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Pathological and Molecular Biological Studies on
Canine Distemper

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CHAPTER V

Comparison of Molecular Analysis and Growth Properties of Two Different Clusters of Canine Distemper Viruses Recently Isolated in Japan

Summary

To compare the molecular and growth properties of two newly isolated canine distemper virus (CDV) strains of Asia 1 and 2 groups, the molecular analysis and growth profiles of these groups were performed. The isolates were classified into Asia 1 and 2 groups based on both P and H gene analysis. In H gene, both groups had 12 cysteine residues. The hydrophobic region of Asia 2 group strains had one amino acid more than Asia 1 group. The strains in Asia 1 group had nine asparagine (N)-linked glycosylation sites, one site (amino acid position 584-586) more than Asia 2 strains. The titers of the released viruses of Asia 2 strains were higher than titers of cell-associated viruses, and were opposite to Asia 1 group strains. These results indicate that the N-linked glycosylation site at amino acid 584-586 is related to the growth properties of CDV and that the virulence showed no significant difference between Asia 1 and 2 groups.

Key words: Asia 1, Asia 2 groups, CDV, molecular character, growth profile

Introduction

Canine distemper virus (CDV) of the *Morbillivirus* genus of the family Paramyxoviridae is an enveloped single-stranded RNA virus that causes lethal systemic disease in dogs and other carnivores (Appel & Summers, 1995; Lamb & Kolakofsky, 1996). Although canine distemper (CD) has been generally controlled well with live attenuated vaccine, the number of typical CD cases has increased in Japan and European

countries (Blixenkroner-Møller et al., 1993; Gemma et al., 1996; Kai et al., 1993; Shin et al., 1995). CDVs in Japan and Asian country have been classified into Asia 1 and Asia 2 groups, which are different from vaccine and known groups in the phylogenetic tree of the only H gene (Hashimoto et al., 2001; Mochizuki et al., 1999), but the viruses could not be isolated. Lan *et al.* (2005d) isolated new CDVs and confirmed Asia 1 and 2 groups by the phylogenetic tree of H and P genes. Understanding details of the characters of each group is necessary and useful to prevent CD or to produce a CDV vaccine.

Our objective was to compare the molecular and growth properties of CDV Asia 1 and Asia 2 groups and to clarify the correlation between molecular and growth characters, clinical signs and pathological findings caused by these CDV groups.

Materials and methods

Viruses: CDV strains 007Lm, 009L, 011C, Ac96I, P94S and S124C isolated from lymph node, lung, cerebrum, large intestine, spleen and cerebellum, respectively, of autopsied dogs showing evidence of CDV infection by pathological findings and immunohistochemistry were used (Table 2 and 5). Samples from 6 dogs were inoculated into Vero-DST cells. CPE development was observed daily by phase contrast microscopy. CPE formation was confirmed by immunocytochemistry and immunofluorescence with monoclonal antibody against NP-CDV.

Results

Sequences and phylogenetic analysis: Sequencing and phylogenetic analysis of the P and H genes of six newly isolated CDV strains were done. Phylogenetic trees were constructed based on the nucleotide sequence of a 390 bp fragment of a P gene (Fig. 8) and a sequence of 607 amino acids of the H gene (Fig. 10) of the new isolates was

predicted. Both phylogenetic trees showed that new isolates Ac96I, P94S and S124C joined to the Asia 1 group that is different from the Asia 2 group that includes CDV strains 007Lm, 009L and 011C. Both Asia 1 and Asia 2 groups were far from the vaccine, Europe and USA groups.

The H gene of all new isolates in Asia 1 and 2 groups has 12 cystein residues (C) that determine the secondary structure of protein at an identical position (Fig.14). The major hydrophobic region containing 20 amino acids from amino acid positions 37 to 56 of the CDV strains in the Asia 2 has one amino acid more than of strains in the Asia 1 group. The H gene of strains P94S, Ac96I and S124C in the Asia 1 group contain nine potential N-linked glycosylation sites at same position: amino acids 19-21, 149-151, 309-311, 391-393, 422-424, 456-458, 584-586, 587-589 and 603-605. Strains 007Lm, 009L and 011C have only eight N-linked glycosylation sites compared with the H gene of the Asia 1 group: no amino acid at position 584-586. That is, Asia 1 and 2 groups have only one different position of N-linked glycosylation site in the H gene.

Growth profiles: The growth kinetics of Asia 1 strains showed that titers of cell-associated viruses were clearly higher than titers of released viruses. The highest titers during the observation period of the Asia 1 group were at the extensive cytopathic effect (CPE) of 90-95%.

A comparison of clinical signs and pathological findings (Table 2 and 5) of dogs 1, 2 and 3 from which CDV strains in Asia 1 group were isolated with those of dogs 4, 5 and 6 from which CDV strains in Asia 2 group were isolated showed no clear difference in virulence between Asia 1 and 2 groups, except for severe diarrhea in the Asia 1 group.

Discussion

N-linked glycans not only regulate folding and intracellular transport of viral protein, but also molecular function (von Messling & Cattaneo, 2003). N-link glycosylation in the H gene has nine sites in the Asia 1 group and eight sites in the Asia 2 group, which may reflect the antigenicity, pathogenicity and growth property. In this study, the growth profiles of two CDV isolated groups in Vero-DST were different according to the number of N-linked glycosylation sites. Growth profiles of strains 007Lm, 009L and 011C of Asia 2 group grew well and the titers of released viruses were higher than titers of cell-associated viruses after 24 hpi for strain 009L and after 28hpi with 011C, or after 50 hpi with strain 007Lm (Lan et al., 2005b). On the other hand, the growth kinetics of Asia 1 strains showed that titers of cell-associated viruses were clearly higher than titers of released viruses as a laboratory strain (data not shown). For clarification, recombinant viruses should be produced to change the site of 584-586 in the H gene. We are now starting to produce infectious clone viruses by using reverse genetic technology.

In this study, we compared the sequences and phylogenetic trees of H and P genes because H protein is the major determinant of tropism and cytopathogenicity (von Messling et al., 2001), and has the highest antigenic variation (Blixenkroner-Møller et al., 1992). The P gene is most conserved within clades of a given CDV lineage (Carpenter et al., 1998). The genetic character of H and P genes are useful for phylogenetic analysis. Hashimoto *et al.* (2001) showed that two groups of new CDVs are in Japan and Asian countries by using phylogenetic trees of the H gene and direct RNA extraction from fresh tissue, but not by using phylogenetic trees of the P gene. Previously, we showed data of only one strain (007Lm) of the P gene of the Asia 2 group (Lan et al., 2005c), so in this study, we clarified the cluster of the Asia 2 group that includes strains 007Lm, 009L and 011C in the P gene phylogenetic tree. Until now, we successfully isolated CDV strains of

Asia 1 and 2 groups and sequenced H and P genes of viruses in fresh tissues and of viruses after isolation in Vero-DST cells. Having isolated viruses with sufficient data of molecular and biological characters of each group seems beneficial for effective CD prevention. To understand clearly the relationship between growth profiles and molecular characteristics, complete genes of CDV strains in Asia 1 and Asia 2 groups should be used in future research.

After isolation in Vero-DST cells, CDV maintains virulence and does not change in H and P genes (Lan et al., 2005c). Therefore, in this study, to compare the possibility of virulence of strains between Asia 1 and Asia 2 groups, we compared clinical signs with pathological findings of dogs 1, 2 and 3 with those of dogs 4, 5, and 6 (Table 2 and 5). Dogs 1, 2 and 3 seemed to show severer clinical signs of diarrhea or bloody diarrhea than dogs 4, 5 and 6. Histopathological findings showed that all dogs in the Asia 1 group had enteritis and inclusion bodies in epithelial cells of the gastrointestinal tract, and in the Asia 2 group only dog 4 had inclusion bodies in epithelial cells of the stomach and intestine. No diarrhea was recorded in dog 4, but when we inoculated 007Lm isolated from dog 4 at a high dose back into the dogs, diarrhea was observed (Lan et al., 2005c). Viruses at different doses have different potential abilities to cause disease. Therefore, although Asia 1 group dogs seemed to have enteritis and all three dogs of the Asia 2 group had no enteritis, we could not conclude that Asia 1 group was more virulent than Asia 2 group. To confirm the difference between virulence of Asia 1 and Asia 2 groups, we should inoculate isolated viruses of Asia 1 and 2 groups back into dogs of the same experimental condition, such as age, breed, virus doses, and observe the ability of the viruses to cause disease.

In conclusion, this study clarified that two CDV groups isolated in Japan have different molecular characters and growth profiles, that the N-linked glycosylation site at amino

acids 584-586 is related to growth properties of CDV, and that the virulence between Asia 1 and 2 groups cannot be shown clearly.