NOTE *Pathology*

Growth Characteristics of Canine Distemper Virus in a New Cell Line CCT Cells Originated from Canine Malignant Histiocytosis

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ABSTRACT. Canine distemper virus (CDV) growth and the morphological characterization were examined in a cell line established from a canine malignant histiocytosis (CCT cell line). The susceptibility of the CCT cells to 3 CDV strains, FXNO, YSA-TC and MD-77 was shown by detection of the antigen in the indirect fluorescent assay. After passaging 4 and 9 times through the CCT cells, only FXNO strain could produce the syncytia where demonstrated the antigens. Titers of 9 passaged viruses through the CCT cells showed slightly higher in the CCT cells than those in Vero cells. Morphological characterization of karyorrhexis and specific DNA ladder by extracted DNA electrophoresis indicated apoptosis in the CDV infected CCT cells.

KEY WORDS: apoptosis, CCT cell, CDV.

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Although many trials for canine distemper virus (CDV) experiments *in vitro* have been performed [10, 12], it was extremely difficult to isolate and propagate the virulent virus using commercial cell lines such as Vero cells [11, 18, 20], because of cell adaptation and easy attenuation of the virulence through propagation in these cell lines. Since it had been observed that CDV could initially infect to macrophages and lymphocytes in the respiratory tracts of the affected animals, it was suggested that there might be a mitogen stimulated canine or ferret peripheral blood lymphocytes, or there might be same potential in the lung and/or peritoneal macrophages to isolate and propagate the virulence of CDVs from clinical specimens [1, 21]. Therefore, cell lines derived from dog macrophages or lymphocytes have been expected to be available for the experiments. Recently, it has been reported that B95a cells, an Epstein-Barr virus-transformed marmoset B lymphoblastoid cell line were highly susceptible to CDV [6] as well as measles virus (MV) [7] and rinder pest virus [8] belonging to genus *Morbillivirus*. It could also succeed to isolate some field CDV from clinical specimens in Japan. Canine cell lines originated from macrophages or other histiocytic cells were extremely rare and there have been no reports on CDV experiments using such cell lines in spite of their potential susceptibility to CDV anticipated. Recently, a CCT cell line established from a canine cutaneous malignant histiocytosis on a 4 year old male dog and demonstrated characteristics of macrophages by immunostaining, cytochemical staining and electron microscopy [13]. We found the CCT cell line was susceptible to CDV. The present study dealt with the morphological characterization of the CCT cells and virus growth after infected with CDV.

Three of FXNO, YSA-TC and MD-77 CDV strains were used. The latter two strains were kindly supplied by Dr Tokiyoshi S (The Chemo-Sero-Therapeutic Research Institute, Japan). FXNO is a vaccine strain strongly adapted in Vero cells, YSA-TC is propagated in chick embryo fibroblast, so called "avianization" and MD77 is a field isolated wild strain and passaged twice in dog kidney primary cells and 5 times in Vero cells [4]. In our laboratory, they were passaged with the CCT cells or Vero cells and stocked at –80°C.

To investigate the morphological changes after virus inoculation, the CCT cells were prepared in 3-cm culture dish with cover slips and inoculated with the CDV strains. On 3 to 5 days post inoculation (dpi.), the cover slips were taken, fixed in cold acetone for 20 min, and stained with hematoxyline-eosin (HE) or Giemsa. The uninoculated CCT cells were used as the negative control. To detect the viral antigens in the cells, the indirect immunofluorescent assay (IFA) was performed. The first anti-CDV NP monoclonal antibody (D110) kindly supplied by Dr Zurbiggen (University of Bern, Switzerland) and second antibody of FITC conjugated anti-mouse immunoglobulins (DAKO, Japan) were used. FXNO-CDV infected Vero cells were used as the positive control.

Each of CDV strains was passaged through the CCT cells 4 times, and named as FXNO-C4, YSA-TC-C4 or MD-77-C4. FXNO-C9, YSA-TC-C9 and MD-77-C9 were provided additional 5 times passages. After inoculation into the CCT cells, the supernatant and the infected cells were harvested at 24, 48, 72, 96, 120 hr post inoculation (hpi). For titration, 20 μl of each serially 10 fold dilution was inoculated into each of four wells of a 96 well plate with the CCT and Vero cells. Virus inoculation was performed by co-cultivation with cell and virus inoculum. Virus titer was expressed as a 50% tissue culture infectious dose ($TCID_{50}$) as described before [23].

The virus inoculated CCT cells were harvested with cell scraper on day 3 and 4. After washing with PBS, cell pellets

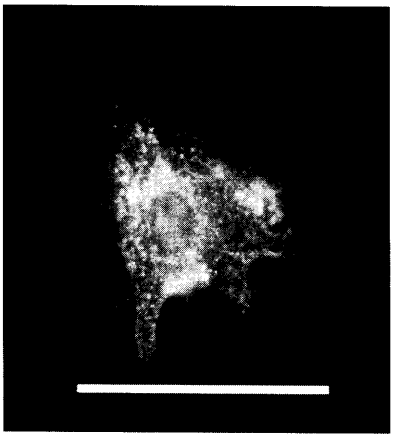

Fig. 1. Indirect immunofluorescent assay. The CCT cells inoculated with FXNO-C4 strain 5 days postinoculation. Bar=50 μm.

were dissolved in 100 μl per microtube of lysis buffer (10 mM Tris-HCl containing 10 mM ethylene diamine tetraacetic acid, pH 8.0, and 0.5% Triton X-100) and placed at 4°C for 10 min. The uninfected cells were used as the negative control. The lysates were centrifuged at 15,000 × g for 30 min. The supernatant was extracted and treated with 2 μl of 20 mg/ml RNase A (Ribonuclease A, Type II-A ; Sigma, England) for 1 hr at 37°C and subsequently treated with 2 μl of 20 mg/ml Proteinase K for 1 hr at 37°C. Then it was mixed with 0.5 M NaCl and 50% isopropanol, and stored at −20°C overnight. After centrifuged at 15,000 × g twice for 30 min, the pellet was resuspended in TE buffer (10 mM Tris-HCl containing 1 mM EDTA, pH 8.0), run on a 2.0% agarose gel in Tris-borate, EDTA (TBE) buffer (89 mM Tris and 89 mM boric acid containing 2 mM EDTA, pH 8.0) added 12 μl of ethidium bromide at 100 V (Mupid, Adovance) for 2 to 3 hr, observed on a UV transilluminator (Funakoshi, Japan), and photographed (Polanoid MP4 Land Camera, U.S.A). A 100 bp DNA ladder (BioLabs, England) was used as a reference.

The CCT cells infected with any strains of CDV including initial stock virus were all positive for IFA. CDV-NP antigens were detected most in the cytoplasm and sometimes in the nucleus (Fig. 1). FXNO virus showed the strongest reactions among 3 strains.

Cytopathic Effect (CPE) in the CCT cells induced by CDV infection was morphologically different depending on the infected virus strains. The virus not passaged through these cells showed no remarkable CPE except for sporadic cytolysis indistinguishable from that in the uninfected cells. FXNO-C4 and -C9 induced the earliest and the most prominent CPE in the cells, and characterized by the syncytium formation demonstrating many nuclei along with flotation of large round cells in the medium (Fig.2 A, B). YSA-C4 and -C9 and MD-77-C4 and -C9 had almost the same CPE characterized by smaller size of rounding cells without any syncytia. The uninfected control cells were detached due to overgrowth. By Giemsa staining, karyopyknosis and/or karyorrhexis of the nuclei, apoptotic figures, were induced following inoculation of all CDV-C9 (9 times passages in the CCT cells) after 3 dpi (Fig. 3). But uninfected negative control cells showed no significant apoptotic figures. It was difficult to distinguish the viral inclusion bodies from eosinophilic granules which were normally distributed in the cytoplasm.

Table 1 shows comparison of virus titration in the CCT cells. The virus titers on -C9 measured in the CCT cells at 48 hpi was clearly higher than that of in Vero cells. FXNO-C4 and -C9 showed also high titers in Vero cells. When compared between several -C4s and -C9s, FXNO-C9, MD-77-C9 and YST-C9 showed higher titers than those of -C4s in the CCT cells.

Figure 4 shows the results of electrophoresis. On 3 dpi, only FXNO-C9 inoculated CCT cells showed a ladder of DNA. But on 4 dpi, all strains of CDV showed the ladders in the CCT cells. The uninfected negative control cells did not show any prominent ladders.

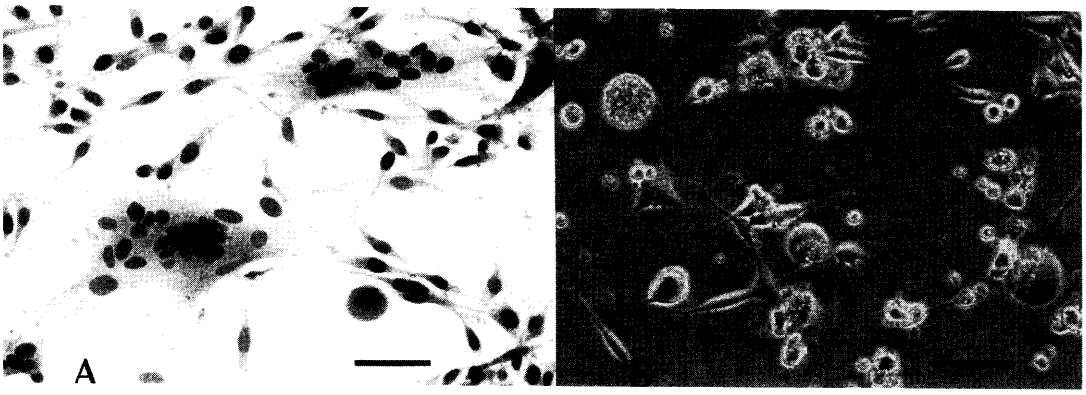

Fig. 2. The CCT cells infected with CDV-FXNO strain. A: Inoculation with FXNO-C4 strain 3 days postinoculation. Two large multinucleus syncytium cells are observed. HE staining. B: Inoculation with FXNO-C9 strain 4 days postinoculation. Some floating round cells are observed. Examined by phase-contrast microscope. Bar=50 μm.

Fig. 3. The morphological change of CDV inoculated CCT cells. The MD-77-CDV passaged in CCT cells inoculated CCT cells. The cell in the center shows karyorrhexis. Giemza staining. Bar=50 μm.

Table 1. Growth of CDVs in CCT cells

	Titrated by CCT cells				Titrated by Vero cells			
	24[a)]	48	72	96	24	48	72	96
FXNO- C4	0.5[b)]	3.0	3.0	3.0	0	1.25	2.75	3.0
FXNO- C9	0	2.75	3.5	3.5	0	0	2.5	3.25
MD77- C4	0	0	2.0	2.7	0	0.75	1.75	2.0
MD77– C9	0	1.5	2.25	2.75	0	0	2.25	2.5
YST- C4	0	0	2.0	2.25	0	1.0	2.0	2.5
YST- C9	0	1.5	2.0	3.0	0	0	2.25	2.5

a) Hrs postinoculation.
b) Log_{10} $TCID_{50}$/20 μl

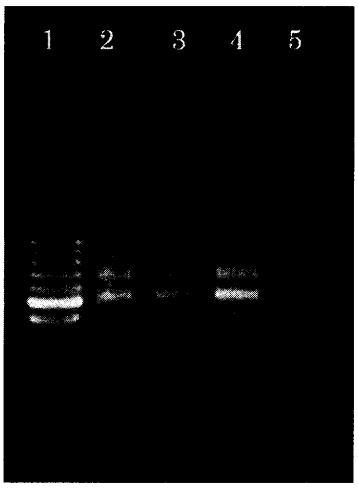

Fig. 4. The DNA fragmentation assay by electrophoresis. Lane 1: 100 bp DNA ladder, lane 2: FXNO-CDV, lane 3: YSA-TC-CDV, lane 4: MD-77-CDV inoculated CCT cells, lane 5: uninfected negative control CCT cells. CDV inoculated CCT cells 4 days postinoculation. All virus inoculated cells show DNA ladder. The negative control shows no DNA fragmentation.

The present study demonstrated that the CCT cells originated from the mononuclear-phagocyte system were susceptible to CDV. Although the initial stock viruses could not induce obvious CPE except for sporadic cytolysis indistinguishable from that in the uninfected cells, virus antigens could be readily detected by IFA. But this phenomenon resembled to that in the blast lymphocytes infected with CDV [1]. After passaging through the CCT cells, only strain FXNO of CDV could produce the syncytia. Hirayama *et al.* [4] reported that all CDV strains used in Japan for examination of the biological and molecular characteristics could induce the syncytium in Vero cells. There would be some mentions about this phenomenon. Firstly, it has been demonstrated that viral envelope proteins, hemagglutinin (H) and fusion (F) proteins are important to attach and invade to the susceptible cells following syncytium formation. These proteins are known to work for attenuation of the virus after adaptation [3, 5, 17]. Secondary, the viral receptors on the CCT cells might be different from those of Vero cells. Vero cells have MV receptor of CD46 which is a complement binding protein belonging to a family known as the regulators of complement activation (RCA) gene function [22]. HeLa and human T cells also have specific protein, CD46. But recently, another molecule, SLAM, signaling lymphocyte-activation molecule also known as CDw150, was demonstrated on the MV infected lymphocytes. B95a cell line which is susceptible to CDV as well as MV also expresses abundant SLAM, suggesting SLAM might play as a receptor for CDV [19]. Whereas, dog SLAM has been known to be a real receptor for CDV and we reported an efficient isolation of CDV in Vero cells expressing canine SLAM [16]. However Vero cells were from monkey kidney. Although the CCT cells were originated from dog histiocytic cells, the same or other types of receptor might exist in these cells. On the other hand, the antibody to CDw150 could not react to dog SLAM. Production of an antibody to dog SLAM, CDV receptor, is a critical issue in future studies. The third, antibodies to CD9, the tetraspan transmembrane protein detected in HeLa, Vero and dog brain cell cultures, which could prevent the syncytium formation and allow virus to release in the infected cultures [14]. It is perceived that a cell-to-virus interaction can play a critical role in the formation of syncytia.

The slight higher titers of the -C9 viruses compared to those of the -C4 viruses may suggest that these viruses adapted to the CCT cells, because the viruses used in the present study were not provided from the fresh samples in the diseased dogs, unfortunately.

The target cells responsible for initial infection and maintenance of virulence of CDV in dogs are thought to be macrophages and/or lymphocytes. Using the CCT cell line, the

isolation and cultivation of virulent wild strains of CDV might be expected. Further examinations including virus isolation, elucidation of mechanism of virus-cell interactions, and other investigations using the CCT cells will be needed.

As mentioned about the nature of CPE observed in the CDV inoculated CCT cells, apoptosis of these cells was also suspected. To confirm this assumption, the DNA extraction to detect the DNA fragmentation was performed, and specific DNA ladder was observed. It has been described that CDV would initially infect to lymphocytes or macrophages *in vivo* and might induce apoptosis of the infected cells [9] resulting in leukopenia, while the mechanism of this event has not been confirmed nor understood, well. MV closely related to CDV is known to induce apoptosis of lymphocytes [2], and some observations exist about CDV-induced apoptosis in lymphocytes, within the lesions of chronic demyelinating encephalitis [15] and lymphocytes in lymph nodes [9].

Our data might indicate a possibility of *in vitro* experiment using the CCT cells, since apoptosis induced by CDV infection. These suggest that CDV has a potential to induce apoptosis in the mononuclear-phagocyte system *in vitro*. Further examinations for understanding the relationship between CDV and the host cells, the mechanism of virus entry, and the possibility or mechanism of apoptosis shall be warranted in the future.

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Trypanosoma congolense 特異的診断用抗原を探索する過程でabc1ファミリー蛋白質ホモログをコードする完全長cDNA(*P74*)がクローニングされた．abc1ファミリー蛋白質は新規のシャペロニンであり，ミトコンドリア内膜bc1複合体における電子伝達系とコエンザイムQ(CoQ)の生合成に必須であることが示唆されている．また，トリパノソーマのCoQ生合成経路は抗トリパノソーマ薬による新たな標的としても期待されている．酵母のabc1蛋白質は核あるいはミトコンドリアに局在するが，cDNA配列から予想されるp74のアミノ酸配列中には既知の核移行シグナルおよびミトコンドリア移行シグナルは確認できなかった．ノザンブロット解析の結果，血流型虫体の*P74*転写レベルはプロサイクリック型虫体の約4倍高いことが明らかとなった．この結果は特異抗体を用いたウエスタンブロット解析によっても裏付けられた．また，予想アミノ酸配列から算出されるp74の分子量は約74 kDaであるが，ウエスタンブロット解析の結果明らかとなった分子量は約56 kDaであった．これらの結果はp74が未知のシグナル配列除去などの翻訳後修飾を受けていることを示唆している．p74の更なる機能解析によって*T. congolense* のCoQ生合成経路に関する新たな知見を得ることが期待できる．

C57BL/6マウスへの実験的 *Eimeria pragensis*（*E. pragensis*）感染に対するクリンダマイシンの治療効果をオーシスト産生の抑制および *E. pragensis.* の細胞内ステージの発育障害の程度によって判定した．クリンダマイシン800 mg/kg/dayの用量での感染後1～4日および4～8日まで短期間投与することにより，血様性下痢，元気消失などの臨床症状の消失とともにオーシスト産生の有意な低下およびシソゴニー期虫体の発育抑制が観察された．興味深いことに，この短期間投与は治癒マウスの再感染に対する防御免疫を強く誘導した．感染後1～12日までのマウスへの同用量のクリンダマイシンの投与は，著しく臨床症状とオーシスト産生を抑制したが，治癒マウスへの再感染に対する防御効果は完全ではなかった．結論として，これらの結果は，抗コクシジウム剤としてのクリンダマイシンの有効性および初期および後期の *E. pragensis* の細胞内ステージ原虫がいずれも再感染防御免疫を誘導できることを示唆している．

病　理　学：

遺伝子診断により *CL-16* 欠損症と診断された2～65ヵ月の黒毛和種牛37症例の腎臓について病理学的検索を行った．症例では若齢で発育不良，腎不全，過長蹄，貧血などの臨床的症状が認められた．発症時期と月齢との相関は認められなかった．体重と腎重量の相関ではほぼ正常牛と同じ分布を示していたが，腎機能障害が重度の症例では，萎縮による腎臓の縮小がみられた．腎臓の病理組織学的検索では糸球体数の減少，糸球体および尿細管の代償性肥大，間質の線維化およびリンパ球の浸潤を伴う糸球体および尿細管の萎縮が認められた．症例牛では病変が軽度な腎臓においても，正常牛と比べて明らかに糸球体数が少なく，未熟な糸球体および未熟な尿細管も確認されており，出生時に形成されている腎単位数が少ないことが示された．この事から，*CL-16* 遺伝子の欠損は，「腎単位の形成異常」に関与していることが示唆された．免疫組織化学的には，正常対照牛のヘンレのワナ上行脚の太い部分の上皮細胞がCL-16蛋白の抗血清に対して陽性染色を示したのに対して，病牛の腎尿細管上皮細胞が陰性の染色性を示し，腎尿細管上皮細胞におけるCL-16の欠損が示された．