

## ISOLATION AND CHARACTERISTICS OF AVIAN NEPHROSIS-INDUCING INFECTIOUS BRONCHITIS VIRUS (CORONAVIRUS)

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Infectious bronchitis virus (IBV) isolates that cause nephrosis with the accumulation of urates have been reported in America<sup>27)</sup>, Australia<sup>7,8)</sup>, Italy<sup>24)</sup>, and England<sup>21)</sup>. In Japan, however, no papers on the isolation of the virus have been published as yet.

WINTERFIELD and HITCHNER<sup>27)</sup> suggested that there might be some cross protection between the "classical" IB types and the nephrosis-inducing types. The immunological relationship between the nephrosis-inducing type and any other known serotype of IBV was studied in detail by HITCHNER et al.<sup>13)</sup> and WINTERFIELD et al.<sup>28)</sup> Quite recently, HOPKIN<sup>16)</sup> reported that about 10 different serotypes were established in cross neutralization tests with the IBV strains isolated up to the present. Identification of nephrosis-inducing IBV is based only on the immunological relationship determined by using cross neutralization tests. No nephrosis-inducing virus has been properly characterized as yet in cell cultures.

In their previous paper<sup>25)</sup>, the authors reported the isolation of infective agents in embryonating eggs from the natural outbreaks of chicken nephrosis. The infective agents were confirmed to be the causal agents of nephrosis from their pathogenicity for chickens experimentally or naturally infected with nephrosis.

This paper deals with the isolation of the agent in primary chicken embryo kidney (CEK) cell cultures, and its biological, physicochemical, and morphological properties.

### MATERIALS AND METHODS

**Virus:** The GN-2 strain of the nephrosis virus isolates was mainly used<sup>25)</sup>. It was prepared from the 10th and 20th passage of the virus in primary CEK cell cultures. The stock of all the strains of viruses was stored in frozen state at  $-20^{\circ}\text{C}$  until use. The Beaudette 42 and Gray strains of IBV used were kindly supplied by Dr. J. FUJITA of the National Institute of Animal Health, Kodaira, Tokyo.

**Cell cultures:** Primary CEK and DEK cell monolayers were prepared from the kidneys of 18-day-old chicken and duck embryos. The cells were grown in Hanks' balanced salt solution containing 0.5% lactalbumin hydrolysate (LH) and 10% calf serum, and maintained in Medium 199 containing 2% calf serum. All the media contained penicillin (100 units/ml) and streptomycin (100  $\mu\text{g/ml}$ ).

**Virus assay:** All the virus samples were assayed by inoculating CEK cell cultures contained in small bottles with their serial tenfold dilutions. Each dilution was inoculated into 4 cultures. The cells were observed for 5 days after inoculation. Virus titer was calculated by the method of REED and MUENCH<sup>23)</sup> and expressed as  $\text{TCID}_{50}$ .

**Growth curve:** Each of the CEK cell cultures contained in small bottles was inoculated with  $10^{1.0}$   $\text{TCID}_{50}$ . After absorption, the cultures were washed 3 times with

LH to remove unabsorbed virus. After addition of maintenance medium, the cultures were incubated at 37°C. At given intervals of time during the period of incubation thereafter, medium was collected from three bottles and frozen. All the harvests were stored at -20°C for the subsequent titration of virus content.

**Exposure to ether:** The virus in capped tubes containing 20% ethylether was allowed to stand at 4°C for 18 hours after shaking. The ether in the virus suspension was evaporated by aspiration. The virus was titrated immediately after treatment.

**Exposure to chloroform:** The virus was exposed to 5.0% chloroform for 20 minutes in the same manner as it was exposed to ether.

**Exposure to heat:** Two 2-ml samples of virus were sealed in small tubes. One of them was placed in a water-bath heated to 56°C, and the other allowed to stand at room temperature. Samples were harvested from both cultures four times at 15-minute intervals. They were titrated for virus content immediately thereafter.

**Exposure to pH 3:** The pH of two equal portions of the virus was adjusted by a dropwise addition of 1.0 N hydrochloric acid, while the virus suspension was manually agitated. The pH was reduced to 3.05 in one portion and to 7.05 in the other. Both samples of virus were maintained at 4°C for 30 minutes and then titrated for viable virus.

**Exposure to 5-bromodeoxyuridine (BUDR):** Cultures of CEK cells in small bottles were incubated in excess for 4 hours with maintenance medium containing 50 µg of BUDR/ml. Control cultures included those incubated with 50 µg of BUDR/ml and those incubated with maintenance medium only. After incubation, each culture was inoculated with 10<sup>1.0</sup> TCID<sub>50</sub> of virus in the three groups. At each interval of time during the period of incubation thereafter, medium was collected from three bottles, pooled, and maintained at -20°C for the subsequent titration of virus content.

**Filtration of virus:** The virus suspension was centrifuged at 2,500 rpm for 15 minutes at 4°C for clarification. The resulting supernatant fluid was divided into 3.5-ml quantities, which were forced to pass through a membrane filter once (Millipore Corp., Bedford, Mass.). The membrane was held in a Swinnex-25 filter holder (Millipore). Filtration was accomplished by manually applied positive pressure in a 5-ml syringe. In the various filters used, the pores were 220, 100, and 50 mµ in average diameter. The filtrates obtained were titrated immediately for virus content.

**Exposure to sodium deoxycholate (SDC):** One per cent stock solution of SDC was made in distilled water and sterilized by boiling. The virus was mixed with an equal volume of 0.2% SDC solution in distilled water. The mixture was incubated in a water-bath at 37°C for 1 hour.

**Antisera:** Immune sera were prepared in rabbits by intravenous injections with the partially purified virus from the allantoic fluid.

**Neutralization test:** The virus suspension was mixed with an equal volume of a 1:10 dilution of control or immune serum and incubated at 4°C for 24 hours. Small-bottle cultures and embryonating eggs were inoculated with the virus-serum mixture.

**Electron microscopy:** Materials for negative staining were prepared by partial purification of virus. For this purpose, virus was cultivated in embryonating eggs by allantoic inoculation. The allantoic fluid was clarified by low-speed centrifugation at 5,000 rpm for 30 minutes, and then centrifuged at 40,000 rpm for 60 minutes in the angle rotor, Hitachi RP 40, of ultracentrifuge. The pellets obtained were resuspended in LH. About 4.0 ml of the resulting suspension was laid on 1.0 ml of 50% sucrose solution contained in a cellulose tube, which was placed in the swinging rotor, Hitachi RPA 40, and spun at 40,000 rpm for 60 minutes. A band formed at the boundary between the sucrose and the suspension was collected from the bottom punctured with a needle. The virus fraction was dialyzed in phosphate-buffered saline at 4°C for 24

hours to remove the sucrose. A drop of the partially purified virus was mounted on a collodion-coated mesh, dried, and negatively stained with 2% phosphotungstic acid (PTA) at pH 7.0. Photomicrographs were taken by a JEM-100 B microscope at 80 kv and a magnification of 50,000 times.

RESULTS

**CPE on CEK cell cultures:** Viruses were recovered from 5 specimens collected from the nephrotic kidneys. No CPE was found in the 5th serial passage in CEK cells inoculated directly with these specimens. All of these viruses, however, produced CPE only after 2 or 3 blind passages in CEK cells starting from the 5th or 6th passage in embryonating eggs. Of the viruses which had produced CPE in CEK cells, the GN-2 strain was chosen as the prototype strain and purified by 3 serial selections at limiting dilutions in CEK cells. In these cells, CPE was shown as the first change at 24 hours of incubation (Fig. 1). At 48 hours, the cell monolayer became granulated or shrunken in appearance (Fig. 2). The appearance of many small vacuoles in the cytoplasm was the first change noted in the stained cells. No inclusion bodies were found in cells stained with Giemsa stain.

**Growth curve in CEK cell cultures:** The growth of virus in CEK cells for 120 hours is plotted in Chart 1. Viral replication was evident within 8 hours after inoculation. No CPE, however, was obvious until 24 hours after inoculation. A peak of concentration of virus was reached some time between 48 and 60 hours after inoculation. The highest virus titer, or  $10^{6.8}$  in the culture medium, was observed at 48 hours.

The virus also replicated in primary duck embryo kidney cell cultures, showing CPE.

**Nucleic acid inhibition:** No replication of the virus was inhibited by the incorporation of 50  $\mu$ g of BUDR in the culture medium for 120 hours (Table 1). By inference, the nucleic acid of the virus is ribonucleic acid (RNA).

Chart 1. Growth curves of the GN-2 strain in CEK cell cultures with and without BUDR, DNA inhibitor

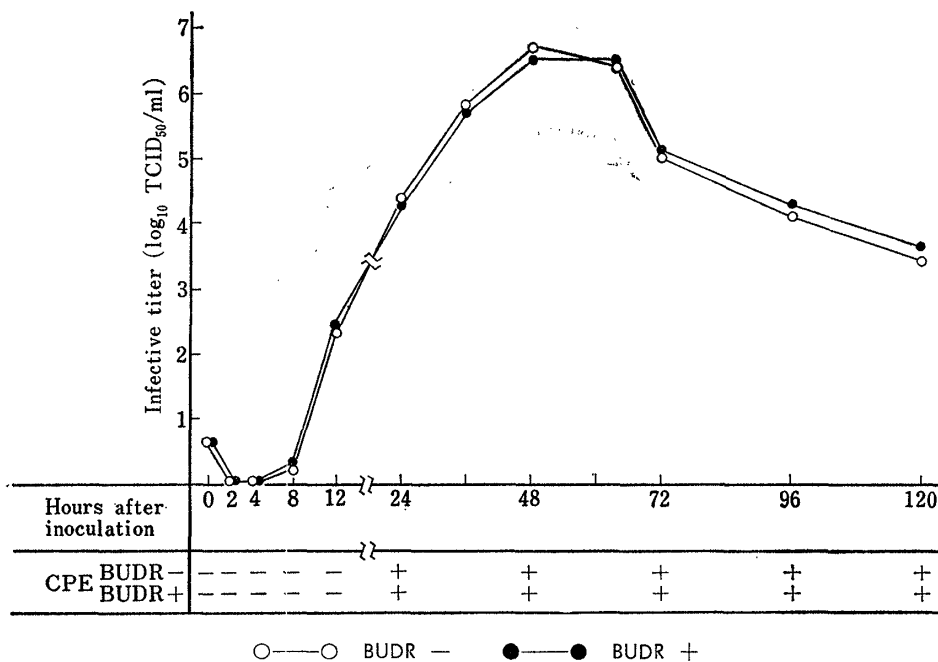


Table 1. Titer of virus following chemical and physical exposure

Type of exposure	Titer of exposed virus (TCID <sub>50</sub> /ml)	Titer of sham-exposed virus (TCID <sub>50</sub> /ml)
20% ether, 4°C, 18 hours	No viable virus	10 <sup>5.0</sup>
5% chloroform, 4°C, 20 minutes	No viable virus	10 <sup>5.0</sup>
0.1% SDC, 37°C, 1 hour	No viable virus	10 <sup>5.0</sup>
pH 3.0, 4°C, 30 minutes	10 <sup>2.5</sup>	10 <sup>5.0</sup> (pH 7.2)
56°C, 15 minutes	10 <sup>1.5</sup>	10 <sup>4.5</sup>
30	10 <sup>1.0</sup>	10 <sup>4.5</sup>
45	No viable virus	10 <sup>4.5</sup>
60	No viable virus	10 <sup>4.5</sup>
Filtration (mμ)		10 <sup>5.0</sup> (unfiltrated)
220	10 <sup>2.5</sup>	
100	10 <sup>0.5</sup>	
50	No viable virus	

Table 2. Neutralization tests in CEK cell cultures and embryonating eggs

Antiserum	Strain No. of isolate									
	GN-2		GN-5		GN-7		GN-10		GN-11	
	CPE	Curling	CPE	Curling	CPE	Curling	CPE	Curling	CPE	Curling
Anti-GN-2	—	—	—	—	—	—	—	—	—	—
Anti-GH-10	—	—	—	—	—	—	—	—	—	—
Anti-Be42	—	—	—	—	—	—	—	—	—	—
Anti-Gray	—	—	—	—	—	—	—	—	—	—

Effect of lipolytics on virus: No viable virus was detectable after exposure to ether or chloroform (Table 1).

Effect of SDC: The virus was inactivated completely with 0.1% SDC (Table 1).

Acid stability of the virus: The virus was not inactivated at pH 3.0, but decreased in titer by 2.5 log (Table 1).

Heat stability of the virus: Exposure to 56°C for 30 minutes destroyed 99.9% of virus. After exposure for 45 minutes, no viable virus was detectable (Table 1).

Filtration of virus: Virus passed through filters 100 mμ or more in average pore diameter. It did not pass through filters 50 mμ in average pore diameter (Table 1).

Neutralization test: Neutralization tests were carried out with the virus isolates and the known IBV strains (Table 2). There was a cross reaction showing no CPE on CEK cells. The curling of embryos was not noticed. The homologous titers determined ranged from 1:128 to 1:256.

Electron microscopy: Negative staining of partially purified, concentrated virus usually revealed round or elliptical particles, many of which were pleomorphic particles (Fig. 3). A characteristic feature of those particles was wide-spaced club- and pear-shaped surface projections, which were narrow at the base, about 10 mμ wide at the outer edge and approximately 20 mμ in length (Fig. 4). The diameters of the particles (including the projections) were within a range of 60 to 220 mμ, averaging 110 mμ. No nucleocapsid structure was seen in the virion.

## DISCUSSION

The evidence obtained from this study indicates that the virus isolates capable of passing in CEK cell cultures are similar to Coronavirus in physicochemical and morphological properties, and that they are immunologically related to the known IBV strains.

The growth and CPE of the classical type of IBV in cell cultures have been reported by several investigators<sup>4,5,6,9,12,18,19</sup>). It is known that IBV, such as the embryo-adapted Beaudette strain, grows in cell cultures, showing CPE after a few serial passages in embryonating eggs. No growth of the nephrosis type of IBV has been reported as yet in cell cultures. As shown in the present study, the growth and CPE of the nephrosis virus were induced by blind passages in CEK cell cultures after the adaptation to embryonating eggs. The nephrosis virus is considered to require more blind passages in cell cultures than the classical type of IBV.

The GN-2 and GN-10 strains of the isolates passed 5, 10, and 20 times in CEK cell cultures. When inoculated intraocularly into susceptible one-day-old chicks, these strains at the 5th and 10th passages in embryonating eggs produced no kidney lesions. The viruses, however, were recovered from the kidneys of chicks 21 days after inoculation. Both of the viruses from CEK and embryonating-egg passages were neutralized by anti-IBV serum of the Be 42 and Gray strains. Therefore, the virus which passed in CEK cell cultures may have lost pathogenicity for chickens. It is known that IBV propagated in the embryonating egg gradually loses its ability to infect chickens<sup>15</sup>).

The biological and physicochemical properties of the nephrosis virus presented here are in general agreement with those of the classical type of IBV described by other workers<sup>15</sup>), except the lability to heating at 56°C for 30 minutes which was reported otherwise in some papers<sup>10,14</sup>). The criteria for the heat stability described above are based on the occurrence of dwarfing in embryos inoculated with exposed virus<sup>10,14</sup>). DUBOSE et al.<sup>10</sup>) reported that of 18 classical-type strains of IBV examined, 15 strains were labile to heating at 56°C for 30 minutes and the remaining 3 strains stable. HOFSTAD<sup>14</sup>) indicated that 60 classical-type strains of IBV had been destroyed after exposure to 56°C for 15 minutes, but that only one such strain had resisted to heating at 56°C for 45 minutes. He suggested that there might be a considerable variation among strains. On the other hand, CUMMING<sup>8</sup>) reported that all the nephrosis-inducing types of IBV had been inactivated when heated at 56°C for 45 minutes. Thus, the heat stability of IBV may be considered to vary noticeably among strains. To determine whether the variation among the strains of IBV is a genetical character or not is an interesting subject for research in future.

The viral particles were sometimes round or elliptical, but many of them were markedly pleomorphic. A characteristic feature was the presence of wide-spaced, club-shaped surface projections, which were narrow at the base, about 10 m $\mu$  wide at the outer edge, and approximately 20 m $\mu$  in length.

The nephrosis-inducing virus showed morphologically characteristic features of coronavirus<sup>1,11</sup>). The virions of the nephrosis-inducing virus is indistinguishable from those of IBV<sup>3</sup>), IBV-like viruses of human beings<sup>2,7,22,26</sup>), or mouse hepatitis virus<sup>20</sup>) in negatively stained preparations.

The authors presented the biological, physicochemical, and morphological properties of avian nephrosis-inducing IBV. Purification of the virus is of value in performing chemical analysis of structural elements and studying the biological significance of the virion. The genetical differentiation of the classical type from the nephrosis-inducing type of IBV will be an interesting essential subject for research in future.

## SUMMARY

The nephrosis-inducing infectious bronchitis virus (IBV) was isolated on primary chicken embryo kidney (CEK) cell culture from the kidney lesion of chickens. The cytopathic effect (CPE) was produced only by blind passages of the virus in CEK cell cultures after the adaptation of the virus to embryonating eggs. The maximum infective titer in culture medium appeared in 48 hours after inoculation.

The virus was sensitive to ether, chloroform, and sodium deoxycholate, stable at pH 3.0, and was inactivated after heating at 56°C for 45 minutes. No replication was inhibited by 5-bromodeoxyuridine. The virus passed through filters 100 m $\mu$  or more in average pore diameter, but not through filters 50 m $\mu$  in such diameter. Its viral particles negatively stained exhibited pleomorphism, were 110 m $\mu$  in average diameter, and had club-shaped projections on their surfaces. No nucleocapsid structure was seen in the virion.

Thus, the virus exhibited such properties as typical of coronavirus. It was neutralized by the known IBV strains. It is therefore concluded that the virus inducing nephrosis in chickens is the nephrosis-inducing type of IBV in Japan.

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## 鶏に Nephrosis をおこす Infectious Bronchitis Virus の 分離と二三の性状について

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著者らは、尿管系における尿酸塩様物質の貯留と、腎の混濁腫張とを主徴とする雛の疾病を研究し、その腎乳剤を発育鶏卵に継代することによって、病原と考えられるウイルス性因子を分離した。雛に対するその病原性についてはすでに報告した。

本報告は、鶏胎児腎初代培養細胞 (CEK) におけるこの分離ウイルスの増殖状況、理化学的性状および形態について報告する。

本ウイルスの CEK における増殖は、感染後 8 時間に培養液相現われ、48~60 時間でほとんど最高値 6~7 log TCID<sub>50</sub>/ml に達した。細胞変性効果 (CPE) の特徴は、原形質の空胞化、感染細胞の球状化、そして円形化細胞の管壁からの脱落である。封入体は認められなかった。アヒル胎児腎初代培養細胞でも、CEK と同様な CPE が認められた。

本ウイルスの理化学的性状は、20% エーテル、

5% クロロホルム、0.1% SDC 処理では不活化され、pH 3.0, 4°C, 30 分処理では安定性を示し、56°C, 30 分までは活性を示すが、45 分以上では不活化された。Millipore filter の 100 m $\mu$  は通過したが、50 m $\mu$  は通過しなかった。CEK 培養液に BUDR を添加しても、増殖に影響しなかった。本ウイルスの構成核酸は RNA と推定される。

本ウイルスは、伝染性気管支炎ウイルス (IBV) の Be 42 株および Gray 株で中和された。

ネガティブ染色試料の電子顕微鏡観察では、ウイルス粒子は大小不同多形態性で、直径の平均は約 110 m $\mu$  であった。ウイルス粒子の外側には、“なし型”の surface projection が観察され、coronavirus の形態を示した。

以上の成績から、今回の分離ウイルスは、IBV の腎炎型と考えられる。

## EXPLANATION OF PLATE I

- Fig. 1. Cytopathic effect of the GN-2 strain in chick embryo kidney monolayer cells. 24 hours after inoculation.  $\times 200$ .
- Fig. 2. Cytopathic effect of the GN-2 strain in chick embryo kidney monolayer cells. 48 hours after inoculation.  $\times 200$ .
- Fig. 3. Viral particles of the GN-2 strain negatively stained with phosphotungstate.  $\times 200,000$ .
- Fig. 4. Single virus particle of the GN-2 strain negatively stained. Club-shaped projections are evident. No internal structure is visible in these preparations.  $\times 200,000$ .



