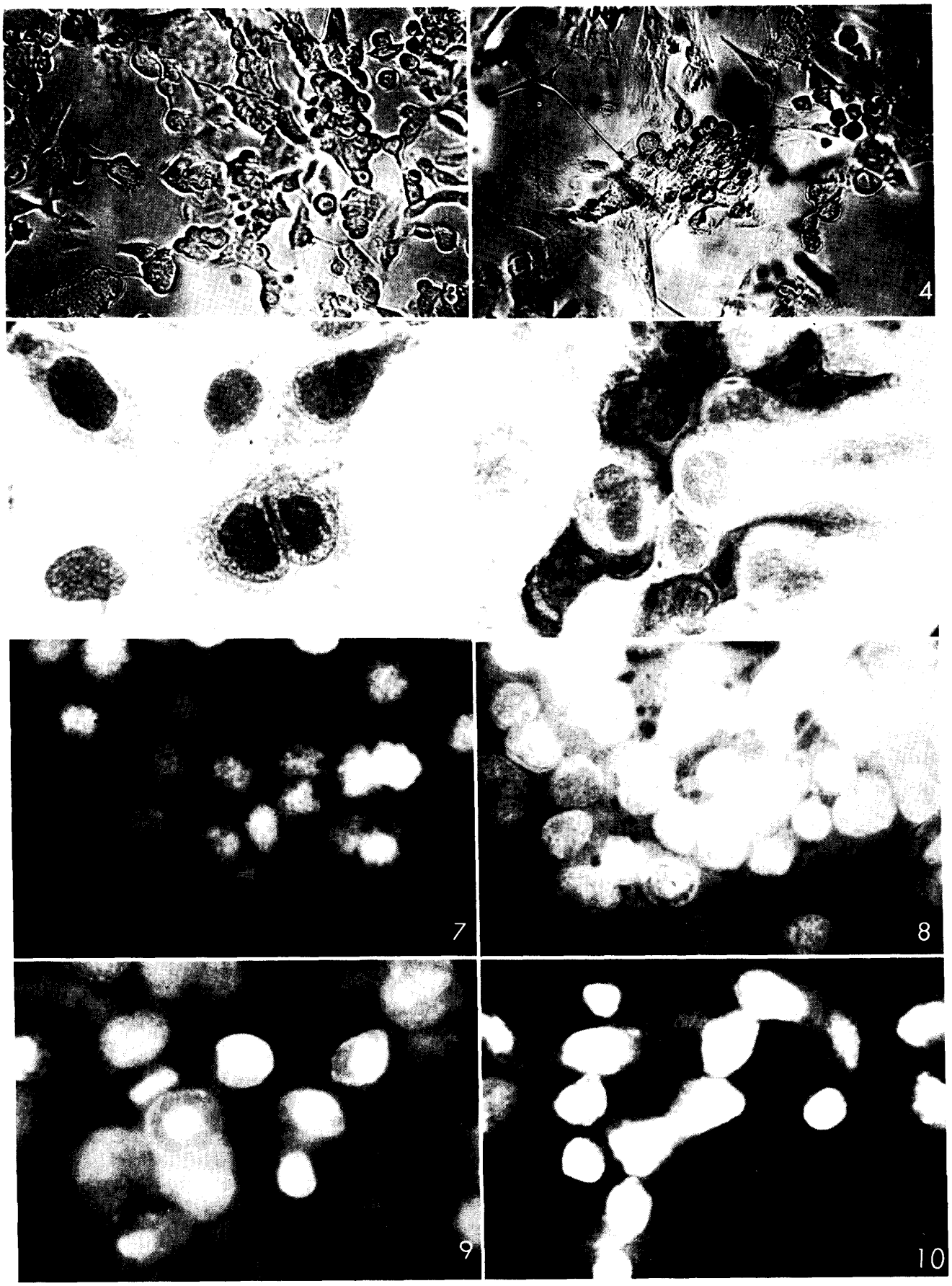
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PLATE II

Figs. 11~15 ICH virus inoculated cells stained with direct fluorescent antibody technique

Fig. 11 HeLa cells 48 hr after inoculation

Fluorescent small granules throughout the nucleus (centre) and inner zone of nuclear margins (both sides)

Fig. 12 Dog kidney cells 24 hr after inoculation

Nuclear fluorescences similar to those of figure 11 can be observed.

Figs. 13 & 14 HeLa cells 72 hr after inoculation

Fluorescent staining is more intense at the margins of nuclei. Nuclear granules as seen in figure 11 are also observed.

Fig. 15 Dog kidney cells 48 hr after inoculation

Large fluorescent granules throughout the nucleus and intense fluorescence at the nuclear margin are observed.

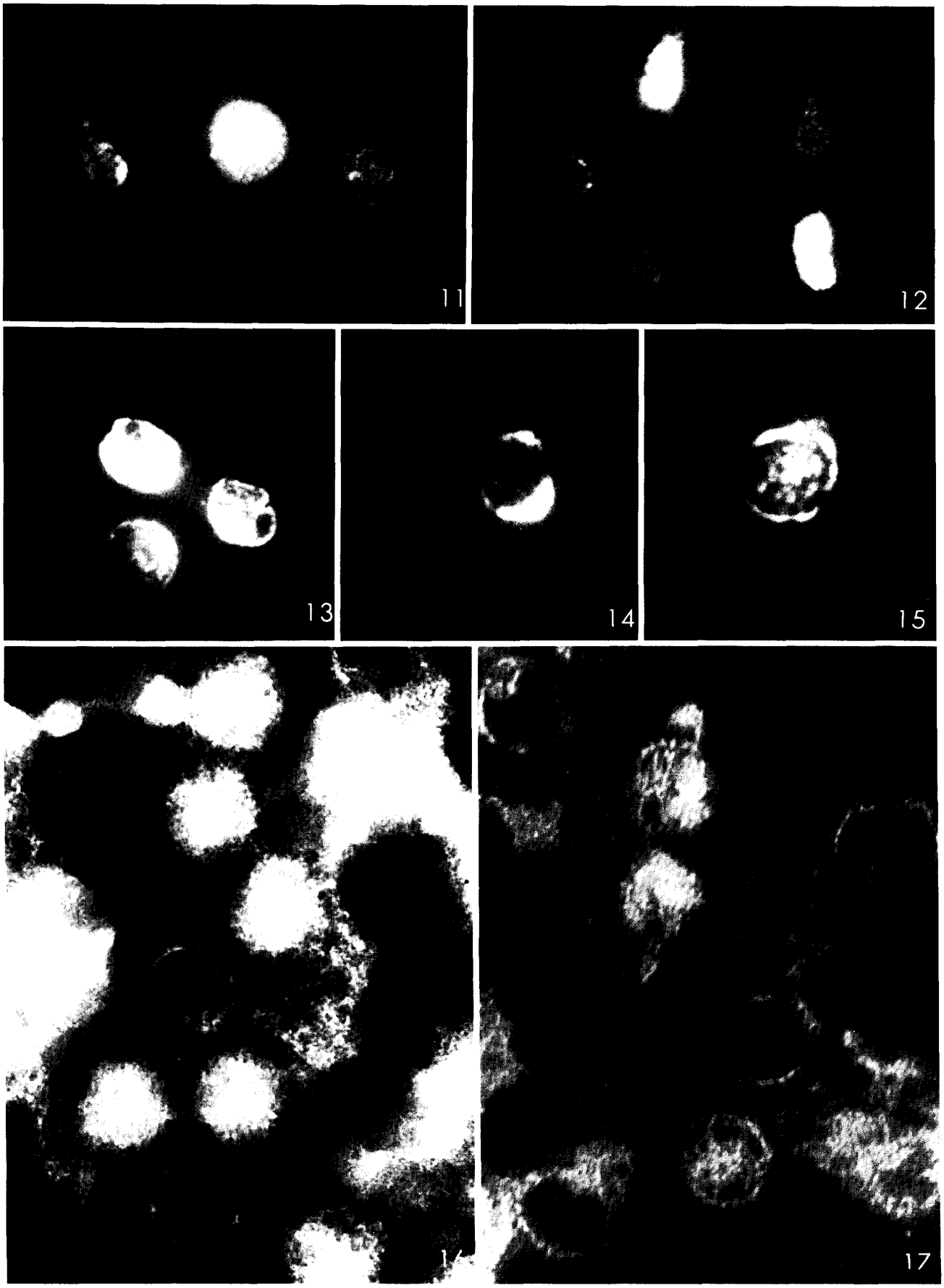
Fig. 16 Electron micrograph of negatively stained ICH virus particles concentrated from culture fluids of HeLa cells 6 days after inoculation

Full and empty particles are observed.

Notice 2 small virus-like particles at upper left × 240,000

Fig. 17 Another field of the same preparation as figure 16

Many empty of irregularly structured particles are observed. × 240,000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