Pathological and Molecular Biological Studies on Canine Distemper

CHAPTER II

Growth Profiles of Recent Canine Distemper Isolates on Vero Cells Expressing Canine Signalling Lymphocyte Activation Molecule (SLAM)

Summary

Fresh samples of lymph node, lung and cerebrum taken postmortem from dogs no.1, 2 and 3 yielded canine distemper virus (CDV) strains 007Lm, 009L and 011C, respectively. These were titrated on Vero cells stably expressing CDV receptor that was canine signalling lymphocyte activation molecule (SLAM; Vero-DST cells). Growth curves of the three strains produced by titration of the released virus and cell-associated virus at various time points. All three isolates, especially 007Lm, grew well on Vero-DST cells. The titres of cell-associated virus of two strains (009L and 011C) were clearly lower than those of virus released into the culture supernate. The results indicate that Vero-DST cells are not only useful for primary isolation but also efficient for titrating virus from fresh tissues and for the study of growth profiles of recent CDV isolates.

Key words: canine distemper; dog; SLAM; Vero-DST cells; viral infection

Introduction

Canine distemper virus (CDV), which cause a serious disease of dogs, is an enveloped virus with negative-stranded RNA genome, belonging to the genus *Morbillivirus*, family *Paramyxoviridae*. The characteristics of CDV strains prevailing in the field have not been investigated due to difficulties in isolation and titration. CDV was isolated, however, by growth in peritoneal macrophages obtained from specific pathogen-free (SPF) ferrets (Poste, 1971; Whetstone *et al.*, 1981), or in mitogen-stimulated canine lymphocytes

(Appel *et al.*, 1992) obtained from SPF dogs. Field isolates failed to propagate in commercial cell lines such as Vero cells derived from African green monkey kidney. Some strains, however, such as the Onderstepoort vaccine strain and cell culture-adapted CDV strains, can replicate in numerous cell lines (Haig, 1956; Appel and Gillespie, 1972; Rzezutka and Mizak, 2002). The use of a canine epithelial kidney (MDCK) cell line or of B95a cells derived from marmoset B lymphoblastoid cells resulted in successful isolation of CDV from clinical samples (Kai et al., 1993; Lednicky et al., 2004). As these cell lines are neither sensitive nor convenient to use, they are not suitable for the isolation and titration of CDV. So far, no cell lines have been adopted for routine titration of fresh tissues from CDV-infected animals.

Recently, signalling lymphocyte activation molecule (SLAM; CD150) from various animals (e.g., human, canine and bovine) was shown to be a proper receptor for *Morbilliviruses* including measles, canine distemper and rinderpest viruses (Tatsuo et al., 2001). Seki et al. (2003) reported that Vero cells stably expressing dog SLAM with tag are highly sensitive for field CDV propagation with apparent CPE. In this study, Vero-DST cells were used for titrating and for examining the growth profiles of CDV strains freshly isolated from clinical samples.

Materials and methods

Tissues selected for this study were collected at necropsy from three dogs pathological changes consistent with canine distemper, supported by the immunohistochemical demonstration of CDV antigens (Table 2). These tissues were stored at -80^{0}C. For use, 0.5 g of each sample was homogenized and sonicated in 5 ml of DMEM containing penicillin 1000 units/ml and streptomycin 1000 μg/ml. The

suspensions were clarified by centrifugation and then used to infect monolayers of Vero-DST cells in 24 well plates. The inoculated plates were incubated in 5% CO_2 at 37^0C.

Total RNA was extracted from CDV-infected Vero-DST cells showing a 90% cytopathogenic effect (CPE) with Trizol reagent (Invitrogen, California, USA) according to the manufacture's instructions. The RNA was used for reverse transcriptase polymerase chain reaction (RT-PCR) with primers within the P gene as forward primer (5'-ATGTTTATGATCACACGGT-3') and reverse primer (5'-ATTGGGTTGCACCACTTGTC-3'). The positive control consisted of cDNA of the Onderstepoort strain propagated in Vero-DST cells; the negative control consisted of the mixture without the matrix of CDV. The electrophoresis of 10 µl of PCR products was performed in 1.2 % agarose gel. The size of amplicons was compared with a 100bp DNA Ladder, marker.

Results

The gross and histological changes in the three dogs (nos 1,2 and 3) were mainly in the lungs, brain and lymph nodes. Pneumonia was characterized by thickened alveolar septa and proliferation of alveolar epithelium in all cases. Non-suppurative encephalitis was observed only in dog 1 (Table 2). Intracytoplasmic and intranuclear inclusion bodies were found in the lung, brain, stomach and urinary bladder. Lymphoid depletion of lymph nodes and spleens was severe in all dogs. CDV antigens were detected immunohistochemically in the brain and lung sections.

Samples from the three dogs were inoculated into Vero-DST cells. At the same time, viral titres in the fresh tissues were calculated. The development of a CPE was observed daily by phase contrast microscopy. The CPE, characterized by syncytium formation (Fig.

3a and b), occurred in first passage samples from all dogs from 18 hpi (Table 3). CPE caused by CDV was confirmed by immunocytochemistry (Fig. 3c and d) and by immunofluorescent assay (Fig. 4a) with monoclonal antibody against the nucleo protein of CDV. Virus strains 007Lm, 009L and 011C were isolated (from the lymph node, lung and cerebrum) from dogs 1, 2 and 3, respectively. These isolates were confirmed as CDV by RT-PCR with P gene (Fig.1). The titres of the new isolates in the first passage were high, especially that of strain 007Lm ($3.16x10^7 TCID_{50}/25\mu l$), as compared with those of laboratory strains (data not shown). Interestingly, in dog 1, the viral titre in the lymph node was higher than in the other tissues. CPE was not found in Vero cells lacking SLAM until 6 days post inoculation.

Study of the growth profile of strain 007Lm (Fig. 5a) showed that, after an eclipse phase (6 to 12 hpi), virus in the cells and supernate increased logarithmically, reaching a plateau at 48 to 96 hpi. The maximum titre of cell-associated virus was $3.16x10^6$ $TCID_{50}/25\mu l$ at 48 h, and that of released virus was $1.48x10^6$ $TCID_{50}$ $/25\mu l$ at 72 h. Before 48 h, the titre of cell-associated virus was higher than that of released virus. The reverse pattern was demonstrated after 48 h. At each harvest after the eclipse phase, a CPE was observed. At 24 hpi, 60% of cells were affected, and at 72 hpi the CPE was extensive (90-95%) indicating the highest titre during the observation. Growth curves of strains 009L and 011C are shown in Figs 5b and 5c, respectively. The titres of released virus were much higher than those of cell-associated virus after 36 h.

Discussion

Vero-DST cells were much more efficient than Vero cells (lacking SLAM) for the titration of CDV from clinical samples. This suggests that SLAM is the main cellular receptor for CDV replication and of importance molecule in establishing binding

between the H proteins of CDV. Mitogen-stimulated canine lymphocytes were found by Appel *et al.* (1992) to be effective for isolation of CDV from field cases, but obtaining lymphocytes from SPF dogs is not easy. Recently, B95a cells (Kai *et al.*, 1993) and MDCK cells (Lednicky *et al.*, 2004) were successfully used for the isolation of CDV. They were not used, however, for the titration of fresh samples.

Johnson *et al.* (1985) reported that CDV could be titrated on the basis of plaque-forming units (PFUs) in Vero cells. We attempted to titrate new isolates on Vero cells and on Vero-DST cells. Plaques were not produced in Vero cells. In contrast, large spreading plaques were formed in Vero-DST cells, but counting was impossible. The adherent nature of Vero-DST cells is probably highly sensitive for forming syncytia probably due to cell to cell infection. In Vero-DST cells, the $TCID_{50}$ method proved to be of value for titrating CDV in clinical samples.

The growth kinetics of virus isolated from the lymph node, lung and brain were demonstrated in this study. So far, it had proved difficult to do this without cell culture adaptation. However, attempts have been made to demonstrate growth curves with primary cultures. Shishido *et al.* (1967) reported that the titre of released virus at no time exceeded that of cell-associated virus. In the present study, in Vero-DST cells, titres of cell-associated virus were lower than those of released virus with newly isolated strains. The laboratory strains showed the higher titres in the associated cells than supernate (data not shown). Three isolates showed a high titre. These indicate that the growth characters of new isolates of CDV maybe different from those of the laboratory strains such as Onderstepoort.

It must be borne in mind that the three dogs in this study had recently been given a live vaccine against canine distemper. Although considered unlikely, the possibility cannot be excluded that the three viral isolates consisted of the recovered vaccine strain. To clarify

this point beyond doubt, a comparison of the vaccine strain with the three isolates by molecular techniques would be required.