

Case Report on Mixed Infection of Canine Parvovirus and Canine Coronavirus—Electron Microscopy and Recovery of Canine Coronavirus

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ABSTRACTS. Occurrence of coronavirus-like virions and associated degenerative changes were seen in the enterocytes of jejunal villi of a mongrel dog which had been diagnosed first as canine parvoviral disease from virological and pathological examinations. A cytopathic agent was isolated in dog kidney cell cultures from intestinal contents of the present case. The isolate, Toda strain, had morphological and physicochemical properties in common with the viruses of the coronavirus group. Toda strain was neutralized by ascites obtained from a cat with feline infectious peritonitis. Thus the case was diagnosed precisely as the mixed infection of canine parvovirus and canine coronavirus.

Canine coronavirus (CCV) [1, 3, 5, 6, 10, 11], one of the agents of canine infectious enteritis first described by Binne *et al.* [3], is being attracted recently much attention. Both CCV and canine parvovirus (CPV) were identified simultaneously in the same stool samples [1, 10], and intestinal contents and certain tissues [5]. Detailed observations on neonatal dogs experimentally infected with CCV 1–71 was described [6, 11]. On the ultrastructural events in enterocytes of dogs naturally infected with CCV, however, no data are available at the present time. The authors [12] previously described electron microscopic findings on enterocytes in natural cases of CPV infection. In the course of further study on the same materials as the previous report, coronavirus-like virions were identified in the enterocytes of jejunal villi in a male adult mongrel dog. These findings prompted the authors to attempt the virus isolation that was suc-

ceeded. Thus the case was precisely diagnosed as the mixed infection of canine parvovirus and canine coronavirus. This paper deals with the electron microscopic observation on the villous enterocytes and the properties of isolated virus.

MATERIALS AND METHODS

The materials used were derived from the case of mongrel dog described in the previous paper [12].

Electron microscopy of villous enterocytes: The method was the same with those described in the previous paper [12]. In the present study, however, tissue blocks containing the villus were exclusively studied.

Cell cultures: Primary and secondary dog kidney (PDK and DK-2) monolayers were prepared from the kidneys of 1-year old healthy beagles by the method as previously described [7]. PDK, DK-2 and Crandell feline kidney (CRFK) cells were propagated in a growth medium

consisting of 80% Eagle's minimum essential medium (MEM), 10% fetal calf serum (FCS) and 10% tryptose phosphate broth (TPB). Maintenance medium used was MEM containing 2% FCS and 5% TPB.

Virus isolation and characterization: Intestinal contents and mucous membrane were collected from the jejunum and used as source material. The specimens were made to a 10% emulsion in phosphate buffered saline (PBS) that contained antibiotics. The procedures used for isolation and characterization of virus were described elsewhere [4, 7]. To check the contamination of CPV, freshly seeded cultures of CRFK cells were inoculated with heat-treated (56°C, 60 min) virus material and subcultured. The isolate, designated as Toda strain, was propagated in DK-2 cells and used for characterization at the eighth passage level.

Virus titration: Virus titration was performed by the inoculation of an aliquot from serial 10-fold dilutions into 4 wells of DK-2 in multi-dish tray. Then, the cultures were observed daily for cytopathic effect (CPE) during 7 days and 50% tissue culture infective dose (TCID₅₀) was calculated.

Virus growth curve: DK-2 cell cultures were inoculated with 0.1 TCID₅₀/cell of Toda strain, washed 3 times with PBS after virus adsorption at 37°C for 1 hr, fed maintenance medium and incubated at 37°C. At intervals, infectivity was determined with culture fluid and infected cells.

Electron microscopy of Toda strain: DK-2 cells were infected with Toda strain and incubated at 37°C for 2 days. The culture medium was harvested and samples were negatively stained after ultracentrifugation. Ultra-thin sections were prepared from the infected cells and examined. The methods were described

elsewhere [7, 8].

Neutralization test: The test was carried out with serum dilution method described previously [7]. Ascites obtained from a cat with feline infectious peritonitis (FIP) showing indirect immunofluorescence (IF) titer of 1:25,600 against FIP virus was kindly supplied by Dr. Toshiharu Hayashi, of the Faculty of Agriculture, University of Tokyo.

RESULTS

1. *Light and electron microscopic findings on the jejunal villi*

Light microscopy made on semi-thin sections of the jejunal mucosa revealed flattening and desquamation of epithelial cells and edema of the middle and apical portions of villi (Figs. 1, 2). Under electron microscope epithelial cells which seemed to be in the early stage of infection exhibited decreased electron opacity of cytoplasmic matrices and slight decrease of the microvilli. Virus particles were scattered among the microvilli and enclosed singly or in small clumps in the cytoplasmic vesicles and Golgi apparatus in these cells (Figs. 3, 4). Structures considered to represent the viral replication by budding were occasionally seen with cytoplasmic protrusion within the vesicles (Fig. 5). These were epithelial cells variously deformed or disorganized near by apex of the villi (Fig. 6). These cells contained many vacuoles, lysosomes and dilated endoplasmic reticula in which virus particles were often observed (Fig. 7). Severely involved cells exhibited clumping and margination of nuclear chromatin, swelling of mitochondria, cytoplasmic vacuolation and deformation or loss of the microvilli. Virus particles were observed as clumps in the cytoplasmic vacuoles (Fig. 8) and freely in the intestinal lumen near the affected villi (Fig. 9). The virions were pleomor-

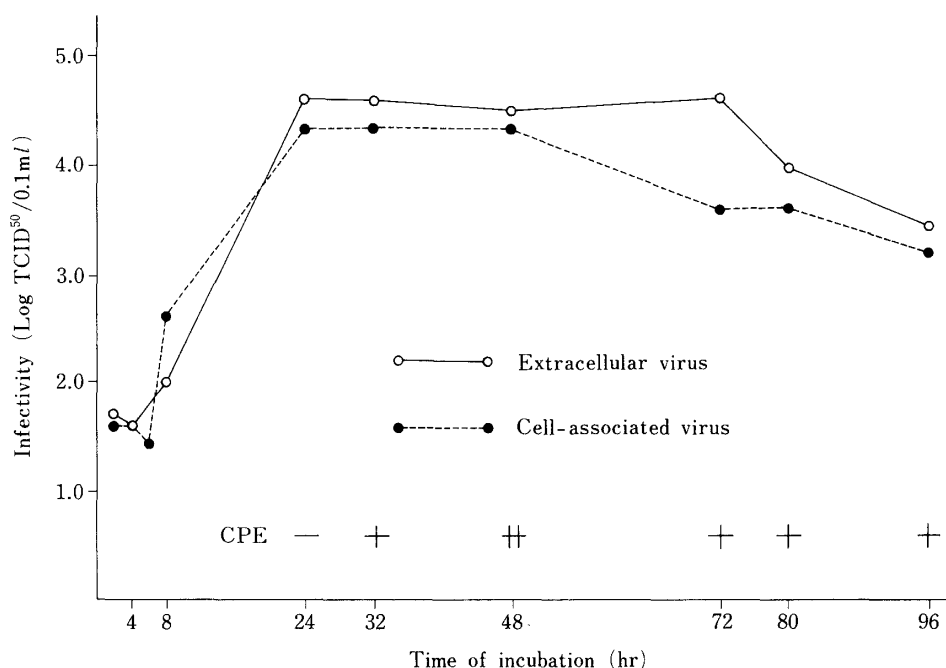


Fig. 13. Growth curve of Toda strain in DK-2 cell cultures.

phic, but mostly spherical or oval and ranged 75 to 140 nm in larger diameter, and contained a dense to translucent core at their center.

2. Virologic findings

Isolation of virus: CPE appeared following the successive blind passages in PDK cell cultures. Observation of unstained cell cultures revealed an increase of round cells and cells detached from the glass surface in inoculated culture. Cell fusion and multinucleated giant cells were not evident in infected cells stained with hematoxylin and eosin. CPE appeared by 2 days after inoculation at 8th passage level of the isolate (Fig. 10). Neither inclusion body nor CPE was observed when heat-treated virus material was inoculated into CRFK to deny the CPV contamination.

Virus growth curve: Figure 13 shows the growth curve of Toda strain in DK-2 cells. The virus titer reached a plateau of $10^{4.5}$ TCID₅₀/0.1 ml after 24 hr postinoculation. The titer of cell-associated virus was always lower than that of fluid

Table 1. Titer of Toda strain following chemical and physical treatment

Treatment	Virus titer*
IUDR**, 10^{-4} M/ml	4.7
BUDR, 10^{-4} M/ml	4.5
Ara-C, 10^{-5} M/ml	4.5
Control	4.7
20% ether, 4°C, 18 hr	0
Control	4.7
10% chloroform, 20°C, 15 min	0
Control	5.5
pH 3.0, 20°C, 3 hr	2.7
pH 7.0, 20°C, 3 hr	4.7
Filtration, 450 nm	5.2
220 nm	5.5
100 nm	3.5
50 nm	0
56°C, 30 min	0

Remarks. *: Infective titer (log TCID₅₀/0.1 ml).

** IUDR: 5-iodo-2'-deoxyuridine
 BUDR: 5-bromo-2'-deoxyuridine
 Ara-C: Arabinosylcytosine

phase and tended to run in parallel with that of the latter.

Physicochemical properties: As shown in Table 1, the presence of 5-iodo-2'-deoxyuridine (IUDR), 5-bromo-2'-deoxyuridine (BUDR) and Arabinosylcytosine (Ara-C) did not inhibit propagation of Toda strain. Toda strain was sensitive to ether as well as chloroform, and affected by treatment at 56°C for 30 min. After exposure to pH 3.0 for 3 hr at room temperature, a 100-fold reduction occurred in infectivity titer. The isolate passed through membrane filters with 220 or 100 nm pores but not with 50 nm pores.

Electron microscopy of the isolate: Negatively stained preparation of Toda strain showed that the virions of the isolate were pleomorphic in shape, and the diameter of virions, including the surface projections, ranged from 115 to 167 nm (mean 140 nm). They had the peculiar club- or petal-shaped long projections with a length of 19.0 nm and the characteristic wide spacing of those projections (Fig. 11).

In ultra-thin section preparations of infected cells, virus particles were observed exclusively within cytoplasmic vesicles. The virus particles were pleomorphic in shape and had generally circular contour and the maximum diameter was in the range of 100 to 200 nm (Fig. 12).

Serum neutralization: Thirteen serum samples from the dogs which had been housed together with the present case were subjected to assay for neutralizing antibody. Six animals showed neutralizing titers of 1:10 to 1:20 against Toda strain. One hundred TCID₅₀ of Toda strain were neutralized by a 1:80 dilution of ascites obtained from a cat with FIP which had a homologous indirect IF titer of 1:25,000.

DISCUSSION

The present and previous studies [12] revealed the mixed infection of CPV and CCV in a mongrel dog suffering from acute intestinal involvement.

The results of present electron microscopic observation on the jejunal villi of the dog are largely coincided with those of the ileum in experimental CCV infection [6, 11]. In the present case the changes caused by CCV infection were recognized mainly in the villi of jejunum, while the changes associated with CPV infection were conspicuous in the crypts of ileum and large intestine as reported in the previous report [12]. Judging from the morphology and mode of occurrence of virions in the villous epithelia, they were regarded as CCV. In the case of CPV infection, occurrence of nuclear inclusion bodies in the crypt epithelia is a change of pathognomonic significance [12]. It seems, on the other hand, that the changes associated with CCV infection are noncharacteristic and histopathological examination has only a limited value in the diagnosis of CCV infection. Actually intestinal changes were essentially restricted to the crypt epithelia [2] in the dog dealt in the present paper. As shown in the present study, electron microscopy first revealed occurrence of CCV which gives a good chance to isolate the agent. Fortunately, the agent was isolated without intervening of CPV when examined at 8th passage level by inoculation into CRFK cell cultures.

The isolate, toda strain, was found to have morphological and physicochemical properties in common with the viruses of the coronavirus group and it was identified as a member of coronavirus. Reynolds *et al.* [9] described that FIP virus was closely related to canine coronavirus as judged by antigen involved in virus neu-

tralization. In the present study, Toda strain was neutralized by ascites obtained from a cat with FIP, suggesting that Toda strain was antigenically related to FIP virus. These characteristics of Toda strain are in general agreement with the properties of canine coronavirus as described by Binn *et al.* [3]. With respect to some characteristics, however, our results differed from that of Binn *et al.* [3]. Namely, they observed multinucleated giant cells in infected primary dog kidney cells. In our experiment, however, cell rounding and detaching were evident, and cell fusion and multinucleated giant cells were not observed. Binn *et al.* [3] further described that the addition of DEAE-dextran to the inocula increased the CPE and viral titer. In our experiment, however, addition of DEAE-dextran in the range of 6 to 50 $\mu\text{g/ml}$ to the inocula did not increase the CPE and viral titer.

The presence of neutralizing antibody in the dogs which had been housed together with affected dog provided evidence of infection with the isolate among these dogs. Present case is the first reported case of mixed infection of CPV and CCV in Japan, to our knowledge, and worthy of note because the entity of canine infectious enteritis are still remained unexplained. The severe clinical signs [12] found in the cases reported in the present paper may be ascribable to the mixed infection of CPV and CCV.

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EXPLANATION OF FIGURES

ER: Endoplasmic reticulum

G: Golgi apparatus

L: Lumen

MV: Microvilli

Bar=200 nm

Fig. 1. Jejunal villus. Marked deformation and edema of the tip of the villus. Semi-thin section stained with toluidine blue. $\times 450$.

Fig. 2. Degeneration of enterocytes and destruction of epithelial lining. Semi-thin section stained with toluidine blue. $\times 450$.

Fig. 3. Part of a infected epithelial cell. Virus particles among the microvilli (arrow heads) and in the cytoplasmic vesicles (arrows). $\times 30,000$. Inset is high-power magnification of the particles. $\times 60,000$.

Fig. 4. Deeper part of a infected epithelial cell. Virus particles in the dilated vesicles (arrows) of the Golgi apparatus. $\times 30,000$.

Fig. 5. Pictures suggesting the budding (arrow).

$\times 60,000$.

Fig. 6. Apex of a villus with deformed epithelial cells. $\times 3,000$.

Fig. 7. High-power magnification of part of Fig. 6. Virus particles (arrows) are scattered in the dilated cisternae of endoplasmic reticulum. $\times 30,000$.

Fig. 8. Clumps of particles in the vacuoles. Surface projection-like structures are noted in some particles (arrows). $\times 30,000$.

Fig. 9. Pleomorphic appearance of virus particles in the intestinal lumen. $\times 60,000$.

Fig. 10. Cytopathic effect in secondary dog kidney (DK-2) cell culture inoculated with Toda strain, 48 hours after inoculation. Phase-contrasted, $\times 250$.

Fig. 11. Particles of Toda strain, negatively stained with ammonium molybdate. $\times 150,000$.

Fig. 12. Viral particles in the cytoplasm of DK-2 cells inoculated with Toda strain. $\times 30,000$.

要 約

イヌ・パルボウイルスおよびイヌ・コロナウイルス混合感染症の症例報告——電顕所見およびイヌ・コロナウイルスの分離：八十島昭・藤波不二雄・土井邦雄・小嶋明広¹⁾・高田 博¹⁾・岡庭 梓¹⁾(田辺製薬株式会社薬理研究所，¹⁾田辺製薬株式会社安全性研究所)——ウイルス学および病理形態学的にイヌ・パルボウイルス(CPV)自然感染と診断された雑種犬1症例の電顕的検索で，主として空腸絨毛先端部にウイルス粒子をみとめ，その増殖様式からイヌ・コロナウイルス(CCV)と考えられた。これに関連して絨毛上皮細胞の変化をみとめた。同例の空腸内容から，イヌ腎培養細胞に細胞病原性を示すウイルス(戸田株)が分離され，形態学および物理化学的性状はコロナウイルスに一致した。戸田株はネコ伝染性腹膜炎罹患ネコの腹水で中和された。

