Molecular and phylogenetic analyses of the haemagglutinin (H) proteins of field isolates of canine distemper virus from naturally infected dogs

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We isolated three strains of canine distemper virus (CDV) – the Ueno, Hamamatsu, and Yanaka strains – from dogs in Japan and analysed the molecular properties of their haemagglutinin (H) proteins. Immunoprecipitation of all three strains with a monoclonal antibody revealed H proteins with molecular masses of 84 kDa, which differs from the molecular mass (78 kDa) of the H protein of the Onderstepoort vaccine strain. However, after tunicamycin treatment immunoprecipitation identified H proteins of identical molecular mass (68 kDa) for all three field isolates and the vaccine strain. Sequence analysis showed nine potential sites for asparagine-linked glycosylation in the H proteins of the new isolates, in contrast to four in the H protein of the Onderstepoort strain. Thus, variation in glycosylation of the H proteins of the isolates and the vaccine strain may cause differences in antigenicity of the viruses. Sequences of the H genes showed that the new Japanese isolates have 99% identity with each other, 95% with other European and American isolates (from seals, a German dog, a ferret and large felids) and 90% with the vaccine strain. Phylogenetically, the new Japanese isolates form one cluster which is separate from recent European or American isolates, all of which are distinct from vaccine strains.

Introduction

Recently, the incidence of canine distemper (CD) both in unvaccinated and vaccinated dogs and in wild racoons has increased in Japan (Kai et al., 1993; Gemma et al., 1995, 1996a, b). Similarly, increasing numbers of cases of typical CD have been reported in European countries (Glardon & Stöckli, 1985; Blixenkrone-Moller et al., 1993). In addition, apparently unrelated enzootic outbreaks of canine distemper virus (CDV) infections among large felids – tigers, lions, leopard and spotted hyaenas – have been noted in different zoos in the United States and Tanzania (Appel et al., 1994; Morell, 1994, 1996; Roelke-Parker et al., 1996; Harder et al., 1995, 1996; Haas et al. 1996). CDV infection has occurred not only in terrestrial but also in aquatic carnivores, such as siberian seals (phocine distemper virus-2; PDV-2), under natural circumstances (Mamaev et al., 1995).

Although differences in individual immunity among dogs or vaccination failure cannot be excluded as the causes, the question arises as to whether a new CDV strain with different properties has evolved. Mamaev et al. (1995) and Harder et al. (1996) reported that CDV wild-type isolates (from seals, a German dog, a ferret and large felids) are significantly different from CDV vaccine strains.

In this study, to isolate wild-type CDVs we used B95a cells, which are susceptible to CDV (Kai et al., 1993) as well as measles virus (MV) and rinderpest virus (RPV) (Kobune et al., 1990, 1991). We co-cultured peripheral blood mononuclear cells (PBMC) from naturally affected dogs with the B95a cells, as infectious CDV is present in T and B lymphocytes in vitro and in vivo (Appel et al., 1992; Friedlander et al., 1985; Iwatsuki et al., 1995). We then analysed the haemagglutinin (H) proteins of field isolates and compared them phylogenetically with the H proteins of CDV vaccine...
strains and of other recent isolates from around the world. Since the H protein is of primary importance as an immunogen against CDV infections (Diallo, 1990) and serves as an attachment protein to the cells, the genetic alteration of the H proteins of isolates may account for the recent CD outbreaks in dogs.

Methods

- **Cells.** B95a cells, an adherent derivative of B95-8 cells (Kobune et al., 1990), were propagated in RPMI-1640 supplemented with 5% foetal calf serum (FCS) and maintained in RPMI-1640 with 2.5% FCS.

- **Viruses.** Viruses were isolated from peripheral blood of three dogs which were suspected of being infected with CDV based on clinical criteria. PBMC were separated from heparinized blood on Ficoll-paque and co-cultured with B95a cells as described previously (Kai et al., 1993). When cytopathic effect (CPE) was observed the cells were frozen and thawed three times, sonicated and centrifuged. The supernatant was further inoculated onto cells and viruses at the third passage were used as field isolates of CDV.

  The Onderstepoort strain was used as a standard laboratory strain.

- **Monoclonal antibodies (MAbs).** MAbs were raised against the vaccine FXNO strain: d-7 to the H protein; k-1, l-7, b-8, a-3 and a-8 to the F protein; h-6, c-5 and f-5 to the NP protein (Hirayama et al., 1991). MAB B-1, raised against the RPV H protein, but which cross-reacts to the CDV H protein, was kindly provided by M. Sugiyama (Sugiyama et al., 1991).

- **Indirect immunofluorescence assay (IFA).** For IFA, the viruses were inoculated onto B95a monolayer cultures in Lab-tek chamber slides (Nunc InterMed). Cells developing CPE were acetone-fixed and reacted with diluted acites containing MAbs followed by fluorescein-isothiocyanate-conjugated rabbit anti-mouse IgG (Cappel Laboratories).

- **Radioimmunoprecipitation (RIP) assay.** Virus-infected cells or mock-infected cells were radio-labelled with 3 MBq/ml [35S]methionine (DuPont NEN) for 12 h. The cells were lysed, reacted with the anti-H protein MAb d-7 for 3 h at 4 °C, followed by incubation with protein A beads. The immunoprecipitates were resolved by 10% SDS–PAGE (Sato et al., 1988). To examine glycosylation of the H proteins, virus-infected cells were maintained in RPMI-1640 containing 5% FCS and 30 µg/ml tunicamycin prior to radiolabelling and analysis.

- **Virus-neutralizing (VN) antibody assay.** Plasma samples from three dogs from which new CDV isolates were obtained, and from a dog experimentally infected with the Onderstepoort strain, were heat-inactivated at 56 °C for 30 min, diluted, mixed with 100 TCID₅₀ of the each virus, and then incubated at 37 °C for 1 h. The mixture was inoculated onto confluent B95a cells in 96-well microplate and cells were examined over a 5 day period for appearance of CPE. The VN antibody titre was expressed as the reciprocal of the highest plasma dilution that inhibited CPE completely.

- **RT–PCR.** Total RNA (2 µg), extracted from virus-infected cells using a commercial reagent (ISOGEN; NIPPON GENE), and an antisense oligonucleotide primer (50 µM) were denatured at 70 °C for 10 min and cooled on ice. To synthesize first-strand cDNA, 10 pM of antisense primer was mixed in a solution containing 50 mM Tris–HCl (pH 8.3), 10 mM dithiothreitol, 75 mM KCl, 5 mM MgCl₂, 15 U ribonuclease inhibitor (Promega), 10 mM of each deoxynucleotide triphosphate (dNTP) and 20 U of Moloney murine leukaemia virus RT (GIBCO BRL). A total of 20 µl of the mixture was incubated at 45 °C for 2 h. PCR was performed in 50 µl of reaction mixture containing 5 µl of the solution containing the cDNA product, 50 pM of a sense primer, 50 pM of an antisense primer, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl₂ and 0.1% Triton X-100 (Perkin-Elmer), with 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 2 min using a DNA thermal cycler (Perkin-Elmer).

**Cloning and sequencing.** For sequencing of the H gene of the Yanaka strain, an RT–PCR product was cloned into TA cloning PCR II vector (Invitrogen) and the plasmid DNA was deleted serially with exonuclease III (Stratagene). Resulting deletion clones were sequenced by means of a dye-primer cycle sequencing system (Applied Biosystems (ABI)). To determine the 5’-terminal sequence of the H gene of the Yanaka strain, rapid amplification of cDNA ends (RACE) was performed as described previously (Oshikamo et al., 1994). Direct sequencing of the RT–PCR products was also performed for the Ueno and Hamamatsu strains with the ABI dye-terminator cycle sequencing system. The primers used for these procedures were CDVL2, CDVH13, CDVH14, CDVH16 (nt 40–21 of the L gene and nt 33–52, 1731–1712, 1690–1671 of the H gene of the Onderstepoort strain, respectively), and CDVF5, CDVH15, CDVH17, CDVH19, CDVH21, CDVH23, CDVH25, CDVH27, CDVH29, CDVH18, CDVH20, CDVH26, CDVH2d, CDVH4d, CDVH6d, CDVH8d (nt 2128–2147 of the F gene and nt 288–307, 1–20, 901–920, 1171–1190, 1725–1744, 1110–1129, 436–455, 1496–1515, 207–188, 152–133, 1430–1411, 1601–1642, 1226–1207, 1013–994, 635–616 of the H gene of the Yanaka strain, respectively).

We used GENETYX-MAC version 7.3.1 (Software Development Co.) for nucleic acid and protein analyses, and BIORESEARCH/SINCA version 2.0 (Fujitsu) for computer-assisted phylogenetic analysis by the neighbour-joining method (Saitou & Nei, 1987) with distance calculation by Kimura’s 2-parameter method (Kimura, 1980).

Results

**Virus isolation**

Three CDV isolates, the Ueno, Hamamatsu and Yanaka strains, were successfully obtained from dogs clinically diagnosed as having CD between June 1992 and February 1994 in different places in Japan. B95a cells inoculated with these isolates at an m.o.i. of 0.0001 exhibited CPE at 1–3 days post-inoculation. The size of the syncytia varied according to the virus isolate (data not shown).

**Virus antigens and the electromobility of the H proteins**

The Onderstepoort strain and the three field isolates were analysed with nine anti-CDV MAbs and one anti-RPV MAb by IFA. The three field isolates were recognized by all the MAbs used (data not shown).

The H proteins of the three field isolates and the Onderstepoort strain were analysed by RIP using d-7, a monospecific MAb against the H protein. As shown in Fig. 1(a), an H protein of molecular mass 84 kDa, larger than the
Haemagglutinin protein of CDV field isolates

Fig. 1. Immunoprecipitation of the H protein of CDV produced in B95a cells. Lysates of B95a cells infected with field isolates or with the Onderstepoort strain of CDV (a) or lysates of infected B95a cells treated with 30 µg/ml tunicamycin (b) were reacted with the anti-CDV MAb, d-7, and analysed by SDS-PAGE. Lanes: 1, Onderstepoort strain; 2, Ueno strain; 3, Hamamatsu strain; 4, Yanaka strain; 5, mock-infected B95a cells.

78 kDa H protein of the Onderstepoort strain, was identified in all three field isolates. When glycosylation was blocked by tunicamycin, the H proteins of all three field isolates were detected as single proteins of molecular mass about 68 kDa, a value identical with that of the Onderstepoort strain (Fig. 1b).

Neutralizing activities

Of the three dogs with typical signs of CD, two had no vaccine history. The remaining animal, from which the Yanaka strain was obtained, was vaccinated at 60 days of age, and died 20 days later. The VN antibody titres in the inactivated plasma of these three dogs were low (< 40) or undetectable against any of the field isolates or the Onderstepoort strain. This indicated that the dogs were in the acute phase of infection, and that the third dog had not been properly vaccinated.

A positive control plasma from a dog immunized with the Onderstepoort strain had a high VN antibody titre against the strain itself (1280). However, it had much lower activities against the field isolates (Ueno, 160; Hamamatsu, 176; Yanaka, 75).

Nucleotide sequence analysis of the H genes of the field isolates

First, we sequenced the H gene of the Yanaka strain. The nucleotide sequence of the cDNA of the H protein from genomic RNA of the Yanaka strain was determined for two clones, H1 and H2. Sequence analysis was facilitated by constructing nested deletions of the H1 clone. By means of RACE with primers CDVH18 and CDVH20, which were based on the sequence of the H1 clone, 32 nt at the 5′ end of the H gene and 82 nt of the end of the F gene were sequenced. The accuracy of the sequence of H1 and the cDNA obtained by RACE was confirmed by sequencing the H2 clone. To determine the sequence of the other two strains (Ueno and Hamamatsu), primers CDVF5, CDVH15, CDVH17, CDVH19, CDVH21, CDVH23, CDVH25–27, CDVH29, CDVHd2, CDVHd4, CDVHd6 and CDVHd8 were designed according to the sequence of the Yanaka strain. The PCR products obtained using CDVF5 and CDVH16, CDVH17 and CDVH16 or CDVH17 and CDVL2 of the Ueno and Hamamatsu strains were directly sequenced. The H gene sequences of the three strains showed that the largest open reading frame (ORF)
Table 1. Nucleotide and predicted amino acid identities between CDV H genes (%)

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<th>Yanaka</th>
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<th>A92-27/4</th>
<th>5804/Han90</th>
<th>1493/Han89</th>
<th>PDV-2</th>
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Fig. 2. Phylogenetic relationships of CDV vaccine strains and CDV-like virus isolates based on the entire protein coding region of the H gene. Sequences of the H genes of the Ueno (D85753), Hamamatsu (D85754) and Yanaka (D85755) strains were generated in this study. Other sequences were extracted from the GenBank or EMBL databases [Onderstepoort strain (D00758), Convac strain (Z35493), 5804/Han90 (German dog isolate X85000), 1493/Han89 (German ferret isolate X84999), A92-27/4 (Chinese leopard Z54156), A92-6 (black leopard Z54166), PDV-2 (X84998)].

Phylogenetic analysis of the H gene of the CDV isolates

The sequences of the three Japanese field isolates were approximately 99% identical to each other (Table 1). Comparison of these sequences with those of the other field isolates from a German dog, a German ferret, large felids and PDV-2 showed approximately 95% identity but only 90% identity with the vaccine strains, Onderstepoort and Convac. The phylogenetic analysis of these sequences and other CDV strains are shown in Fig. 2. The recent CDV isolates are clearly separated from the vaccine strains as indicated by the high bootstrap values. The Japanese field isolates appear to be more closely related to PDV-2 than to the CDV vaccine strains.

Amino acid sequences of the H proteins of the field isolates

The predicted amino acid sequences of the H genes of the Japanese field isolates were aligned with those of the other CDV strains (Curran et al., 1991; Kövamees et al., 1991; Mamaev et al., 1995; Harder et al., 1996) (Fig. 3). The identities between the amino acid sequences of the Japanese field isolates and those of the vaccine strains were approximately 90%, whereas identities between the Japanese field isolates and other...
Haemagglutinin protein of CDV field isolates

Fig. 3. Alignment of deduced amino acid sequences of the H proteins of CDV vaccine strains [Onderstepoort (D00758) and Convac (Z35493)], Japanese field isolates [Ueno (D85753), Hamamatsu (D85754) and Yanaka (D85755)], two isolates obtained from captive large felids [black leopard (Z54166) and Chinese leopard (Z54156)], recent German field isolates from a dog (X85000) and a ferret (X84999), and PDV-2 (X84998). These sequences were aligned using GENETYX-MAC version 7.3.1. The major N-terminal hydrophobic region (amino acids 35–55) is overlined. The potential glycosylation sites are shaded. Asterisks indicate conserved cysteine residues. Dots represent identical amino acids.
field isolates were approximately 95% (Table 1). Within the proposed H protein of the Japanese field isolates 12 cysteine residues are located at positions identical to those of the other strains. One major hydrophobic region which is conserved in morbilliviruses (Alkhatib & Brièdis, 1986; Tsukiyama et al., 1987; Curran et al., 1991, 1992) was also observed in the Japanese field isolates. Nine potential glycosylation sites for asparagine N-linked glycosylation were found at amino acid positions 19–21, 149–151, 309–311, 391–393, 422–424, 456–458, 584–586, 587–589 and 603–605. Of the nine sites, the seventh (584–586) is additional to those reported for other strains (Fig. 3).

Discussion

Recently, the number of dogs in Japan diagnosed as having CD has increased (Gemma et al., 1995, 1996a, b; Okita et al., 1997). A recent survey from two animal hospitals in the Tokyo area revealed that more than two-thirds of dogs diagnosed as having CD had been vaccinated, and most of them had high VN titres to the Onderstepoort strain (Gemma et al., 1996a). In addition, some sera from the affected dogs had low VN but high ELISA titres (Gemma et al., 1995). These phenomena suggest the appearance of CDVs with antigenicities different from those of the vaccine strains, and implicate antigenic alterations in the recent increase of CD in Japan.

All the virus antigens recognized by MAbs against a laboratory strain of CDV were identified in the three field isolates by means of IFA. However, the inactivated plasma of a dog immunized with the Onderstepoort strain, which had high VN antibody titres against this strain, had significantly lower titres against the field isolates. This correlates with our recent findings of cross-reactivities, as determined by modified VN-ELISA. Thus, we showed that anti-Hamamatsu and anti-Yanaka sera have high VN titres against both Hamamatsu and Yanaka field isolates, but much lower titres against the Onderstepoort strain (Gemma et al., 1996b). These results suggest antigenic differences in the epitopes of the envelope proteins involved in virus neutralization between the field CDV isolates and the Onderstepoort strain. Since the H protein is considered to play a role in the attachment of the virus to cells, and is most likely to undergo changes under immunological pressure, the alteration in neutralizing reactivity implies antigenic alteration of the H protein.

The H protein of the Japanese field isolates has nine potential glycosylation sites. Four of them (19–21, 149–151, 422–424, 587–589) are in the same positions as all four glycosylation sites of the Onderstepoort strain (Curran et al., 1991). Another vaccine strain of CDV, the Convac strain, and one of the field isolates (from black leopard), have seven N-linked glycosylation sites (Kövamees et al., 1991; Harder et al., 1996) whereas other field isolates have eight sites (Mamaev et al., 1995; Harder et al., 1996), all of which are shared with the Japanese field isolates. The seventh glycosylation site in the Japanese field isolates was discovered in this study.

The H genes of the Japanese field isolates and the Onderstepoort strain encode 607 and 604 amino acids and the calculated molecular masses of the deduced proteins are approximately 68.4 and 67.3 kDa, respectively. The molecular masses of the unglycosylated H proteins of both strains are about 68 kDa according to SDS–PAGE (Fig. 1b), which agree with the calculated molecular mass. By contrast, the molecular mass of the glycosylated H protein of the Onderstepoort strain is about 78 kDa, which probably corresponds to the 76 kDa protein described elsewhere (Örvell, 1980; Rima, 1983). The molecular masses of the H proteins of the Japanese field isolates are estimated to be 84 kDa according to SDS–PAGE (Fig. 1a). Therefore, the carbohydrate constituents of the Japanese field isolates and the Onderstepoort strain may contribute differences of about 16 kDa and 10 kDa, respectively. Taking the molecular mass of one oligosaccharide chain to be 2000–3000 kDa (Keil et al., 1979; Horisberger et al., 1980), all three of the extracytoplasmic glycosylation sites in the Onderstepoort strain are likely to be used. In contrast, the H protein of the Japanese field isolates should have six to eight oligosaccharide chains. Therefore, all nine glycosylation sites are probably not used. Interestingly, PAGE analysis of the H proteins of recent isolates of wild-type MV indicates that these proteins consistently migrate more slowly than those of the vaccine strains (Saito et al., 1992; Rota et al., 1992; Sakata et al., 1993), and an explicit clustering of predicted amino acid changes occurs around potential N-linked glycosylation sites (Rota et al., 1992; Sakata et al., 1993).

The genetic relationships shown in Fig. 2 indicate that current wild-type CDV isolates and vaccine strains are clearly distinguishable, and that Japanese isolates form one cluster which is distinguishable from European and American isolates. The recent field isolates have a tendency to cluster according to geographical factors. The virus which contributed to recent outbreaks in Japan is, therefore, probably not the same virus as the European or American ones. These changes in the H gene suggest a progressive evolution in wild-type CDV isolates. The changes shared among recent isolates are suspected to have accumulated in healthy vaccinated dogs.

It is interesting that similar changes in the H genes have been observed in isolates from different areas of the world obtained over the past decade. It is important to investigate whether sequence variation of H protein is accompanied by antigenic changes which may affect the efficacy of CDV vaccine. Additional wild-type isolates from this period and from years prior to the resurgence of CD need to be analysed to measure accurately the degree of genetic variation occurring in circulating CDV and the distribution of the recent variants of CDV.

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