Multiplication of Canine Coronavirus in CRFK Cells

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ABSTRACT. Canine coronavirus produced CPE in CRFK cells without any blind passages. Syncytium formation was one of the most prominent cytopathic changes after inoculation at a low multiplicity of infection (MOI) of 1 $TCID_{50}$/cell. At a high MOI of 50 $TCID_{50}$/cell, however, rounding and detachment of the cells occurred, but no syncytium formation was found. Specific immunofluorescence and immunoperoxidase staining were confined in the perinuclear cytoplasm at 4 hr postinfection when infective titers of culture fluid began to increase. Virus titers reached their maxima of $10^{4.2}$ and $10^{5.3}$ $TCID_{50}$/0.1 ml after infection at an MOI of 1 and 50 $TCID_{50}$/cell respectively. Electron microscopic observation revealed that virus particles occurred in the restricted area of the perinuclear cytoplasm, mainly at the Golgi field, in the early stage of infection. Localization of virus particles as well as positive immunofluorescence and immunoperoxidase staining for specific CCV antigen was seen in the same area of infected cells, i.e., around the centriole and Golgi complex and perinuclear cytoplasm in an indentation in the early stage of infection.—KEY WORDS: canine coronavirus, CRFK cell.

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Canine coronavirus (CCV) infection in Japan was first reported by Yasoshima *et al.* [18] in a mongrel dog suffering from acute intestinal involvement diagnosed as the mixed infection of canine parvovirus and canine coronavirus. The Toda isolate in the above had morphological and physicochemical properties in common with the coronavirus group [18]. In *in vitro* study on Toda strain of CCV, the authors found that CRFK (Crandell feline kidney) cells were susceptible to the virus and provoked to induce the cytopathic changes in the infected cells.

This paper deals with the electron microscopic and virological observations on CRFK cells infected with Toda strain of CCV.

MATERIALS AND METHODS

Cell culture: CRFK cells, ATCC CCL 94, 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 consisted of 93% MEM, 2% FCS and 5% TPB.

Virus: History of isolation and characterization of Toda strain of CCV were described previously [18]. The virus was propagated in secondary dog kidney (DK-2) cells and used at the 11th passage level. The virus ($10^{5.0}$ $TCID_{50}$/0.1 ml) was concentrated to $10^{7.0}$ $TCID_{50}$/0.1 ml by ultracentrifugation at 86,500×g at 4°C for 2 hr.

Virus assay and growth curve: Virus titration was performed by the inoculation of an aliquot from serial 10-fold dilutions into 4 wells of CRFK cells in 24-well tissue culture plate. Virus growth curve was obtained by the methods as previously described [18].

Cytopathology and Immunocytochemistry: Cover slip samples of CRFK cell cultures infected at an MOI of 1 or 50 $TCID_{50}$/cell with Toda strain were collected as scheduled (Table 1). They were stained with Mayer's hematoxylin and eosin (HE), in-

Table 1. Observation of cytopathic changes in CRFK cells inoculated with Toda strain

Inoculum size ($TCID_{50}$/cell)	Method	Hours after infection											
		0	2	4	6	8	10	13	16	24	32	40	48
1	Unstained	−	−	−	−	−	−		−	−		+s[a)	+s
	Phase contrast	−	−	−	−	−	−		+s	+s		+s	+s
	HE stain	−	−	−	−	−	+s		+s	+s		+s	+s
	Immunofluorescence	−	+	+	+	+	+		+	+		+	+
50	Unstained	−	−	−	−	−			+r[b)	+r			
	Phase contrast	−	−	−	−	−			+r	+r			
	HE stain	−	−	−	−	−			+r	+r			
	Immunofluorescence	−	−	+	+	+			+	+			
	Immunoperoxidase staining	−	−	+	+	+			+	+			
	Electron microscopy	−	−	+	+	+			+	+			

a) With syncytium formation.
b) With rounding of cells.

direct immunofluorescence and immunoperoxidase technique. Anti-CCV serum was prepared in mature rabbit of the KBL:JW strain by 3 times of i.v. inoculation at 2 weeks' intervals with partially purified Toda strain harvested from infected DK-2 cell culture. Neutralizing antibody titer of anti-CCV serum was 1:2560 by the method described previously [9]. For indirect immunofluorescence, cover slip samples were fixed in aceton at room temperature for 10 min and treated with anti-CCV serum (1:160) at 37°C for 30 min applying the indirect immunofluorescent technique [8]. Fluorescein-conjugated IgG fraction goat anti-rabbit IgG (Cappel Laboratories, USA) was used for the second antibody (1:5) after purification with Sephadex G-25 and DEAE cellulose [8]. For immunoperoxidase staining, $F(ab')_2$ fragments of anti-CCV serum were prepared from IgG by digestion with pepsin in 0.1 M acetate buffer (pH 4.5). Cover slip samples were fixed in aceton 10 min and treated with $F(ab')_2$ fragments solution (1:10) at 37°C for 30 min. Peroxidase-conjugated affinity-purified goat anti-rabbit IgG (Cappel Lab.) was used for the second antibody (1:150) at 37°C for 30 min. The substrate solution consisted of 0.05% H_2O_2 and 0.02% 3,3'-diamino benzidine tetrahydrochloride in 0.05 M tris-HCl buffer (pH 7.6) [17].

Electron microscopy: Cells were harvested at 0, 2, 4, 6, 8, 16 and 24 hr after infection at an MOI of 50 $TCID_{50}$/cell. They were pelleted by centrifugation at 700 rpm for 10 min. The pellets were fixed at 4°C in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) and post-fixed at 4°C in 1% osmium tetroxide in cacodylate buffer. Ultra-thin sections were prepared and examined by the methods described elsewhere [9, 12].

RESULTS

Findings by light microscopy: Light microscopic observation of CRFK cells inoculated at an MOI of 1 or 50 $TCID_{50}$/cell of Toda strain is summarized in Table 1. At an MOI of 1 $TCID_{50}$/cell, specific immunofluorescence was first observed at the restricted area of perinuclear cytoplasm in an indentation in a few cells 6 hr postinoculation (p.i.) (Fig. 2). Small syncytia were formed at 16 hr p.i. and increased in size
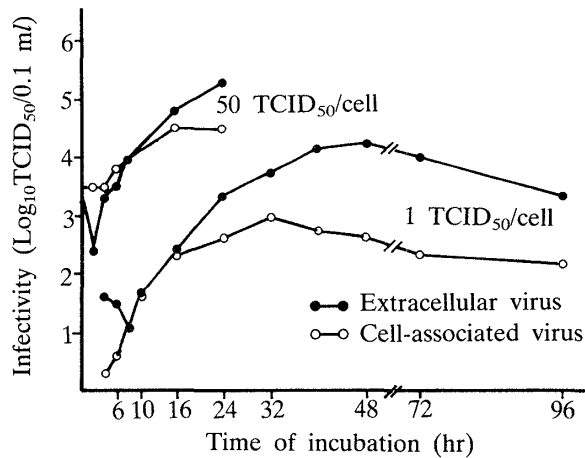
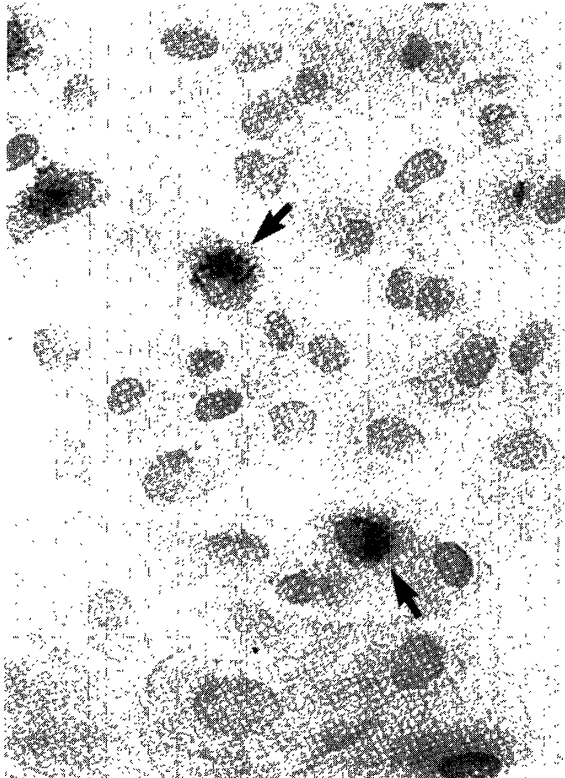Fig. 1. Growth curve of Toda strain in CRFK cell culture.

Fig. 3. CRFK cells, 6 hr p.i., stained with immunoperoxidase technique. Specific immunoperoxidase staining is seen in perinuclear cytoplasm (arrows). ×400.

and number thereafter. Huge syncytia began to lyse and detach from the dish at 40 hr p.i. The CPE was focal and did not spread from the initial foci.

At an MOI of 50 $TCID_{50}$/cell, specific immunofluorescence and specific immunoperoxidase staining (Fig. 3) were first

Fig. 2. CRFK cells, 6 hr p.i., stained with immunofluorescence technique. Specific immunofluorescence is seen in perinuclear cytoplasm (arrow). ×800.

detected at 4 hr p.i. at the cytoplasm in an indentation, and they spread further into the cytoplasm thereafter. Rounding and detachment of the cells were observed as CPE in both the stained and unstained preparations at 16 hr p.i., but syncytium formation was not recognized in either of the stained and unstained preparations throughout the observation period.

Virus growth curve: Growth curve of CCV in CRFK cells is shown in Fig. 1. The virus titers in the fluid phase began to increase 4 and 8 hr p.i. and reached their maxima of $10^{4.2}$ and $10^{5.3}$ $TCID_{50}$/0.1 ml by the inoculation with 1 and 50 $TCID_{50}$/cell respectively.

Findings by electron microscopy: In the early stage of infection, virus particles were localized restrictedly in the perinuclear area in an indentation, but a few particles were seen in the other part of the cytoplasm. Fig.
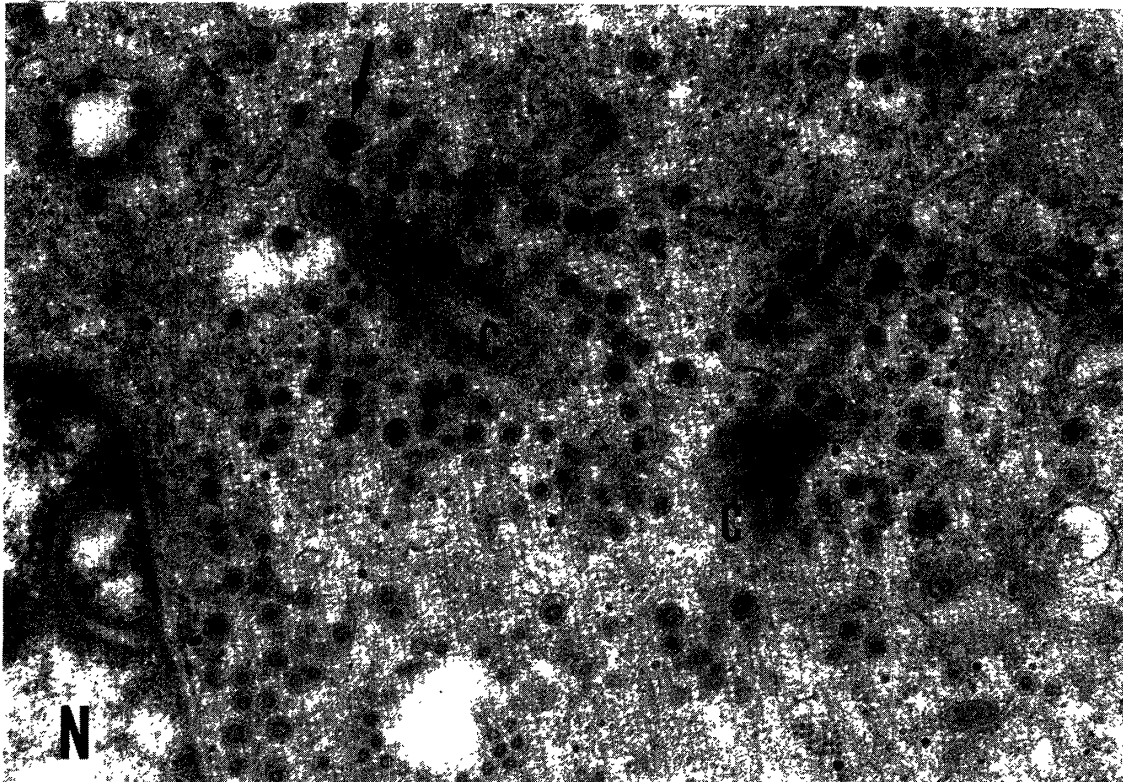
Fig. 4.   Part of a CRFK cell at 8 hr p.i. Small smooth vesicles containing viral particles assemble around centriole (C). Arrow indicates viral particles in a small cluster. ×35,000.
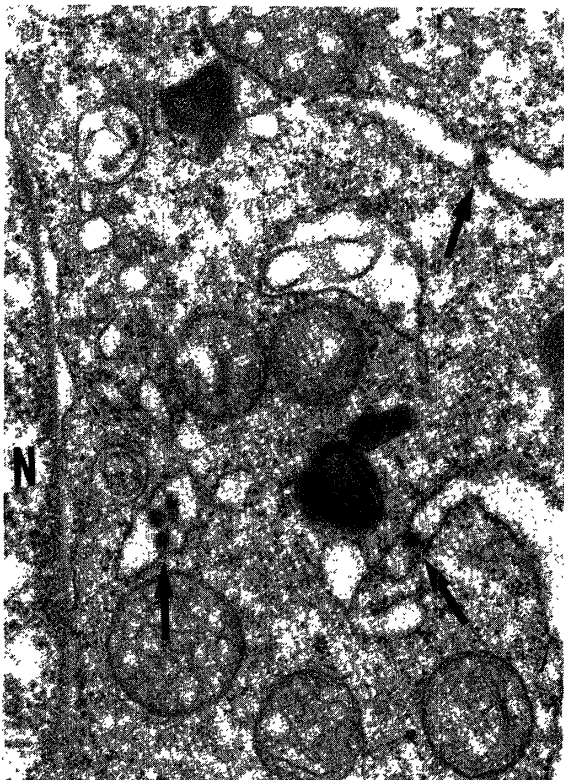

Fig. 5.   Viral particles (arrows) are present inside the distended rough endoplasmic reticulum. 8 hr p.i. ×32,000.

4 shows characteristic pattern of viral localization in the early stage. Virus particles occurred densely around the centriole. They were enclosed singly or in small clumps in small smooth vesicles. Particles were also seen in small vesicles and in vacuoles in the Golgi region and in the cisternae of the Golgi apparatus (Fig. 6). Some particles were present in the distended rough endoplasmic reticulum (Fig. 5). It was not rare that the virus particles were present together with the centriole.

In the late stage of infection, virus particles were found in the whole cytoplasm. Most of the particles were observed in the small vesicles, in the vacuoles, and in the cisternae of distended smooth endoplasmic reticulum. In addition, electron-dense lysosome-like vacuoles containing many virus particles as their main contents were seen in the cytoplasm near the plasma membrane (Fig. 7).
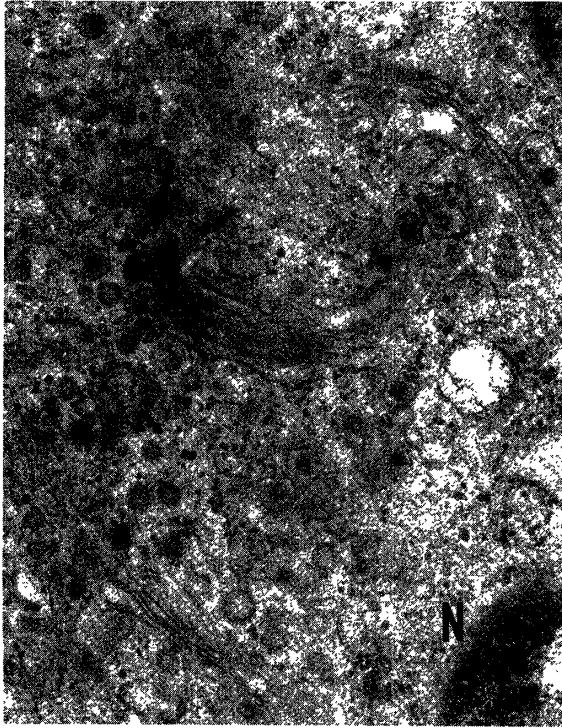
No   definite   budding   figures   were

Fig. 6. Viral particles are present in Golgi sacs, in which two particles (arrows) are pinched-off at the end of the Golgi sacs. 6 hr p.i. ×30,000.

observed at any part of the membrane-bound structures throughout the observation period. The virus particles were generally circular in shape with some pleomorphism and the maximum diameter was in the range of 80 to 140 nm (mean 100 nm). They had dense core, sometime being doughnut-shaped, in the center.

Syncytia were found in the specimens harvested at 40 hr p.i. Electron-lucent nuclei, atrophied mitocondria and lysosome-like structures were observed in the syncytia and a few virus particles were scatteringly seen in the cytoplasm and on the cellular membrane.

DISCUSSION

The viruses of the coronavirus group generally have fastidious nature of their growth requirement, and cultivation of the virus has usually been limited to their growth in the host animal or cells and tissue
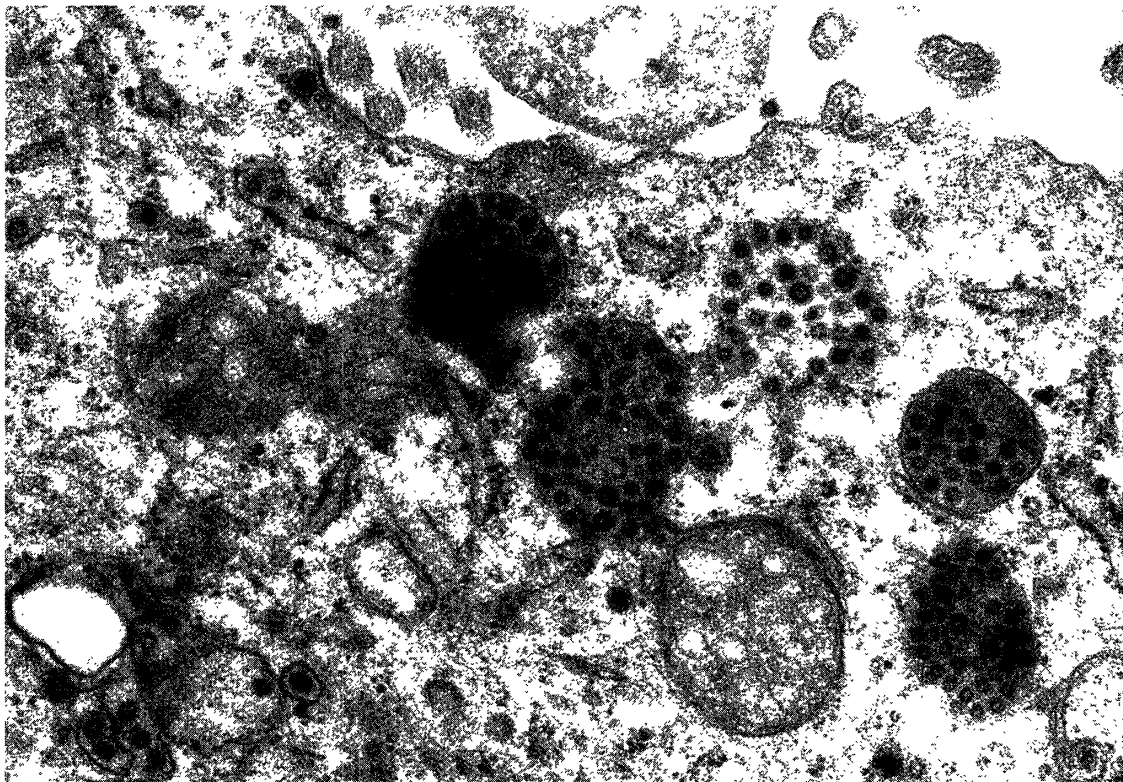

Fig. 7. Electron-dense, lysosome-like cytoplasmic vacuoles are filled with viral particles. 6 hr p.i. ×35,000.

derived from that host [10]. Binn et al. [1, 2] reported that the 1–71 isolate of CCV produced CPE only in dog cell cultures. Woods [16], however, reported the feline cell line FC supported the growth of CCV. Growth of CCV in CRFK cells has been reported and put to practical use by Evermann et al. [5], Helfer-Baker et al. [7] and Miller et al. [11], but it has never been fully investigated up to the present. The present study revealed the multiplication of CCV in CRFK cells which were established from kidney cells of a non-host animal.

CPE appeared in CRFK cell cultures without successive blind passages by the inoculation with CCV which was propagated in DK-2 cells. Yasoshima et al. [18] reported that rounding and detachment of the cells were the most prominent cytopathic changes in DK-2 cells inoculated with Toda strain of CCV, and that syncytium formation was not evident. In CRFK cells, however, syncytium formation was remarkable and rounding of the cells was observed only when concentrated virus ($10^{7.0}$ $TCID_{50}$) was inoculated.

The virus titers began to increase in parallel with the appearance of the virus specific immunofluorescence and immunoperoxidase staining. The virus harvest was comparatively poor and the maximum virus yield was almost the same as that reported in DK-2 cells [18].

Immunocytochemical study revealed that the virus-specific immunofluorescence and immunoperoxidase staining appeared at 4 hr p.i. at the restricted perinuclear cytoplasm in an indentation. Tooze et al. [15] reported that, during infection of sac⁻ cells by murine coronavirus MHV A59, viral glycoprotein E1 was confined at early stage of infection to the perinuclear region by immunofluorescence, and that the intracellular site where progeny virions budded correlated with the distribution of the viral glycoprotein E1.

Electron microscopic observation revealed that virus particles occurred restrictedly around the centriole and in the Golgi complex in the early stage of infection. Most of the viral particles were enclosed singly in small vesicles. It was very similar to that described in porcine enteric coronavirus by Ducatelle et al. [4]. They suggested that these small vesicles were formed by the pinching-off from the end of the Golgi sacs.

Perinuclear cytoplasm in an indentation is known as the site of centriole and Golgi complex or, in other word, the cell center. Thus, close correlation between occurrence of virus particles and positive reaction in immunofluorescence and immnoperoxidase tests was demonstrated in the present study. Association of virus particles with the centriole was observed frequently and seemed to be one of the most characteristic features in CRFK cells infected with Toda strain of CCV. It is possible that the close relation between virus particles and the centriole facilitated the detection of the centriole, which is rather hard to find in routine electron microscopic examination.

Takeuchi et al. [14] reported the replication of CCV by budding on membrane of dilated vesicles of enterocytes in the study of experimental enteric infection in neonatal dogs. Budding structures, however, could not be found at any part of the intracellular membrane-bound structures in the present study. Ducatelle et al. [4] have mentioned that the frequency of observation on budding structures not only varies markedly from one coronavirus to the other, but also depends on the host or cell type. Therefore, the combination of CCV vs. CRFK cells may be an experimental system which exhibits budding condition in a mode of multiprication very occasionally.

The process of multiplication of CCV in CRFK cells coincided roughly with what had been proposed as the hypothetic

scheme of coronavirus morphogenesis: budding into the endoplasmic reticulum lumen, transport to the Golgi complex, formation of virus-containing vesicle, transport through the cytoplasm and release [4, 15].

Syncytium formation was one of the most prominent cytopathic changes in CRFK cells infected at a low MOI of 1 $TCID_{50}$/cell with CCV. In these syncytia, there were no findings suggesting the multiplication of the virus, and these cells lost almost all of the organelle and contained only a few free virus in the cytoplasm and on the cellular surface. Since syncytium formation was not observed at a high MOI of 50 $TCID_{50}$/cell, this type of cell fusion may occur by the "late" form of polykaryocytosis [13]. In the cells infected with murine hepatitis virus, envelope-associated viral glycoprotein, E2, was supposed to induce cell fusion and syncytium formation [3, 6, 15].

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要　　約

CRFK 細胞でのイヌコロナウイルスの増殖：小嶋明廣・高田　博・岡庭　梓（田辺製薬株式会社安全性研究所）——イヌコロナウイルスは CRFK 細胞で盲継代を経ずに CPE を発現した。 $1 TCID_{50}/cell$ の接種で合胞体の形成がみられたが，$50TCID_{50}/cell$ の接種では細胞の円形化と脱落を主体とする CPE が認められた。感染 4 時間後に免疫蛍光法および酵素抗体法で，核近傍細胞質にウイルス特異抗原の局在が観察された。抗原の出現に一致してウイルス感染価の上昇がみられ，$10^{4.2}TCID_{50}/0.1ml$ ないし$10^{5.3}TCID_{50}/0.1ml$ の最高値に至った。感染初期の細胞では核近傍のゴルジ野周辺に限局してウイルス粒子が観察された。ウイルス粒子の局在部位は免疫蛍光法および酵素抗体法によるウイルス抗原の局在部位と一致し，中心体およびゴルジ装置の周辺ならびに核近傍の細胞質などに認められた。