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PROPERTIES OF A SMALL VIRUS ASSOCIATED WITH INFECTIOUS CANINE HEPATITIS VIRUS

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INTRODUCTION

The adeno-associated satellite virus (ASV)* is a recently described group of small defective DNA viruses (picodnaviruses) that can replicate only in cells coinfecting with helper adenoviruses^{1,2,7,11,18,28}. It is characteristic of ASV that they interfere with the replication of the adenovirus^{9,21}. Electron microscopy^{1,3,17} and fluorescence studies^{4,17} suggested that ASV replicated in the nucleus, but not in the cytoplasm. Four types of ASV have been described serologically^{11,23}. Isolation of ASV and distribution of the antibodies have frequently been found in man and monkey^{6,8,24}. However, detection of ASV from infectious canine hepatitis virus (ICHV) and other adenoviruses, except human and simian adenoviruses, has not been reported. Ability of ICHV to help the replication of the defective human ASV has been shown in canine cells¹¹ and human cells²⁷.

SUGIMURA & YANAGAWA (1968) found a small virus associated with ICHV, Matsuda strain, and reported its morphology. Attempts were made by the authors to isolate this small virus and to clarify its properties. The results are described in this paper, which suggest that the small virus is a member of ASV.

MATERIALS AND METHODS

Virus Five strains of ICHV, Matsuda, FD, C-I, N-IV and Woc-4, were used in this study. Of these, Matsuda was originally isolated in this department by OSAMURA et al. (1957).

Tissue culture and virus inoculation The growth medium used was HANKS' balanced salt solution supplemented with 10% calf serum and 0.5% lactalbumin hydrolysate. Maintenance medium used was the same medium containing 1% calf serum. These media were added to 200 u/ml of penicillin and 200 µg/ml of streptomycin.

Primary dog kidney cell cultures (DKC) were prepared as described by YOUNGNER (1954).

* AAV (Adenovirus-associated virus) was proposed by ATCHISON et al. (1965) and ASV (Adeno-associated satellite virus) was proposed by MAYOR et al. (1966). In this paper we prefer ASV.

Usually, secondary cultures of DKC were used in this study. The cells of primary culture were washed twice with phosphate-buffered saline (PBS) (pH 7.2) and finally resuspended in a growth medium so as to contain 10^5 cells per ml. The cells, suspended in the growth medium, were dispensed 10 ml per bottle ($4 \times 10 \times 4$ cm) and 0.5 ml per tube (1.2×12 cm) and incubated at 37°C . Cell sheets grown in bottles were used for the propagation of the virus. Each bottle was inoculated with 1 ml of the virus material, and after 2 hr for the adsorption of virus, maintenance medium was added. The inoculated virus material was discarded and the cells were washed with HANKS' balanced salt solution 3 times. Then the maintenance medium was added and incubated at 37°C .

Detection and isolation of the small virus When cytopathic effect (CPE) appeared maximally, usually 4~5 days after inoculation, the cells in bottles were frozen at -20°C . Then the cells were thawed at room temperature. The fluid and cells in the bottles, about 200 ml, were removed and centrifuged at 3,000 rpm for 30 min. The supernatant fluid obtained was centrifuged at 8,000 rpm for 30 min, and the resultant supernatant fluid was again centrifuged at 30,000 rpm for 90 min in an ultracentrifuge (Hitachi, model 40P). The upper portion, about $3/4$, of the supernatant fluid was removed, and the rest of the supernatant fluid was mixed with the pellet, by pipetting, which was then centrifuged at 8,000 rpm for 30 min. The procedure of these centrifugations was repeated again. The pellet was mixed with the lower portion, $1/4$, of the supernatant fluid or, after removing the supernatant fluid, was suspended in 0.5 ml PBS. The former pellet was used for isolation of the associated small virus from ICHV by filtration through 50- μ millipore filter, and the latter for electron microscopic study.

Electron microscopic examination of the negatively stained viruses The material for electron microscopic examination was mounted on the carbon-coated collodion membrane grid for 2~3 min. After absorbing the excess amount of material with filter paper, the specimen was negatively stained for 1~2 min with 1% phosphotungstic acid which was adjusted with 1N potassium hydroxide to pH 7.0, and was examined with a JEM-7 electron microscope at instrumental magnification up to 100,000 times.

Infectivity titration Ten-fold serial dilutions of virus materials were made with maintenance medium. The infectivity titer was determined by inoculation 0.5 ml of each ten-fold dilution into sets of 4 tubes containing DKC. Following a reincubation of 5 days, the 50% tissue culture infective doses (TCID₅₀) were calculated utilizing the method of BEHRENS-KÄRBER.

Acridine orange staining of the small virus The 50 μ -millipore filtrate of ICHV Matsuda was concentrated 10 times by centrifugation. The concentrated filtrate was found to contain observable small viruses by electron microscopy, but no ICHV particles. One drop of the concentrated filtrate was mounted on a cover glass, dried at room temperature and fixed in CARNOY's fixative for 5 min. The specimen was then washed thoroughly in McIlvaine's buffer (pH 3.8), and stained with 0.01% acridine orange for 5 min. The stained preparation was washed with the same buffer and examined under Nikon fluorescence microscope. Saturated solutions of herring sperm DNA (Daiichikagaku) and yeast RNA (Sigma) were stained as controls.

Ultra thin sectioning Cells in bottle culture were inoculated with strain Matsuda

$10^{3.5}$ TCID₅₀. This virus material contained the small virus, which was larger in number than ICHV particles under the electron microscope. The cells were harvested at various times after inoculation. Before harvesting, the culture medium was removed from the inoculated cell cultures and PBS was added in order to wash the cells. Then the cells were scraped from the glass with the rubber policeman. The cell suspension thus obtained was centrifuged at 1,000 rpm for 5 min. Cell pellets were fixed in 3.5% glutaraldehyde for 20 min as described by SABATINI et al. (1963) and postfixed in 1% osmium tetroxide for 30 min at 4°C. After dehydration in serial ethanol and infiltration with propylene oxide, the cell blocks were embedded in Epon 812 (LUFT). Ultra thin sections were cut by JUM 5A type ultramicrotome. The sections were stained with uranyl acetate and lead citrate. Sections were examined with a JEM-7 electron microscope at instrumental magnification up to 30,000 times.

RESULTS

1 Detection of the small virus from ICHV

Five strains of ICHV were examined electron microscopically. The results shown in table 1 indicate that only the Matsuda strain contained small virus. The small virus particle was hexagonal, without envelope and 20~25 m μ in diameter (fig. 1). The ratio of ICHV to the small virus particles in Matsuda strain was from 1:60 to 1:70 by repeated electron microscopic examinations. The other 4 strains contained ICHV particles but no small virus (fig. 2).

Matsuda strain which contains the small virus, Matsuda (+), and FD strain containing no small virus, FD (0), are described below.

TABLE 1 *Detection of the small virus from ICHV*

PARTICLES*	ICHV STRAINS				
	Matsuda	FD	C-I	N-IV	Woc-4
ICHV	+	+	+	+	+
Small virus	+	-	-	-	-

* Detected by electron microscopic examination

2 Comparison of the infectivity titer between Matsuda (+) and FD (0)

Infectivity titers (TCID₅₀) of Matsuda (+) and FD (0) were, as shown in table 2, $10^{3.29}$ and $10^{7.25}$ per ml respectively. It was noted that the infectivity titer of Matsuda (+) was distinctly lower than that of FD (0). In experiment II shown in this table, the same inoculum sizes were used but the appearance of CPE was earlier in the cultures inoculated with FD (0) than those inoculated with Matsuda (+).

3 Evidence of defectiveness of the small virus

Possibility of the autonomous replication of the small virus was examined using DKC. The multiplication of ICHV and the small virus was examined by electron microscopy.

TABLE 2 *Infectivity titers of Matsuda (+) and FD (0)*

VIRUSES	EXP I	EXP II* ¹	AVERAGE
Matsuda (+)	2.75* ²	3.50	3.29
Matsuda (+)	3.50	3.00	
FD (0)		7.25	7.25

*¹ Inoculated with 100 TCID₅₀/ml in DKC from the same puppy

*² Expressed as log TCID₅₀/ml

(+) Contained the small virus

(0) Contained no small virus

a) Experiment using the small virus separated from ICHV by filtration through 50-m μ millipore filter

For this purpose, the small virus in Matsuda strain was separated from ICHV by filtration through 50-m μ millipore filter. The filtrate was found to contain observable small virus by electron microscopy but no ICHV particles. Matsuda (+) produced ICHV and the small virus while FD (0) produced only ICHV. As shown in table 3, filtrate containing the small virus alone caused no CPE, and no multiplication of the small virus and ICHV was found. However, when the small virus were inoculated in DKC simultaneously with FD (0)

TABLE 3 *Multiplication of the small virus in the presence and absence of infectious ICHV*

a)* Experiment using the small virus separated from ICHV by filtration through 50-m μ millipore filter

VIRUSES	CPE	ICHV	SMALL VIRUS
Matsuda (+)	+	+	+
Small virus	-	-	-
Small virus + FD (0)	+	+	+
FD (0)	+	+	-

b)* Experiment using the small virus whose helper ICHV was inactivated by heating

VIRUSES	CPE	ICHV	SMALL VIRUS
Matsuda (+)	+	+	+
Matsuda (+) 60°C 10 min	-	-	-
Matsuda (+) 60°C 10 min + FD (0)	+	+	+
FD (0)	+	+	-

* Infectivity titer of Matsuda (+) and FD (0) used was 100 TCID₅₀/ml.

multiplication of the small virus and ICHV could be found. Therefore, it was proved that the small virus could not replicate in DKC, but could replicate when coinfecting with ICHV. These findings suggest that, in DKC, the small virus is defective and requires ICHV as helper for its replication.

b) Experiment using the small virus whose helper ICHV was inactivated by heating

Another evidence of the defectiveness of the small virus was given in the following experiment. A test was made of heating Matsuda (+) with the expectation that heating at a certain degree of temperature would inactivate ICHV but not ASV. It has been known that ICHV was inactivated by heating at 60°C for 5 min, while ASV was more heat resistant. Matsuda (+) and FD (0) heated at 60°C for 5 or 10 min caused no CPE, and no multiplication of ASV and ICHV were found. However, when Matsuda (+) heated at 60°C for 10 min was inoculated in DKC simultaneously with FD (0) multiplication of the small virus and ICHV was found (tab. 3). The above findings show that the multiplication of the small virus could be supported only by infectious ICHV and not by heat-inactivated ICHV. The small virus was also found to be resistant to heating at 70°C for 10 min.

These experiments suggest that the small virus with Matsuda strain is a member of adeno-associated satellite viruses.

4 Acridine orange staining of the small virus

Matsuda culture fluid, 200 ml amount, was concentrated by centrifugation in an amount of 20 ml, which was filtered through 50-m μ millipore filter. Details of these procedures with those of acridine orange staining were described in MATERIALS AND METHODS. Dried dropped-preparation of concentrated and purified small virus, fixed and stained with acridine orange, was examined under fluorescence microscope. RNA from yeast and DNA from herring sperm were used as controls. The result was shown in table 4. The small virus fluoresced yellow-green, indicating that the small virus contained double stranded nucleic acid, probably DNA.

TABLE 4 *Acridine orange staining of the small virus*

PREPARATIONS	STAINING PROPERTIES
Small virus	Yellow-green
Matsuda (+)	Yellow-green*
DNA (from herring sperm)	Yellow-green
RNA (from yeast)	Red-orange

* A few small aggregates of red-orange color were seen, which were considered to be contaminated cellular debris.

5 Multiplication of the small virus by electron microscopy

Electron microscopic examinations were carried out on DKC infected with Matsuda (+). The small virus particles, 20~25 m μ , were found in the nucleus from 18 hr after inoculation. At the same time ICHV particles also appeared. The small virus particles were usually found as close packed aggregates throughout the nucleus (figs. 3 & 4). Particles scattered

in the nucleus were also found (fig. 4). As described by ARCHETTI et al. (1966) 3 different conditions were observed: a) nuclei which apparently contained only the small virus particles; b) nuclei which apparently contained only ICHV particles; c) nuclei which contained both the small virus and ICHV particles (fig. 3). In the case of c, the small virus and ICHV particles formed aggregates, which were separated respectively.

It was noticeable, from 48 hr after inoculation, that the small virus particles were visible in the cytoplasm. The intracytoplasmic array of the small virus particles was usually like a cord, where the small virus particles were mostly arranged between two membranous structures (figs. 5~7). Crystalline array was also found but less frequently, in the cytoplasm (fig. 8).

The existence of the small virus in the cytoplasm, found in this study, is considered to be the result of their own multiplication in the cytoplasm. The reasons are 1) the intracytoplasmic array of the small virus, as described above, is quite characteristic and distinct from their intranuclear array; 2) no rupture of the nuclear membrane was observed. It was found that ICHV also multiplied in the nucleus and cytoplasm (fig. 9). Therefore, the cells infected with Matsuda (+), that is the infection with the small virus and ICHV, could be grouped into the following 9 patterns: a) cells whose nucleus contained only the small virus particles (fig. 4); b) cells whose cytoplasm contained only the small virus particles (figs. 5 & 8); c) cells whose nucleus contained only ICHV particles; d) cells whose nucleus contained both the small virus and ICHV particles; e) cells which contained only the small virus particles in the nucleus and cytoplasm (fig. 7); f) cells which contained only ICHV particles in the nucleus and cytoplasm (fig. 9); g) cells which contained the small virus particles in the cytoplasm and ICHV particles in the nucleus (fig. 6); h) cells whose cytoplasm contained only ICHV particles; i) cells which contained both the small virus and ICHV particles in the nucleus, and only ICHV particles in the cytoplasm (fig. 10).

DISCUSSION

Attention has been focused on ASV, which is a defective virus and requires adenovirus as a helper. Such ASV has been known to be associated with human and simian adenoviruses, but not with adenoviruses of other animal origins.

A small virus associated with ICHV Matsuda, described by SUGIMURA & YANAGAWA (1968) was found as a defective virus which required ICHV as a helper. This is the first report of a defective virus associated with ICHV.

Defectiveness of the small virus was proved by isolating it from ICHV particles by filtration through 50-m μ millipore filter or inactivating ICHV by heating, and then inoculating into DKC. No replication of the small virus was found as long as infectious ICHV was not coinfecting. Cell cultures other than DKC were not used in this study. However, recent study using HeLa, Vero (a cell line from monkey) indicates that the small virus alone can not replicate in these cells (authors' unpublished work). Further studies using other cell cultures

(particularly those of dog origin) should be used to confirm the defectiveness of the small virus.

BINN et al. (1968) reported the isolation of minute viruses recovered from fecal specimens of asymptomatic dogs. These viruses, which belonged to picodnaviruses, produced CPE in dog cell lines but not in primary DKC, and were serologically different from H-1, RV and MVM. Differences between these minute viruses and the small virus associated with ICHV Matsuda will be studied later.

There are also picodnaviruses of rodent origin. These rodent picodnaviruses, H-1, H₃, RV, X14 and MVM, are distinguishable from ASV because they are capable of autonomous replication. Of these, H-1 multiply in the presence of human adenovirus type 12 in human embryonic lung cells, in which H-1 alone can not multiply¹⁴). Comparative studies among the rodent picodnaviruses, 4 types of ASV and the authors' small virus will also be a further problem for investigation.

When infected in DKC with the same amount of ICHV Matsuda (+) and FD (0), respectively, infectivity titer of Matsuda (+) was lower than that of FD (0). And Matsuda (+) produced CPE, grape-like aggregates of cells, and destruction of cells more slowly than FD (0) did. It seems that such low infectivity titer and delayed appearance of CPE of Matsuda (+) are the results of the small virus. Similar phenomenon has been reported in human and simian adenoviruses associated with ASV^{9,21}).

Almost complete destruction of cells was caused by Matsuda (+), which was nearly equal to the destruction of cells caused by FD (0). Ultra thin section studies showed that only small virus particles appeared in some cells. Similar findings were also reported by other workers^{1,3,17}). Also MAYOR et al. (1967) by fluorescent antibody study and BEREZKY & ARCHETTI (1967) by acridine orange staining showed that multiplication of ASV alone was common. Therefore, there might be a possibility that the small virus alone could produce destruction of cells.

ASV is known to contain DNA. ATCHISON et al. (1965), MAYOR et al. (1965) and JAMISON & MAYOR (1965) reported from the results of acridine orange staining that ASV associated with SV15 possessed double-stranded DNA. Later MAYOR & MELNICK (1966), using the same method, showed that this ASV contained single-stranded DNA. BEREZKY & ARCHETTI (1967) observed the inclusion body of ASV associated with SV11, SV15 and SV39 fluoresced red. They considered that these ASV contained single-stranded DNA. From the results of chemical analysis, density gradient centrifugation and thermal melting experiments, ROSE et al. (1966) and PARKS et al. (1967²²) reported that type I ASV and type 4 ASV

contained double-stranded DNA. Guanine plus cytosine (G+C) content of type 4 ASV was determined as 58~62% which was higher than that of type 1 ASV (54.2%). Ito et al. (1967) proposed by investigating formaldehyde treated DNA of type 4 ASV that DNA in the virion was single-stranded while extracted DNA was double-stranded. The small virus separated from ICHV Matsuda fluoresced yellow-green by acridine orange staining, indicating that the small virus contained double stranded nucleic acid, perhaps DNA. The properties of DNA of the small virus associated with ICHV Matsuda should be studied further using a more concentrated and purified virus preparation.

ASV has been observed to multiply in the nucleus but not in the cytoplasm^{1,3,4,17}. Electron microscopic studies on the cells infected with ASV have been done only after the helper adenovirus produced CPE^{1,3,17}. Whole stages of ASV infection have never been clarified by electron microscopy. In the present experiment, the small virus particles first appeared in the nucleus 18 hr after inoculation. At the same time ICHV particles also appeared.

Sometimes only the small virus was found in the cells. It may be possible that the helper effect of ICHV is not produced by the virion of ICHV. Some subviral substances might have helper effect in the multiplication of the small virus.

It should be especially emphasized that multiplication of the small virus was found in the cytoplasm. Intracytoplasmic array of the small virus particles was observed from 48 hr after inoculation which was characterized by their cord like arrangement between two membranous structures. Another arrangement of the small virus particles, crystalline array, was also found. The membranous structures were never observed in uninfected cells. The small virus particles within the membranous tubules, were uniform in size, shape and density. They were 20~25 m μ in diameter, comparable to the size of the small virus particles determined by negative staining. Membranous structures containing virus particles have been well known in plant and insect cells infected with their viruses. However, this type of virus arrangement within membranous structure has rarely been known only twice in animal viruses. Recent studies have shown that the development of simian virus 40 (SV 40) appeared within cytoplasmic tubules¹⁵ or cytoplasmic membranes²⁰, and vesicular exanthema of swine virus, type H₅₄, particles within cisternae in the cytoplasm³¹. We were unable to explain the significance and the origin of the membranous structures found in the present study.

It was difficult, in thin section studies, to distinguish the small virus from ribosome. So, in this study, the small virus was morphologically identified only when the above described characteristic arrays were recognized. There is a possibility that more small virus particles are present in the cytoplasm.

The pathogenicity of ASV is unknown. Newborn hamsters inoculated with ASV showed no disease development 2 months after inoculation²⁾. No sign of tumor development 6 months after inoculation and no malignant transformation have been reported in any of the cell lines infected with ASV¹⁷⁾. GILDEN et al. (1968) reported that inclusion of ASV types 1 and 2 in adenovirus type 12 inocula did not influence oncogenicity of this adenovirus in newborn hamsters. It will be necessary in the future to determine the pathogenicity and to know the distribution of the small virus associated with ICHV Matsuda.

SUMMARY

The small virus contained in infectious canine hepatitis virus (ICHV) Matsuda was studied. Morphologically, the particles of the small virus were cubic, without envelope and 20~25 m μ in diameter. In the culture fluid of dog kidney cells (DKC) infected with Matsuda, the ratio of ICHV particles to the small virus particles was from 1 : 60 to 1 : 70, as observed through the electron microscope. Infectivity titer of Matsuda, containing the small virus, was distinctly lower than that of another ICHV, FD, which contained no small virus. The small virus alone inoculated in DKC caused no CPE and did not multiply. Multiplication of the small virus was found only when ICHV was coinfecting. The multiplication of the small virus was dependent on infectious ICHV but not on heat-inactivated ICHV. The small virus was resistant to heating at 70°C for 10 min, and fluoresced yellow-green with acridine orange staining. From the above findings, the small virus, detected for the first time from ICHV, was found to be a member of the adeno-associated satellite viruses. Ultra thin section studies suggested that this small virus multiplied not only in the nucleus but also in the cytoplasm.

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EXPLANATION OF PLATES

PLATE I

Fig. 1 ICHV, Matsuda (+); ICHV (indicated with arrows) and many small virus particles are seen.

The small virus particles, hexagonal, include empty particles.

Fig. 2 ICHV, FD (0); only ICHV particles are seen.

Figs. 1 and 2 were the preparations negatively stained with 1% phosphotungstic acid.

Scales indicate 100 m μ .

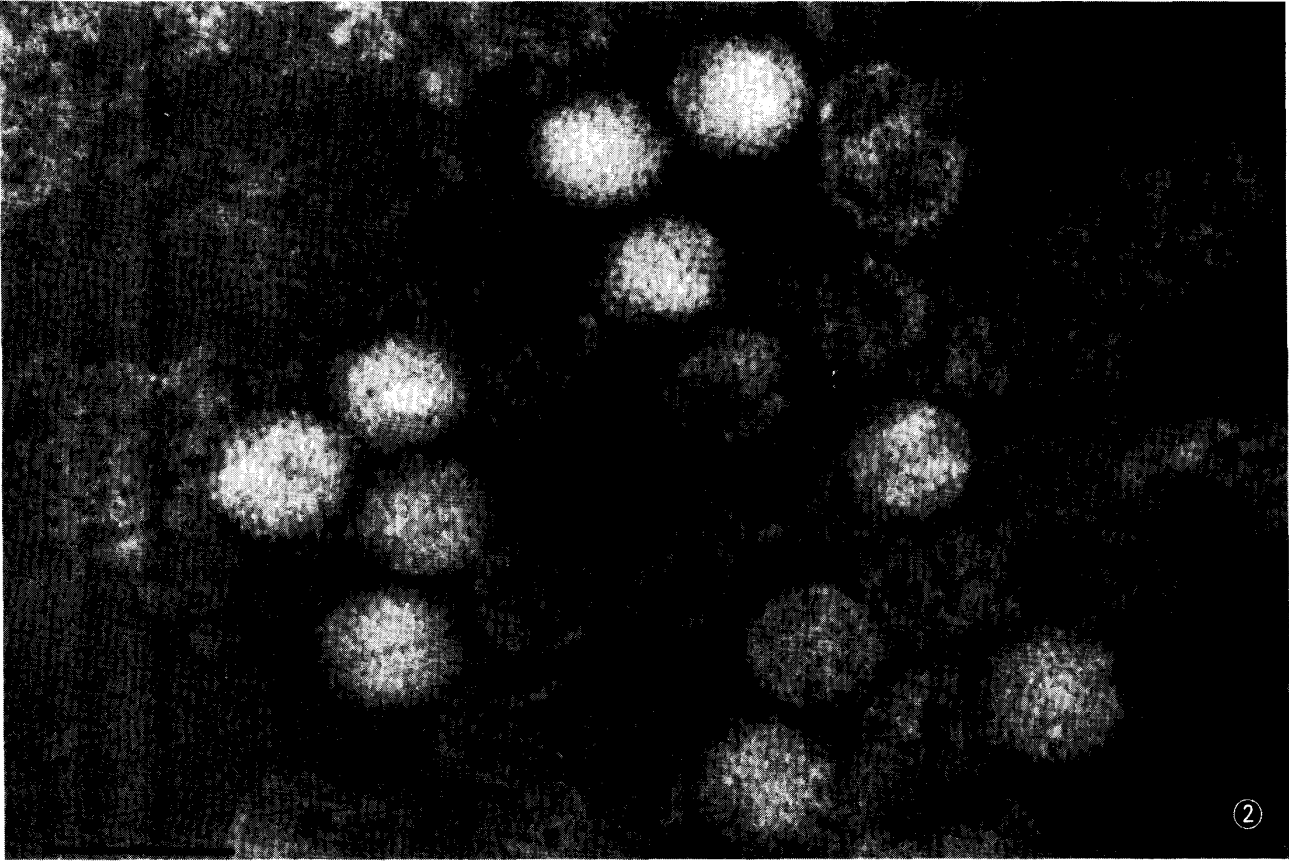
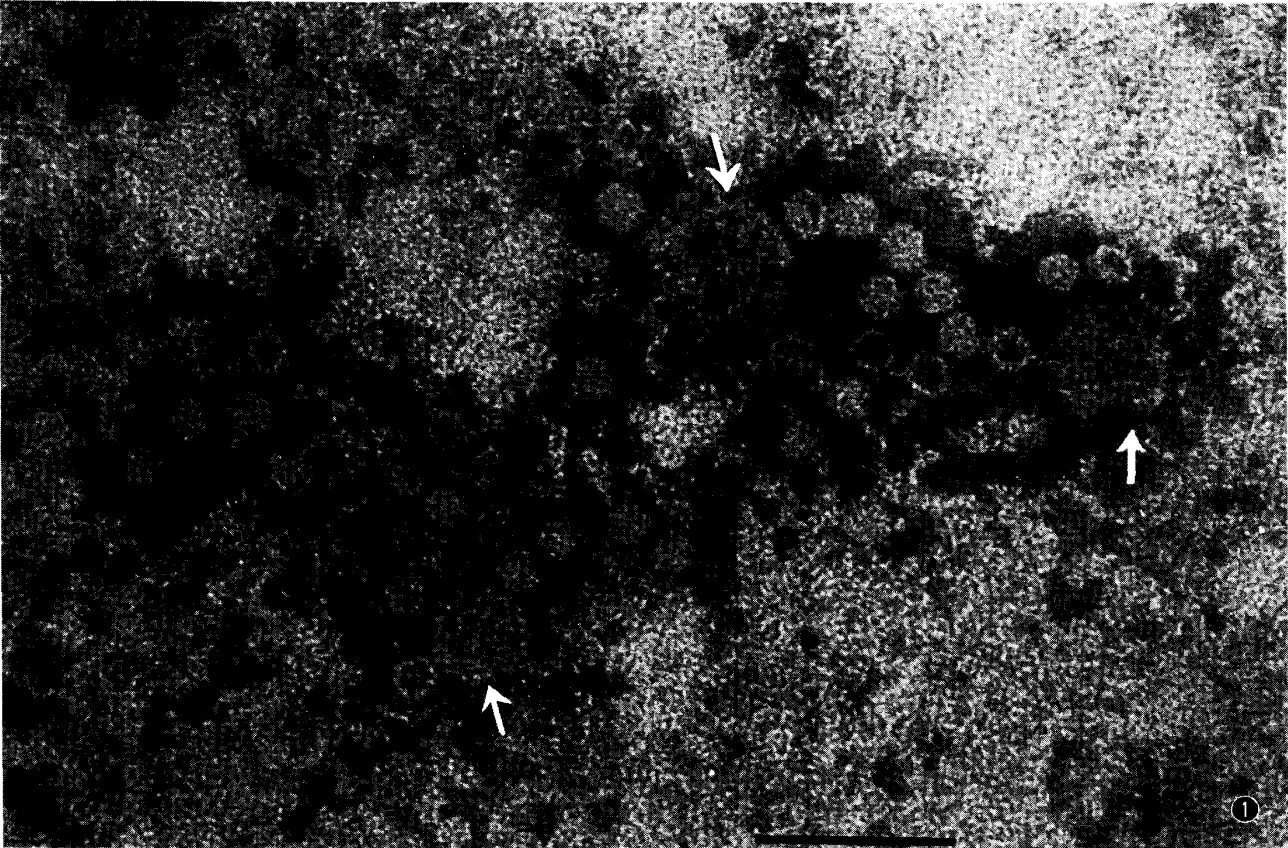


PLATE II

Fig. 3 The small virus and ICHV particles in the same nucleus (18 hr after inoculation)

The small virus and ICHV particles are located separately from each other. ICHV particles attached to cell surface are also seen.

Scale indicates 1 μ .

Fig. 4 The nucleus containing only small virus particles, which aggregate and scatter (72 hr after inoculation)

Scale indicates 250 m μ .

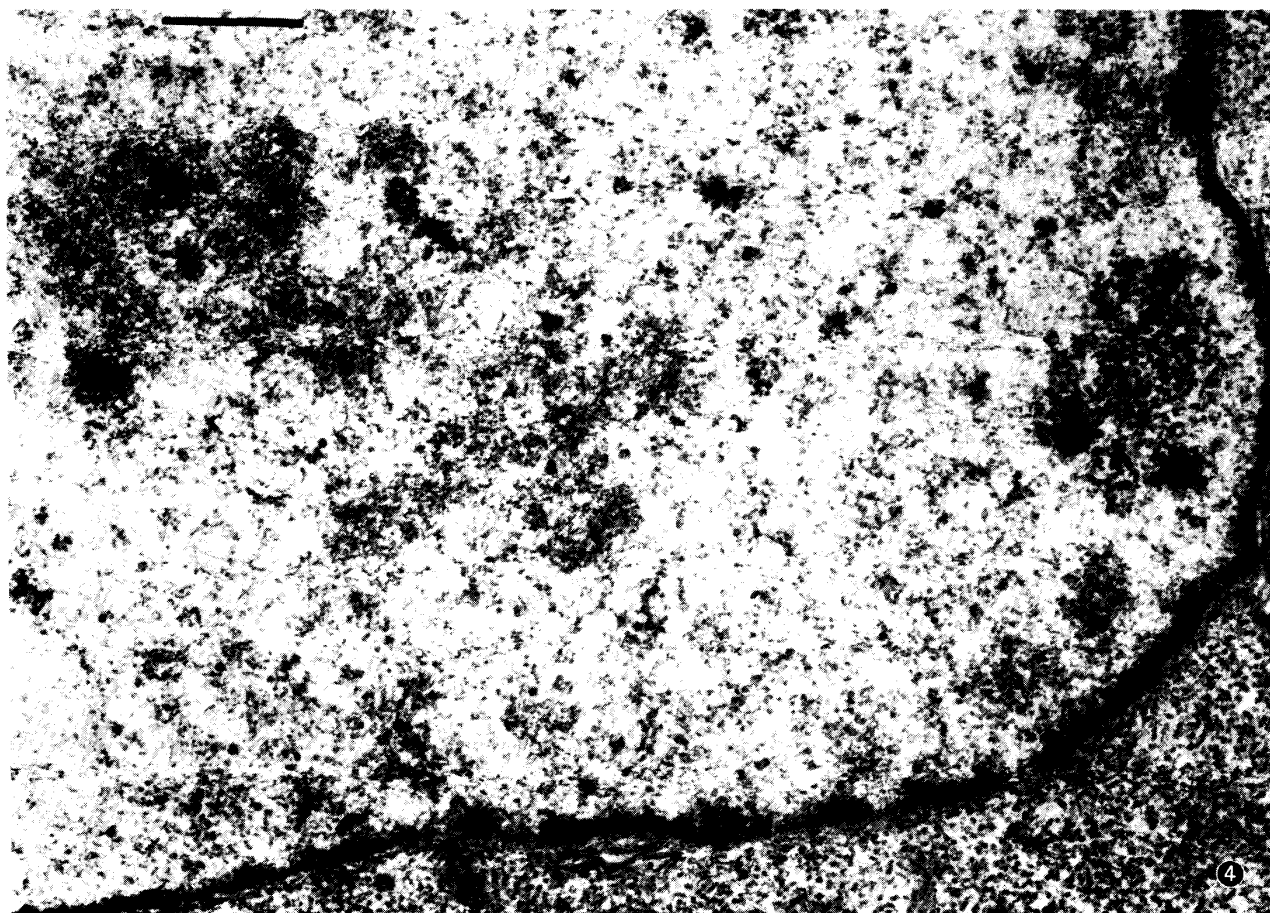
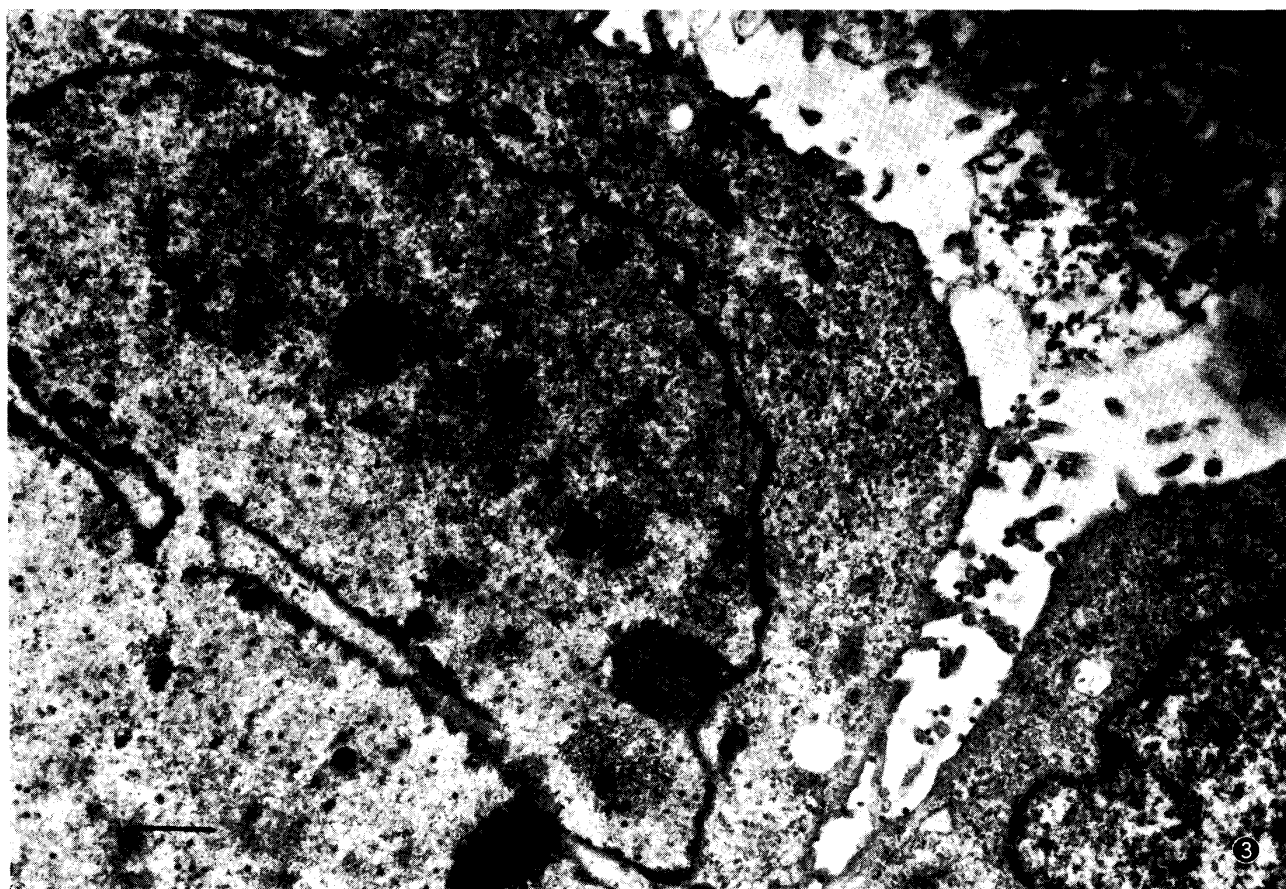


PLATE III

Fig. 5 Part of the cytoplasm containing only small virus particles, between two membranous structures
The array of the small virus particles appeared as a cord (48 hr after inoculation).

Fig. 6 The cell contained the small virus particles in the cytoplasm (arrayed as a cord) and ICHV particles in the nucleus (48 hr after inoculation)

Scales indicate 250 m μ .

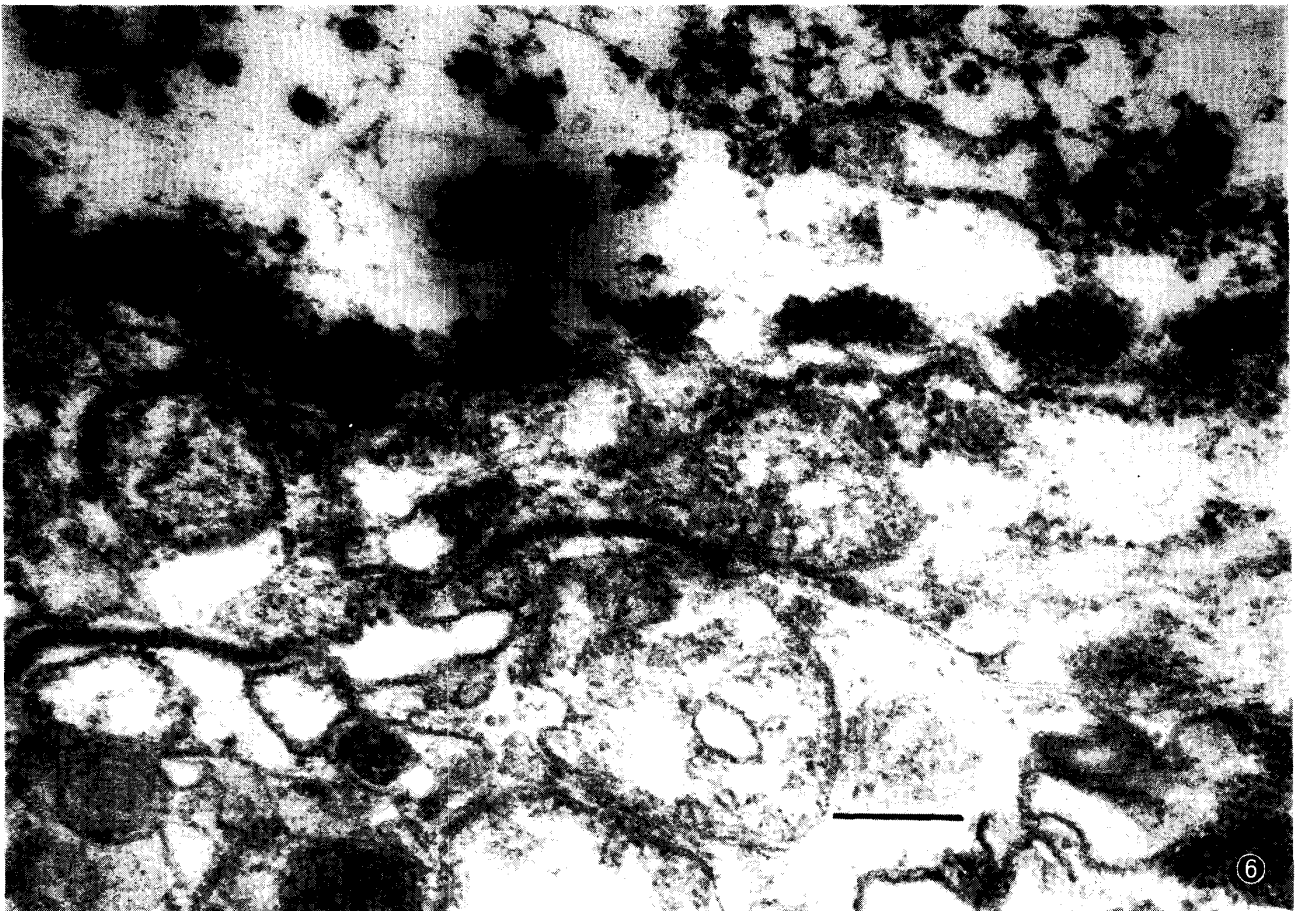
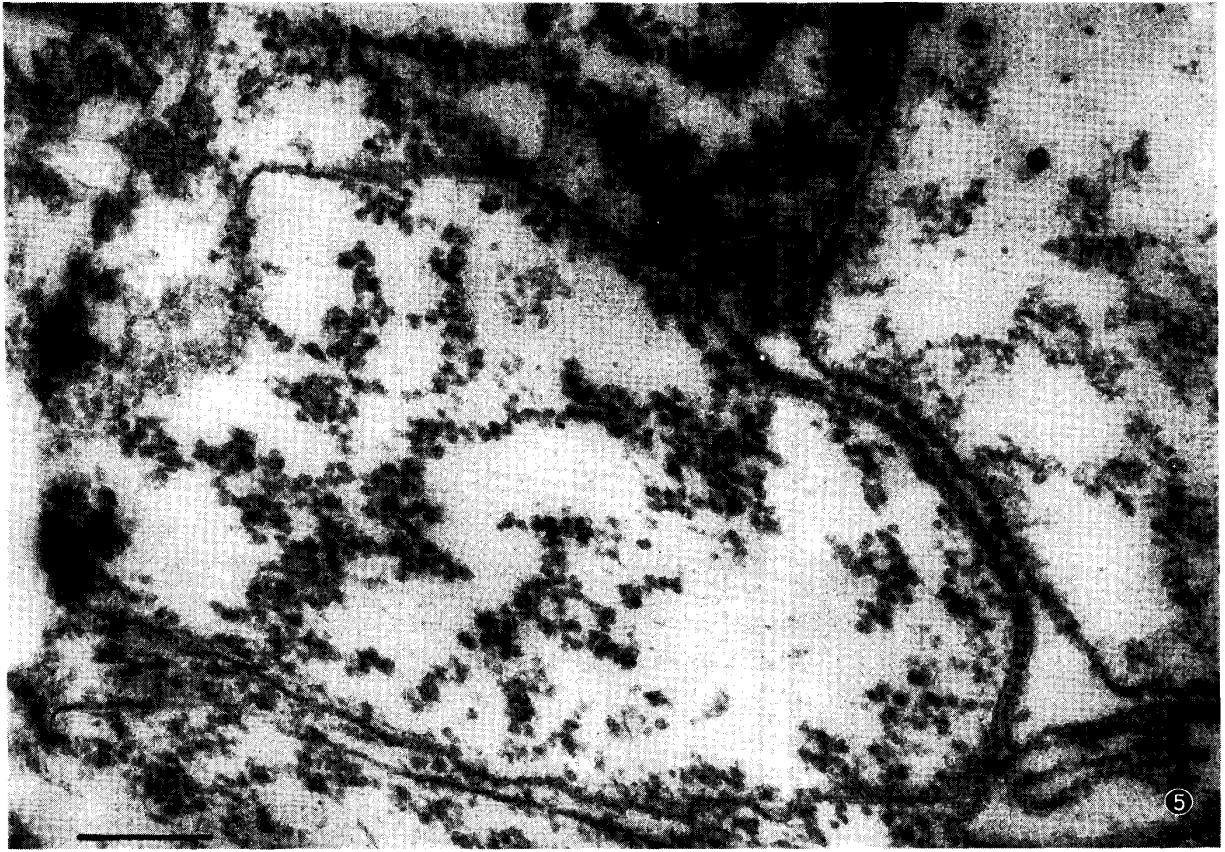


PLATE IV

- Fig. 7 The cell contained only the small virus particles both in the nucleus and cytoplasm
Intracytoplasmic small virus particles, between two membranous structures, appeared as a cord.
Intranuclear small virus particles are seen as a large packed aggregate (48 hr after inoculation).
Scale indicates 500 m μ .
- Fig. 8 Crystalline aggregate of the small virus particles in the cytoplasm (48 hr after inoculation)
Scale indicates 250 m μ .

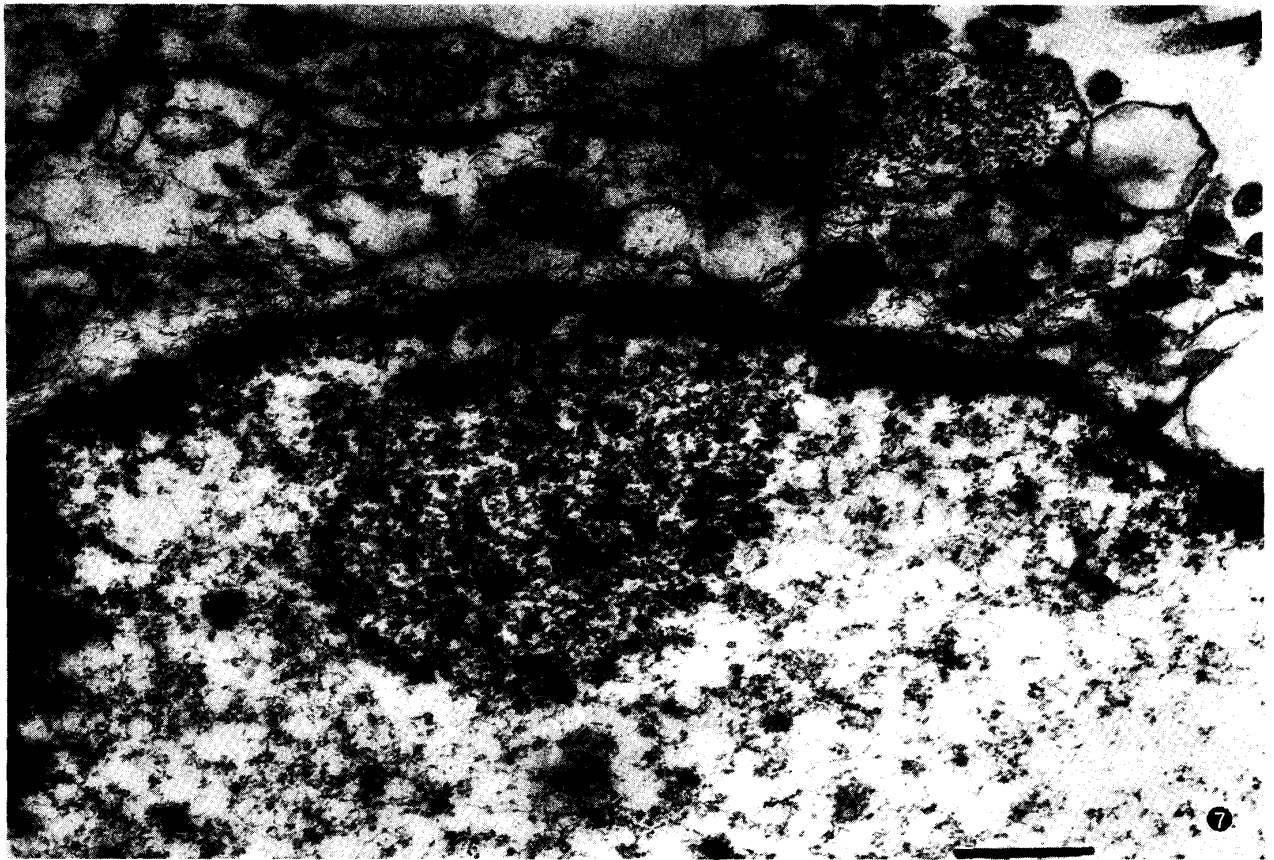


PLATE V

Fig. 9 The cell contained a small number of ICHV particles in the nucleus and numerous ICHV particles (crystalline array) in the cytoplasm

Note ICHV multiplied in the cytoplasm (18 hr after inoculation).

Fig. 10 The cell contained a small number of ICHV and a mass of small virus particles in the nucleus, and numerous ICHV particles in the cytoplasm (18 hr after inoculation).

Scales indicate 1 μ .

